CHAPTER II

LITERATURE REVIEW

2.1 Roselle

The scientific name of roselle is *Hibiscus sabdariffa* L. The genus *Hibiscus* belongs to the same family as the cotton plant and okra. In the genus *Hibiscus* there are about 300 species which are distributed in tropical and subtropical regions around the world consisting of ornamental plants, vegetables, medicinal plants and forest trees. Therefore, there is a great diversity in the shape and size of plants, ranging from scrambling herb to a tall plant 30 meters in height. Roselle belongs to Malvaceae family (Table 2.1) and it is probably a tetraploid (Wilson, 1994; Halimaton, 2005).

Table 2.1: Roselle (*Hibiscus sabdariffa* L.) taxonomy

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Dilleniidae
Order	Malvales
Family	Malvaceae
Genus	*Hibiscus*
Species	*Hibiscus sabdariffa* L.

Source: US Department of Agriculture (USDA)-Natural Resource Service 2008

Vernacular names in addition to roselle, in English-speaking regions are rozelle, sorrel, red sorrel, Jamaica sorrel, Indian sorrel, Guinea sorrel, sour-sour, Queensland jelly plant, jelly, jelly okra, lemon bush and Florida cranberry. In French, roselle is called 'oseille rouge' or 'ossille de Guinee'. In other languages, roselle is known as Jamaica (Spanish), karkade (Arabic) and bissap (Wolof). Table 2.2 shows the common names given to this plant in various countries (Ross, 1999).

Table 2.2: Common names given to *Hibiscus sabdariffa* in various countries

Countries	Common names	Countries s	Common name
Bangladesh	Mesta	Iraq	Roselle
Congo-Brazzaville	Abuya	Japan	Roselle
	Ibuya	Malaysia	Asam paya
	Inkulu		Asam susur
East Africa	Sudan tea		Roselle
Egypt	Karkade	Mexico	Roselle
	Karkadesh	Nicaragua	Hamaiga
	Red sorrel	Senegal	Basap
	Roselle		Bisap
Germany	Jericho rose		Bondio
	Karkade		Dakouma
	Red sorrel		Fasab
Guinea-Bissau	Baquitche		Indian sorrel
	Cutcha		Kuges
	Folere		Red sorrel
Guatemala	Rosa de Jamaica		Roselle
India	Gogu		Roselle hemp
	Lal ambri		Senegal bisap
	Patwa	Sierra Leone	Satui
	Red roselle		Sawa sawa
	Red sorrel	Somaliland	Karkade
	Roselle	Sudan	Karkadeh
Indonesia	Susur	Thailand	Krachiap daeng
Italy	Karkade	Thailand	Roxella-red sorrel

Source: Modified after Ross 1999

There is a considerable uncertainty regarding its origin, but apparently it is not Asiatic, though it must have been in Asia for at least three centuries. In Malaysia it has very few names. The Malay name, 'asam susur' has a recent use. It is almost certain that the plant has not been in Malay Peninsula for long (Burkill, 1993). *Hibiscus*

sabdariffa possibly originated in tropical Africa where it is known by different synonyms and vernacular names. It was introduced to the West Indies in the 18th century where it was cultivated mainly as an ornamental plant and partly for fibre (Omobuwajo et al., 2000).

2.1.1 Roselle in Malaysia

Roselle is a relatively new crop in Malaysia. It was introduced into Malaysia in early 1990s probably from West Africa, and its commercial planting was first promoted by the Department of Agriculture in Terengganu. The crop has now spread to other States. The planted area was 12.8 ha in 1993 but had steadily increased to 506 ha in 2000. Today, the planted area is around 100 ha annually. Only very few companies are involved in processing, product development and marketing, largely for local market. Roselle grows well in a warm and humid tropical climate. It is considered a hardy crop, and does quite well in poor soils, such as bris or sandy soils. Fresh roselle calyces are usually harvested 85-100 days after sowing. Essentially, there are two varieties available for growers to plant, namely "Terengganu" and "Arab" varieties. Present varieties are reported to yield up to 8 t/ha of fruits, or up to 4 t/ha of calyces. The calyces from the plant are used to produce a pro-health drink due to its high contents of vitamin C and anthocyanins.

Roselle research at UKM began in 1999 (Chang, 2006; Mohamad & Abd. Rahman, 2006; Mohamad, et al., 2004; Mohamad, et al., 2002a; Mohamad, et al., 2002b; Mohamad, et al., 2005; Wong, 2006). Starting with only three accessions at that time, the number of accessions in its germplasm collection has grown to more than 40 accessions in the 1999-2007 periods. The available roselle accessions, although particularly useful in crop improvement programme, are still considered inadequate and limited in terms of genetic variation. Since conventional hybridization is very difficult and is not practical to carry out, a mutation breeding programme was started to generate new traits for breeding purposes, and to develop improved selections to increase crop productivity of roselle. From the mutation breeding programme, few potential lines with desirable characteristics have been selected (Mohamad et al., 2005). Assessment of the morphological, agronomic and physico-chemical

characteristics of the accessions and mutants in the field and lab conditions are essential in order to further gather important data on the desirable and other attributes of roselle. Roselle is a tetraploid species, thus segregating populations require longer time to achieve fixation as compared to diploid species. In April 2009, UKM successfully launched three new varieties named UKMR-1, UKMR-2 and UKMR-3 respectively. These three new varieties were developed using variety Arab as the parent variety in a mutation breeding programme which started in 2006.

2.1.2 Botanical description

The plant is an annual, erect, bushy, herbaceous sub-shrub with a height of 1.5 to 2.4m tall (Figure 2.1). The stems are reddish in colour whichsmooth or nearly smooth and cylindrical. The leaves are dark green to red, alternate, glabrous, long-petiolate and palmately divided into 3-7 lobes with serrate margins. Leaves of young seedlings and upper leaves of older plants are simple. Flowers, borne singly in the leaf axils are up to 12.5 cm wide, yellow or buff with a rose or maroon eye, and turn pink as they wither at the end of the day. At this time, the typically red calyx consisting of 5 large sepals with a collar (epicalyx) of 8-12 slim, pointed bracts (or bracteoles) around the base, begins to enlarge, becomes fleshy, crisp but juicy, 3.2-5.7 cm long and fully encloses the velvety capsule 1.25-2 cm long which is green when immature, 5-valved with each valve containing 3-4 kidney-shaped light brown seeds (3-5 mm) long (Figure 2.2). The capsule turns brown and splits open when mature and dry. The calyces stems and leaves are acidic and closely resemble the cranberry in flavor (Morton, 1987).

Figure 2.1: Standing crop on bris soils in Rhu Tapai, Terengganu

Figure 2.2: Roselle fruits of variety Terengganu (aka UMKL-1)

2.1.3 Ecology

Roselle prefers a well-drained humus rich fertile soil in full sun. Roselle requires a permeable soil, a friable sandy loam with humus being preferable, however it will adapt to a variety of soils. It is not shade tolerant and must be kept weed-free. It will tolerate floods, heavy winds or stagnant water. Roselle is reported to tolerate an annual precipitation of 64 to 429cm, an annual temperature in the range of 12.5°C to 30°C and a pH of 4.5 to 8.0. Plants are sensitive to the length of daylight and do not flower if there are more than 13 hours of light in the day. Roselle is widely cultivated in the tropical and sub-tropical zones for its fibre and edible calyx, there are some named varieties. Roselle is best suited to tropical climates with a well-distributed rainfall of 1500 - 2000 mm yearly, from sea-level to about 600 m altitude. It tolerates a warmer and more humid climate than kenaf (*Hibiscus cannabinus*). In the United States, plants exhibit marked photoperiodism which means it will not be flowering at shortening days of 13.5 hours, but flowering at 11 hours. Plants are also do not flower until short days of late fall or early winter. Since flowering is not necessary for fibre production, long light days for 3 - 4 months is the critical factor. Plants have a deep penetrating taproot (Duke, 1983).

2.1.4 Food uses of roselle

Many parts of roselle including seeds, leaves, fruits and roots are used in various foods. Among them, the fleshy red calyces are most popular. In the United States, they are used fresh for making wine, juice, jam, jelly, syrup, gelatine, pudding, cakes, ice cream and flavours and also dried and brewed into tea, spice, and used for butter pies, sauces, tarts and other desserts (Qi et al., 2005). For stewing as sauce or pies, they can be left intact, making them almost indistinguishable from cranberry sauce. In Africa, the calyces are frequently cooked as a side-dish eaten with pulverized peanuts while dried roselle is pressed into solid cakes or balls (Lagenhoven et al., 2001). In Senegal, the dried calyces are squeezed into great balls weighing 175 lbs (80 kg) for shipment to Europe, where they are utilized to make extracts for flavouring liqueurs (McCaleb, 1996). The calyces possess pectin that makes a firm jelly (Qi et al., 2005). The calyces possess over 3% pectin, and in Pakistan roselle has been recommended as a source of

pectin for the fruit-preserving industry (Morton, 1987). The calyces are used to make cold and hot beverages in many of the world's tropical and sub-tropical countries. In the West Indies and tropical America, roselle is prized primarily for the cooling, lemonade-like beverage made from the calyces. In Egypt, roselle 'ade' is consumed cold in the summer, hot in winter (Morton, 1987). Calyces are used in the West Indies to colour and flavour rum. The brilliant red colour and unique flavor make it a valuable food product. The anthocyanin pigments that create the colour are responsible for the wide range of colouring in many foods (Tsai et al. 2002). The young leaves and tender stems of roselle are eaten raw in salads or cooked as green alone or in combination with other vegetables and/or with meat. They are also added to curries as seasoning. They have an acid, rhubabrb-like flavour (Qi et al., 2005). The leaves of green roselle are marketed in large quantities in West Africa (Morton, 1987). The seeds which are high in protein can be roasted and ground into a powder then used in soups and sauces (Qi et al., 2005). Seeds have been used as an aphrodisiac coffee substitute. Seed has property similar to those of cotton seed oil, and is used as a substitute for crude castor oil. The seeds are considered excellent feed for chickens. The residue after oil extraction is valued as cattle feed when available in quantity. The young root is also edible, but very fibrous.

2.1.5 Food value of roselle

Roselle is well known for its rich contents of vitamin C and anthocyanins (Mat Isa et al., 1985). Nutritionists have found roselle calyces sold in Cetral American markets to be high in calcium, niacin, riboflavin and iron. Table 2.3 to Table 2.6 shows the nutritional value in roselle calyces, leaves and seeds.

Table 2.3: Nutritional value of roselle calyces

Food value per 100g of edible portion

Calyces, fresh	
Moisture	9.2g
Protein	1.145g
Fat	2.61g
Fibre	12.0g
Ash	6.90g
Calcium	1.263mg
Phosphorus	273.2mg
Iron	8.98mg
Carotene	0.029mg
Thiamine	0.117mg
Riboflavin	0.277mg
Niacin	3.765mg
Ascorbic acid	6.7mg

Source : Morton, 1987

Table 2.4: Nutritional value of roselle leaves

Food value per 100g of edible portion

Leaves, fresh	
Moisture	86.2%
Protein	1.7-3.2%
Fat	1.1%
Carbohydrate	10%
Ash	1%
Calcium	0.18%
Phosphorus	0.04%
Iron	0.0054%
Malic acid	1.25%

Source : Morton, 1987

Table 2.5: Nutritional value of roselle seeds

Food value per 100g of edible portion

Seeds, fresh	
Moisture	12.9 %
Protein	3.29%
Fatty oil	16.8%
Cellulose	16.8%
Pentosans	15.8%
Starch	11.1%

Source : Morton, 1987

Table 2.6: Amino acid composition of fresh roselle calyces

Arginine	3.6
Cystine	1.3
Histidine	1.5
Isoleucine	3.0
Leucine	5.0
Lysine	3.9
Methionine	1.0
Phenylalanine	3.2
Threonine	3.0
Tryptophan	-
Tyrosine	2.2
Valine	3.8
Aspartic acid	16.3
Glutamic acid	7.2
Alanine	3.7
Glycine	3.8
Proline	5.6
Serine	3.5

Source : Morton, 1987

The dried calyces contain the flavonoids gossypetine, hibiscetine and sabdaretine. The major pigment, formerly reported as hibiscin has been identified as daphniphylline. Small amounts of delphinidin 3-monoglucoside, cyanidin 3-monoglucoside (chrysanthenin) and delphinidin are also present. Flowers contain phytosterols. Root contains saponins and tartaric acid. Aspartic acid is the most common amino acid. Dried fruits also contain vitamin C and Calsium oxalate (Watt & Breyer-Brandwijk, 1962).

2.1.6 Therapeutic values of roselle

2.1.6.1 Traditional uses

Roselle is an aromatic, astringent, cooling herb that is much used in the tropics. In India, Africa and Mexico, all above-ground parts of the roselle plant are valued in native medicine (Morton, 1987). It is said to have diuretic effects to help lower fevers and is antiscorbutic[1]. The leaves are good against scurvy, emollient, diuretic[2], refrigerant, and sedative. The leaves are very mucilaginous and are used as an emollient and as a soothing cough remedy. They are used externally as a herb for treatment of abscesses. The fruits are antiscorbutic. The flowers contain gossypetin, anthocyanin, and the glycoside hibiscin. These may have diuretic and choleretic[3] effects which experimentally have been showed that an infusion decreases the viscosity of the blood, reduces blood pressure and stimulates intestinal peristalsis (Duke, 1983).

The leaves and flowers are used internally as a tonic tea for digestive and kidney functions. The seeds are diuretic, laxative and tonic. They are used in the treatment of debility[4]. The bitter root is aperitif[5] and tonic[6]. It is used as a folk remedy in the treatment of abscesses, bilious conditions, cancer, cough, debility, dyspepsia[7],

[1] Antiscorbutic – against scurvy
[2] Diuretic – agent to increase the volume of urine excreted
[3] Choleretic – promoting bile secretion by the liver
[4] Debility – the state of being week and feeble
[5] Aperitif – a small alcoholic liquor taken to stimulate the appetite before a meal
[6] Tonic – a medicine that invigorates or strengthens
[7] Dyspepsia – impaired digestion

dysuria[8], fever, hangover, heart ailments, hypertension, neurosis[9], scurvy, and strangury[10]. One report says that the plant has been shown to be of value in the treatment of arteriosclerosis and as an intestinal antiseptic, though it does not say which part of the plant is used. Simulated ingestion of the plant extract decreased the rate of absorption of alcohol, lessening the intensity of alcohol effects in chickens (Duke, 1983).

The calyx infusion called "Sudan tea" which is famous in East Africa is taken by the local to help relieve cough. As for the treatment of biliousness, a formulation of roselle juice with salt, pepper, asafetida[11] and molasses is prepared. The heated leaves are believed to help ease cracks in the feet and are also used on boils and ulcers to speed maturation. While a lotion made from leaves is also used on sores and wounds. The seeds are said to be diuretic and tonic in action and the brownish-yellow seed oil is claimed to heal sores on camels. In India, a decoction of the seeds is given to relieve dysuria, strangury and mild cases of dyspepsia and debility. Brazilians attribute stomachic[12], emollient and resolutive[13] properties to the bitter roots.

2.1.6.2 Medicinal uses

Roselle is a folk remedy for abscesses, hypertension (Onyenekwe et al., 1999) and to prevent cardiovascular and hepatic diseases (Ali et al., 2003). Roselle tea has been shown to lower blood pressure in patients with hypertension (Farayi & Tarkhani, 1999). Studies show that roselle tea may be effective against low-density lipoprotein oxidation and hyperlipidemia (Chang-Che et al., 2004; Suboh et al., 2004).

[8] Dysuria – difficult or painful urination
[9] Neurosis – mild personality disorder typified by excessive anxiety
[10] Strangury – painful urination due to muscle spasms of the urethra or urinary bladder
[11] Asafetida – a soft, brown, lumpy gum resin having a bitter, acrid taste and obnoxious odour obtained from the roots of several Near Eastern plants belonging to the genus *Ferula*, of the parsley family
[12] Stomachic – beneficial to the stomach
[13] Resolutive – having the ability to dissolve

2.2 Obesity

2.2.1 Obesity in Malaysia

Malaysia has been experiencing a rapid phase of industrialisation and urbanization in recent decades and has often been recognized as a role model for developing economies. Statistics available from several Ministries for the last two decades suggest that as the population achieves affluence, their intake of energy, fats and sugars increase as reflected in the rising and now substantial size of the food importation bills. The 'westernisation' of global eating habit has also brought about an increase in the number of fast-food outlets in Malaysia during the last decade (Ismail, 2002).

At the population level, a high prevalence of obesity results from a complex interaction between changes in the population's lifestyle involving a higher energy and fat consumption and an increasingly sedentary existence (WHO, 2000), the effects of these changes, being particularly severe if the population has an inherited metabolic predisposition to fatness. Several previous studies, albeit not representative of the population have reported that obesity is prevalent in all age-groups namely in children, male 12.5% and female 5.0% (Ismail & Tan, 2000), in male adolescents 1.0% in 1990 to 6% in 1997 (Ismail & Wickeswary, 2000) and in adults 21.0% overweight and 6.2% obese using World Health Organisation criteria (Ismail et al., 1995).

2.2.2 Obesity Treatments

Obesity is recognised as a social problem and has become the focus of attention by public and health institutions since it is associated with serious health risks and increased mortality. Hypertension, hyperlipidemia, insulin resistance and glucose tolerance are known as cardiac risk factors that cluster in obese individuals. It has been reported that the statistic of death related to obesity is approximately 200,000 individuals around the world annually (WHO 2003). It is necessary to treat obese individuals by both lifestyle interventions and pharmacological therapy. Successful obesity treatment plans incorporate diet, exercise, behavior modification with or without pharmacologic therapy and surgery. Many therapeutic agents are available for

the management of obesity, but adverse effects have been reported with almost all of them (Wells et al., 2003).

The general public uses many other methods for weight loss including herbs, vitamins, nutritional supplements and meal replacement preparations. Complementary and alternative therapies have long been used in the Eastern world but recently these therapies are being used increasingly worldwide (Hasani-Ranjbar et al., 2008). When conventional medicine fails to treat chronic diseases and conditions such as obesity efficaciously and without adverse events, many people seek unconventional therapies including herbal medicines (Liu et al., 2004).

2.2.3 Herbal medicines

Herbal medicines are defined as raw or refined products derived from plants or parts of plants (e.g. leaves, stems, buds, flowers, roots or tubers) used for the treatment of diseases. The synonyms of herbal medicines are herbal remedies, herbal medications, herbal products, herbal preparations, medicinal herbs and phytopharmaceuticals. In the US, herbal and food supplements are also employed to promote weight loss. The Food and Drug Administration does not strictly regulate these products, so the ingredients may not be active and safe (Wells et al., 2003). However in many developed countries certain traditional or complementary and alternative medicines are becoming more and more popular. These approaches include pharmacological therapies such as herbal medicines.

The world market of herbal medicines based on traditional knowledge is estimated at USD 60 billion (WHO, 2003). In fact the use of medicinal plants contributes significantly to primary health care especially in developing countries (WHO 2003). Furthermore, the role of medicinal plants and traditional medicine for developing new drugs is incontestable (Rates, 2001). Moro & Basile (2000) have reviewed the use of plants that are claimed to be useful in the treatment of obesity all over the world and concluded that some of them could be useful when associated with diet therapy. Dickel et al. (2006) reported that *Hibiscus sabdariffa* as one of the seven species studied which present data that indicates a potential role in the control of

certain conditions which are associated with obesity such as hyperlipidimia. The compound which controls this activity is known as hydroxycitric acid.

2.3 Hydroxcycitric Acid

Hydroxycitric acid (HCA) which is γ-hydroxy acid, is the principal acid found in the fruit rinds of *Garcinia cambogia*. HCA has been known to be beneficial for the control and reduction of body weight. In particular, HCA isomers and derivatives thereof are found to prevent the conversion of excess carbohydrates to fatty acids by inhibiting the actions of cytoplasmic (cystolic) ATP citrate lyase enzyme which plays a key role in the conversion of carbohydrates to fatty acids and cholestrol (Sullivan et al., 1974). This compound also functions as a natural anorectic[14] agent in mammals. This particular stereoisomer of HCA occurs in the free acid form and in the lactone form. However, only the free acid form of HCA is found to exhibit bio-activity. HCA has been widely used and incorporated in a wide range of pharmaceutical preparations in combination with other ingredients for the claimed purpose of enhancing weight loss, cardioprotection, correcting conditions of lipid abnormalities and endurance in exercise (Jena et al., 2002). Extracts of *Garcinia* fruits have become popular worldwide as an ingredient in diet supplements with a market value exceeding USD400 million annually (Yamada et al., 2007).

2.3.1 Sources of Hydroxcycitric Acid

2.3.1.1 Hydroxcycitric Acid From *Garcinia*

Hydroxcycitric acid is found mostly in the fruits rinds of certain species of *Garcinia*, which include *Garcinia cambogia*, *G. indica*, and *G. atroviridis* (Lewis et al., 1965 & Lewis., 1969). These species thrive prolifically on the Indian subcontinent and in western Sri Lanka (Watt, 1972). The dried rinds of the fruit of *G. cambogia* popularly knwn as "Malabar Tamarind" is extensively used all over the West Coast of South India for culinary purposes and commercially for "Colombo curing" of fish. These *Garcinia* species are mostly used for culinary purposes and are available commercially

[14] Anorectic – having no appetite

in India. HCA presents to the extent of 20-30% in the dried fruit rinds (Lewis, 1969). In recent times, *Garcinia* has received worldwide attention as a nutriceutical for effective obesity control. Among the most famous company who manufactures and markets herbal extracts from *Garcinia* is Sabinsa Corporation Sabinsa, 2007). Several scientists have established that HCA, the active ingredient in the fruit prevents the conversion of excess carbohydrates to fat in animals. Interestingly, HCA from *Garcinia* has been shown to inhibit the action of ATP-citrate lyase, the key enzyme which converts citrate into fatty acids and cholestrol in the primary pathway of fat synthesis in the body (Sullivan et al., 1973; Yamada et al., 2007). The actions of HCA increase the production and storage of glycogen, a readily usable form of energy while reducing both appetite and weight gain. HCA also causes calories to be burned in an energy cycle similar to thermogenesis.

2.3.1.2 Hydroxycitric Acid From *Hibiscus*

Tropical plants are a rich source of valuable secondary metabolites including alkaloids, terpenoids and polyphenols, some are useful as medicines and food additives. HCA is also enriched in the calyces of *Hibiscus sabdariffa*. It is also found in the acidic leaves of *H. cannabinus* and *H. furcatus* which are cultivated in several tropical and semitropical countries (Yamada et al. 2007). The first report of HCA from *H. sabdariffa* was from a sample obtained from a crop grown in India which contained 28% (dry weight basis) of the acid by a German scientist named Grieble (Lewis, 1969). Some roselle UKM accession was also reported as having HCA (Wong, 2006). HCA from *Hibiscus* inhibits pancreatic α-amylase and intestinal α-glucosidase leading to the reduction in carbohydrate metabolism (Yamada et al., 2007).

2.3.1.3 Hydroxycitric Acid From Microorganism

Many organis acids are produced by microorganisms in sufficient yields that they can be manufactured commercially. There have been attempts to isolate microorganisms that produce HCA (Hida et al., 2005). The screening from two strains, *Streptomyces*

species U121 and *Bacilllus megaterium* G45C showed that the purified metabolites indicated that they are identical to the HCA derived from *Hibiscus* (Hida et al., 2005).

2.3.2 Chemistry of Hydroxcycitric Acid

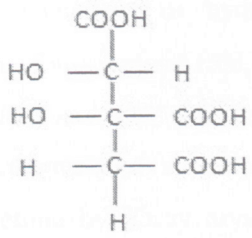
2.3.2.1 Discovery of Hydroxycitric Acid

The organic acids present in the fruit of *Garcinia sp*. have been held responsible for the bacteriostatic effect of the pickling medium by lowering the pH. The fruit contains about 30% acid calculated as citric acid on dry weight basis. The organic acids present have been mistakenly identified as tartaric acid and citric acids (Sreenivasan & Vankataram, 1959; Kuriyan & Pandya, 1931). The aqueous extracts of the fruit showed two predominant acid spots on paper chromatograms run on different solvents, which were very near to tartaric and citric acids, but there was always a significant small difference in the R_f values in all solvent systems. Analysis of the *Garcinia* fruit juice by use of an ion exchange column as described by Palmer (1955) showed the largest peak to be the citric acid region, but the acids failed the cream of tartar test for tartaric acid and the pentabromoacetone test for citric acid.

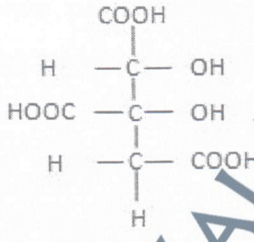
Lewis and Neelakantan (1965), isolated the principal acid in the fruit rinds of *G. cambogia* and identified it as (-)-HCA on the basis of chemical and spectroscopic studies. Identification and separation of the hydroxycitric acid on Whatman No. 1 paper were performed using *n*-butanol/acetic acid/water (4:1:5) and *n*-propanol/formic acid/water (4:1:5). The acid spots were identified by spraying with 5% metavanadate. Upon saponification of the acid mixture with excess alkali and passing it through a column of ion exchange resin (Zeocarb 215), the eluate showed only one lower spot (R_f =0.34) corresponding to the free (-)-HCA. On concentration, the eluate gave only one upper spot (R_f =0.46) corresponding to the lactone. Fruit extracts showed two predominant acid spots on chromatograms with two different solvent systems. On titration of this material with alkali, using phenolphthalein, two different end-points were obtained, in the cold and after boiling, showing the characteristics of lactones. The two spots on chromatograms were identified as hydroxycitric acid and its lactone (Figure 2.3 and Figure 2.4). It was thus clear that the two spots on the chromatograms

are those of γ-hydroxy acid and its lactone and not those of tartaric acid and citric acids.

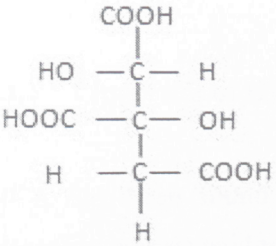
Figure 2.3: Structures of hydroxycitric acid isomers (Jena et al. 2002)



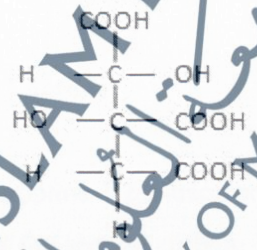
(-)-Hydroxycitric acid (I)



(+)-Hydroxycitric acid (II)

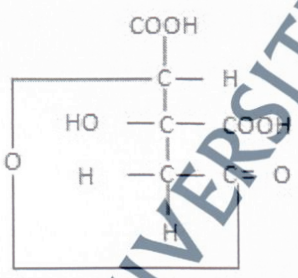


(+)-allo-Hydroxycitric acid (III)

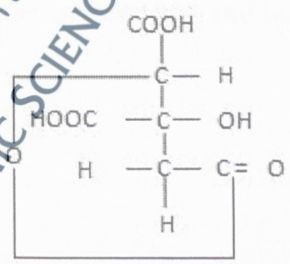


(-)-allo-Hydroxycitric acid (IV)

Figure 2.4: Structures of hydroxycitric acid lactones (Jena et al. 2002)



(-)-Hydroxycitric acid lactone



(+)-allo-Hydroxycitric acid lactone

2.3.2.2 Stereochemistry

Hydroxycitric acid (1,2-dihydroxypropane-1,2,3-tricarboxylic acid) has two asymmetric centres hence, two pairs of diastereoisomers or four different isomers are possible (Figure 2.3). Martius and Maue (1941) have synthesised the four possible stereoisomers of hydroxycitrate. The absolute configuration is determined from Hudson's lactone rule, optical rotary dispersion curves, circular dichrosim curves, and calculation of partial molar rotations. Glusker et al., (1969 & 1971) have reported the structure and absolute configuration of the calcium hydroxycitrate and (-)-HCA lactone by X-ray crystallography. Stallings et al., (1979) have reported the crystal structures of the ethylene-diamine salts of diastereoisomeric hydroxycitrates.

2.3.2.3 Properties of Hydroxycitric Acid and Lactone

HCA has been found to be 1,2-dihydroxypropane-1,2,3-tricarboxylic acid. HCA comprises a citric acid with a hydroxyl group at the second carbon. Being a γ-hydroxy acid, it crystallises readily to the corresponding lactone (Figure 2.4) (Lewis et al., 1969). HCA exists in two forms, the free acid form and the lactone form. The free acid form is biologically active and the lactone form is in active. However, the free acid form is not stable and gets converted to its lactone form which is stable but inactive (Majeed et al., 2005). The physical properties of free HCA and lactones from *Garcinia* and *Hibiscus* are presented in Table 2.7.

Table 2.7: Comparison of physical properties of HCA and lactones from *Garcinia* and *Hibiscus*

Properties	*Garcinia*		*Hibiscus*	
	Free acid	Lactone	Free acid	Lactone
mp(°C)		178		183
$[\alpha]^{20}_{D}$ (deg)	-20	100	122	31
Crystal shape		Needles		Needles
Hygroscopicity		Slight		High
Solubility		High in alcohol and water; fair in ether		High in alcohol and water; slight in ether
Paper chromatography (R_f)				
Butanol/Formic acid/H_2O	0.24	0.42	0.15	0.39
Propanol/Acetic acid/H_2O	0.26	0.36	0.35	0.26
Metavanadate spray	Yellow	Reddish orange	Yellow	Yellow

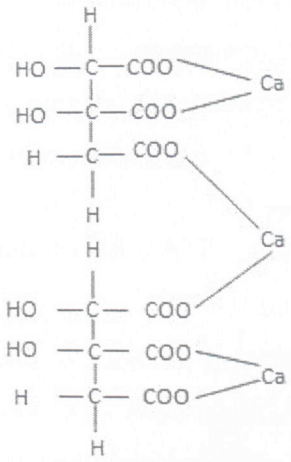
Source: Jena et al., 2002

2.3.2.4 Hydroxycitric Acid Derivatives

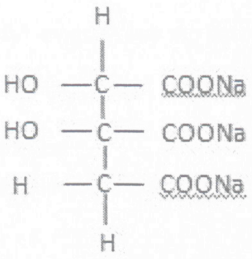
The fruit rinds of *Garcinia cambogia* and *Garcinia indica* contain 20-30% HCA. It is thus the prime source of HCA (Lewis & Neelakantan, 1965). HCA is susceptible to lactonisation during evaporation and concentration. Hence, stable derivatives of HCA namely lactone, ester (Lewis & Neelakantan, 1965; Krishnamurthy et al., 1982), sodium and potassium salts of HCA (Lewis, 1969) and calcium salt (Singh et al., 1995) have been reported (Figure 2.5). In commercial samples of *Garcinia cambogia* extract, HCA is present as its calcium salt for the reason of stability. Free HCA can

easily be generated from the *Garcinia cambogia* extract samples for further analysis by passing an aqueous solution of the calcium salt through a cation exchange resin.

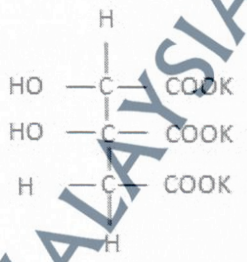
Figure 2.5: Structures of hydroxycitric acid derivatives



Calcium hydroxycitrate



Sodium hydroxycitrate



Potassium hydroxycitrate

Majeed et al., (1998) have reported the preparation of potassium hydroxycitrate from *Garcinia*. It involves the extraction of HCA from *Garcinia* fruit using alkyl alcohol, and the combined extract was treated with potassium hydroxide and refluxed to form potassium hydroxide precipitate. Balasubramaniam et al., (2000) have reported the preparation of a new soluble metal salt of group IA and IIA of HCA. It involves the aqueous extraction of HCA and treating the extract with different metal hydroxides and metal chlorides to get a double salt. Ibnusaud et al., (2000) have reported the isolation of HCA from the fresh or dried rinds of the fruits of *Garcinia cambogia*, *G. indica* and *G. atroviridis*. It involves four or five extractions of *Garcinia* fruits with boiling water for 20 hours. The combined extract was concentrated and treated with methanol to remove pectin and filtered. The filtrate was treated with aqueous sodium hydroxide at 80°C to obtain sodium hydroxycitrate.

2.3.2.5 Physiological Properties of Hydroxcycitric Acid

Scientific reports suggest that HCA found in *Garcinia* has tremendous effect on biochemical and physiological systems of animals and man.

2.3.2.5.1 Inhibition of Citrate Cleavage Enzyme by HCA

HCA from *Garcinia* reduces the conversion of carbohydrate calories into fats. It does this by inhibiting the action of ATP-citrate lyase, the enzyme that converts citrate into fatty acids and cholestrol in the primary pathway of fat synthesis in the body (Watson et al., 1969; Loweinstein, 1977). Acetyl coenzyme A is the precursor of fatty acids. By inhibiting the formation of of acetyl coenzyme A, fatty acid synthesis and lipogenesis are repressed.

$$\text{Citrate} + \text{CoA} + \text{ATP} \xrightarrow{\text{ATP Citrate lyase}} \text{Acetyl CoA} + \text{Oxaloacetate} + \text{ADP} + P_i$$

Watson et al. (1995) encountered the powerful inhibition of ATP-citrate oxaloacetate lyase by HCA with purified enzyme from rat liver. In that experiment, HCA had a much greater affinity for the purified enzyme than the natural substrate that is, citrate and the K_i of HCA for citrate cleavage enzyme was between 0.2 and 0.6 µM depending on the conditions and that of the human enzyme is 300 µM (Hoffman et al., 1980) . Later on, Cheema-Dhadli et al. (1973) found inhibition of citrate cleavage enzyme by both free HCA (K_i = 8 µM) and HCA lactone (K_i = 50-100 µM). The lactone of HCA was found to be very less effective inhibitor of citrate cleavage enzyme. Animal studies have reinforced this findings and further showed that HCA suppresses food intake (Rao & Sakariah, 1988) thereby inducing weight loss (Sullivan et al., 1979).

2.3.2.5.2 Inhibiting Lipogenesis

HCA inhibits the actions of ATP-citrate lyase. HCA reduces the availability of acetyl coenzyme A, the building block for fatty acid and cholestrol synthesis (Greenwood & Robinson, 1999). This may also cause the body to remove low density lipoprotein (LDL) from the blood. Effect of HCA on fatty acid synthesis was studied by Sener and Malaisse (1991). The reduction in cholestrol synthesis is greater than the fatty acid sythesis. Animal trials have resulted in the reduction of triglycerides, cholestrol, food consumption and weight gain. Similar results were obtained when chromium was added to HCA in the diet (Greenwood & Robinson, 1999; McCarty, 1994).

2.3.2.5.3 Suppresing the Appetite

Tests to establish the appetite suppressing effects of HCA revealed that a single large oral dose or two divided oral doses resulted in a 10% or greater reduction in food consumption in experimental animals fed on a high sugar diet. The results continued over many weeks with the chronic intake of HCA. The appetite control mechanism of HCA did not involve any conditioned aversion for food, which HCA did not alter taste, cause gastric distress or illness. Rather, this control stems from the increased production of glycogen and concommitant stimulation of glucoreceptors in the liver which results in early satiety through signals sent to the brain via the vagus nerve.. It also enhances fat burning by interfering with malonyl coenzyme A, an enzyme involved in fat synthesis (Greenwood & Robinson, 1999).

Another mechanism suggested for the appetite suppression is its effect on serotonin, which is a natural vital neurotransmitter. Serotonin is involved in a wide range of behavioral functions in the body including mood, sleep and appetite control. Increased plasma levels of serotonin are associated with decreased food intake, reduced weight gain and increased energy expenditure. Scientific studies showed that HCA produced significant increase in serum serotonin levels (45-70%) which in turns resulted in decreased food intake, reduced weight gain and increased energy expenditure. Two preliminary human trials suggest that HCA may work better when combined with chromium or other insulin potentiators or mimics. Diets high in fat and alcohol will reduce the lipogenesis inhibiting and appetite suppressing effects of HCA (Greenwood & Robinson, 1999).

2.3.2.5.4 Diabetes Treatment

HCA is also found to be useful for reducing genetic obesity (Rothaikerda and Waitman, 1997). This is achieved by decreasing serum leptin level (Hayamizu et al., 2003). Leptin is a 167 amino acid protein hormone encoded by the obesity regulatory gene synthesised and secreted by adipocytes (fat cells). HCA has also been shown to inhibit pancreatic α-amylase and small intestine α-glucosidase (Hansawadi et al., 2000). In a human cell model system, it reduced carbohydrate digestion (Hansawadi,

2001). Administration of these α-amylase and α-glucosidase inhibitors reportedly suppresses increases in blood glucose and insulin levels (Jenkin et al., 1981; Tattersall, 1993; Bischoff, 1994). Acarbose, an α-glucosidase inhibitor is widely used as a therapeutic agent for diabetes treatment. However, it must be used with care because of the side effects it produces. In animal experiments, oral administration of HCA did not show any side effects or abnormal changes in tissues or internal organs. Therefore, natural HCA may be a possible safe food additive for diabetes and obesity treatment (Kasai et al., 2000).

2.3.2.6 Toxicology of HCA

Animal studies indicate that HCA is no more toxic than citric acid itself, which is present in many foods in addition to being a normal intracellular compound. Also HCA is a component of a natural product, which has been used in Indian cuisine as well as for medical purposes. Cloutre and Rosenbaum (1994) pointed out that HCA has extremely low levels of toxicity. For example, recent oral toxicity studies performed at Wil Research Laboratories in Ashland, Ohio found that 5000 mg/kg of body weight of HCA resulted in no visible symptoms of toxicity or deaths in laboratory animals. This roughly equivalent to 350 g or 233 times the dosage of 1.5 g/day of HCA that might be consumed by an average–sized person.

Garcinia cambogia and its extracts have proved safe for human consumption. Neither acute nor chronic toxicity is reported with regular consumption of *Garcinia* products as foods or as dietary supplements (Greenwood and Cleary, 1981). The side effects and toxicity study of HCA concentrate was conducted by Sulllivan and Triscari (1985). According to their investigations the LD_{50} (lethal dose for 50 percent of the animals tested) was greater than 2000 mg/kg for intra pentoneal administration and greater than 4000 mg/kg for oral administration (Sullivan and Triscari, 1973). This level is more than the LD_{50} value of citrate, which is 975 mg/kg as indicated in the Merck index (Merck index, 1996). This shows that HCA is safer than citric acid. Safety of HCA on other tissues like liver, blood and brain were also studied and proved (Anthony et al., 1998).

Since *Garcinia cambogia* has a long history of usage as a flavouring agent, preservative and herbal tonic and there are no reports of toxicity regarding traditional use of the *Garcinia* extract, it is highly unlikely that there may be any possibile negative effect that may occur due to excess intake. The possibility of bowel intolerance can be easily reversed by simply reducing the dosage. However, this problem has not been reported in animal studies at the levels that were necessary to reduce appetite. However, despite its inherent safety HCA like any other diet product, is not recommended for certain group of people. HCA has impacts on the body's production of fatty acids and cholestrol therefore it may directly influence the production of sterols thus restricting the production of steroid hormones. As pregnancy is a time of extreme sensitivity of steroid hormones, products containing HCA should not be recommended during pregnancy. Likewise, women who are breast-feeding should also avoid HCA. Although experience with fruit sources of HCA shows that they are not dangerous to young children, they are advised not to consume HCA in large amounts for extended periods.

2.3.2.7 Dosage of HCA

The typical daily dosage necessary for weight management is equivalent to half of a dried fruit. But it is very difficult to consume as raw. The presence of other materials present make it bitter. Hence, it is ideal to take HCA as the different salt forms. One of the study involving 200 subjects, 1500 mg HCA daily was supplied in addition to carnitine and chromium and the subjects lost twice as much weight as controls (Kaats et al., 1997). In another study 1320 mg daily intake of HCA was given and the weight reduced was twice as much weight as controls (Thom, 1996). Hence dosage recommended is 1500 mg of HCA in 2-3 divided dosages per day one hour before meals (Heymsfield et al., 1998). The main advantage of using HCA is that this does not affect Central Nervous System (CNS) by reacting with the catecholamine neuro trasmitters dopamine and norepinephrine which in turn releases adrenaline (Ohia et al., 2002).

2.4 Hydroxycitric Acid Extraction Methods

The purpose of an extraction technique is physically to separate components of a mixture (solutes) by exploiting differencs in their relative solubilities in two immiscible liquids or between their affinities for a solid sorbent. Substances reach an equilibrium distribution through intimate contact between the two phases, which are then physically separated to enable the species in either phase to be recovered for completion of the analysis. An equilibrium distribution of the solutes between the two phases is established by dissolving the sample in a suitable solvent, then shaking the solution with a second immiscible solvent or by passing it through a sorbent bed or disk. Where the equilibrium distributions of two solutes differ, a separation is possible. The principle factors that determine how a solute will distribute between two phases are its polarity and the polarities of each phase. Degree of ionisation, hydrogen bonding and other electrostatic interactions also play a part.

Lewis and Neelakantan, (1965) were the first who extracted HCA on a large scale from the dried rinds of *Garcinia cambogia*. They used the aqueous extraction method. The method consisted of extracting the acid by cooking the raw material with water under pressure (10 lb/in.^{2} for 15 min). The extract was concentrated, and pectin was removed by alcohol precipitation. The clear filtrate was neutralized with alkali, passed through cation exchange resin for recovery of the acid, which was concentrated and dried. The crude dried mass was extracted with ether and recrystallized to give small needleshaped crystals of lactone. Lewis (1969) reported another method for the isolation of HCA from *Garcinia cambogia* using acetone. The acetone extract was concentrated and the acid taken up in water. The aqueous solution yielded the crystalline lactone on evaporation. But, these extraction technologies have a few drawbacks. It employs expensive tedious ion-exchange chromatography process. The extraction method is only useful for preparations in the milligram or gram scale and is not suitable for large-scale extraction. Finally, the crystallisation process of the lactones takes several days to complete.

Moffett et al (1996) have developed a process for the aqueous extraction of HCA from *Garcinia* rinds. The extract was loaded onto an anion exchange column for

adsorption of HCA and it was eluted with sodium/potassium hydroxide for release of HCA. The extract was passed through a cation exchange column to yield a free acid. Guthrie and Kierstead (1977) and Moffett et al. (1997) have reported the preparation of HCA concentrate from *Garcinia* rinds with 23-54% HCA and 6-20% lactone. It has been found, however, that the free acid form of HCA is unstable, forming lactones which generally do not possess the desired bioactivity.

2.4.1. HCA Extraction Method Using Methanol

In the past, it has been difficult to extract hydroxycitrate in a form which is both stable and biologically active. Hydroxycitric acid exists in two forms, the free acid form and the lactone form. The free acid form is biologically active and the lactone form is inactive. However, the free acid form is not stable and gets converted to its lactone form, which is stable but inactive. The extraction of HCA in pure acid form involves the chemical modification of HCA to afford chemically stable product, which will not convert into lactone form, which will not be hygroscopic and which is soluble in aqueous solutions and easily absorbable by the gastrointestinal tract. This provides HCA by combining it with potassium into potassium hydroxycitrate which is a water soluble salt. Potassium is an ion primarily found in the cell cytoplasm and it can easily cross from outside the cell to inside the cell. The cell membrane permeability for potassium is 100 times higher than for chloride. Potassium salt of HCA acts as a transporter of HCA inside the cell, where the biochemical action of HCA is exerted (Majeed et al., 2005).

Potassium hydroxycitrate is prepared by involving the extraction of HCA from the fruit using alkyl alcohol. Preferred alcohols include methyl alcohol, ethyl alcohol, propyl alcohol and isopropyl alcohol. Especially preferred is methanol. The extract is treated with a suitable alkali to precipitate potassium hydroxycitrate. Preferred alkali include potassium hydroxide, potassium carbonate etc. Most preferred alkali is potassium hydroxide. The general process includes the following steps. The fruit is extracted with alkyl alcohol at above ambient temperature. This is done at or above atmospheric pressure. The extract is collected. The extraction step is repeated at least three times. The extracts are combined and treated with an alcoholic solution

containing alkali. The resultant mass is heated above ambient temperature and pH is adjusted to make the solution alkaline. The pH of the solution is normally between 8 to 11.5. The product is dried and kept for further use (Majeed et al., 2005).

2.5 High-Performance Liquid Chromatography (HPLC)

Free HCA leads to the formation of HCA-lactone during concentration and evaporation. The presence of minor organic acids such as citric acid, tartaric acid and malic acid in the *Garcinia* fruits and the lack of official methods for the assay of HCA have created confusion and disagreement among analysts (Anthony et al., 1998). The existing method for the determination of HCA content in *Garcinia cambogia* extract involves acid-base titration, which gives the total acidity of the extracts. However, in this method the concentrations of HCA and lactone cannot be estimated separately.

Lowenstein and Brunengraber, (1981) have estimated the hydroxycitrate content of the fruit of *Garcinia cambogia* by gas chromatography (GC). GC estimation involves the conversion of acid to volatile silyl derivative. They used an OV-27 GC column (3m x 3mm). The column was run at 145°C using nitrogen as carrier gas (40 mL/min) with an injection port temperature of 250°C and a detector temperature of 300°C. HCA lactone is the major constituent of the extract, and the recrystallised compound contains 0.5% of impurities. Silylation requires completely dried samples but HCA has a tendency for lactonisation during drying because of the highly hygroscopic nature of HCA. It is rather difficult to dry the sample. For this reason, the free HCA content cannot be estimated.

Recently Jayaprakasha and Sakariah (2000; 1998 & 2001) have developed high-performance liquid chromatography (HPLC) methods for the determination of organic acids in the fruits of *Garcinia cambogia,* commercial samples of *G.cambogia* extracts and leaves and rinds of *G. indica*. In these HPLC methods, dilute extracts can be quantified without concentration, drying and derivatisation. Here, the advantage is that the HCA and its lactone can be quantified separately.

High Performance Liquid Chromatography is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phases through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvents used.