ANTHOCYANINS STABILITY, ANTIOXIDATIVE ACTIVITIES, ANTIDEPRESSANT AND CYTOTOXICITY OF *CARISSA CARANDAS* SYRUP: EFFECTS OF COPIGMENTATION REACTION

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by

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ABSTRACT

In the present study, an attempt has been made to achieve anthocyanins' stability and colour retention through copigmentation reaction. Ten copigments were added separately to pasteurized syrup (56°Brix), which was prepared from the ripe fruits of *Carissa carandas*. The copigments selected for this study included: quercetin, naringin, rutin, (+)-catechin, L-tartaric acid, caffeic acid, aluminium chloride, iron (II) sulphate, iron (II) chloride and gum arabic. The variables studied were storage, thermal and light stabilities. Results point out that the addition of copigments at 0.2mg/ml conferred stability to anthocyanins in syrups compared to the non-copigmented syrup. Highest stability was obtained through the formation of anthocyanins-caffeic acid complexes, while rutin was found to be the second best copigment to stabilize anthocyanins. Another observed effect of copigmentation reaction were the slight bathochromic shift and evident hyperchromic effect of the visible maximum absorption spectrum of pelargonidin 3-glucoside at 519nm by rutin and caffeic acid copigmented syrups with the latter showed greater effects and this phenomenon presumably caused by the increased stability of anthocyanins. The colour changes of caffeic acid copigmented syrup and rutin copigmented syrup compared to the non-copigmented syrup during storage was expressed as colorimetric coordinates in the CIELAB scale using the L^* (lightness), a^* (redness-greenness), b^* (blueness-yellowness) and h_{ab} (hue angle) notations. A marked increase of lightness and colour shifts towards red and yellow tonalities were observed and this was true to all three samples examined. Caffeic acid copigmented syrup's h_{ab} value (9.00°) was the closest to the $+a^*$ axis, indicates that its colour was the most red at the end of storage period. The antioxidative activities of copigmented and non-copigmented

syrups were evaluated in terms of their free radical scavenging activity, as well as their ability to inhibit lipid peroxidation. Hence, the 2,2'-di-phenyl-1-picrylhydrazyl (DPPH) free radical scavenging, ferric thiocyanate (FTC) and thiobarbituric acid (TBA) assays were employed. Butylated hydroxytoluene (BHT), a synthetic antioxidant was added in syrup and act as a reference in the FTC and TBA assays. (+)-catechin added syrup displayed an excellent radical scavenging activity with 35.70% (56°Brix) and 54.28% (9.8°Brix) of activity. Whereas rutin showed a remarkable inhibition of lipid peroxidation as measured in the FTC and TBA assays. Caffeic acid copigmented syrup proved to be the best radical scavenger with 35.70% (56°Brix) and 59.47% (9.8°Brix) of activity and exhibited the greatest antioxidative activity in both FTC and TBA assays. Copigmented syrups exhibited comparable, or in some samples, greater activity than the BHT copigmented syrup while sugar was found to decrease the activities. The present study was also designed to evaluate the antidepressant effect of syrups and juices in comparison to imipramine. Mice were treated with samples of study (0.01ml/kg body weight) intraperitoneally and submitted to the forced swimming (FST) and tail suspension tests (TST). All samples resulted in a significant reduction ($P<0.001$) of the immobility time in both models of depression, with the unripe and ripe fruits' juices, along with caffeic acid copigmented syrup exhibited the best antidepressant effect comparable with that of the tricyclic antidepressant, imipramine. Copigmented and non-copigmented syrups were further assayed for cellular toxicity against brine shrimp and sheep erythrocytes in the brine shrimp lethality assay and hemolytic assay. Syrups were found to be cytotoxic and lethal towards brine shrimp and in contrary, induced no hemolysis to sheep erythrocytes.

CHAPTER 1 INTRODUCTION

1.1 Beverages and consumers

Beverages are consumed regularly around the world and therefore constitute an important pillar in the daily diet of humans. Their popularity is due mostly to the different kinds of pleasant tastes and sensations aroused, such as sweet, cool or refreshing. However, beverages are also drunk for health reasons, for example, for the supplementation of vitamins, like in fruit juices, or the intake of minerals and trace elements, which can be found especially in mineral water. Furthermore, there is growing evidence that the constituents of different beverages seem to possess anticarcinogenic, antioxidative or antimutagenic effects.

Polyphenols and flavonoids, for example, in green tea or fruit juice and also black tea were described as exerting inhibitory effects on cancer and tumorigenesis (Wang *et al.*, 1992; Yang & Wang, 1993; So *et al.*, 1996), whereas the coffee constituents, caffeic and ferulic acid, were reported to be potent antioxidants (Graf, 1992; Stadler *et al.*, 1994). In contrast to these desired effects, the ingredients of some beverages can also be harmful to human cells. Coffee, for instance, also contains high amounts of dicarbonyls, like methylglyoxal, whose mutagenic potential is enhanced by hydrogen peroxide (H_2O_2), which is generated in freshly prepared coffee depending on duration of steeping time (Nagao *et al.*, 1986). Moreover, the ingredients that give flavour to cola drinks have been reported to induce the formation of deoxyribonucleic acid (DNA) adducts in mice liver, which is an indicator of genotoxicity (Randerath *et al.*, 1993). Recently, various soft drinks termed 'energy drinks' or 'power drinks' become very popular among young generation. Possible positive or negative health effects of these beverages especially those containing taurine and caffeine are not clear at the moment (Ekmekcioglu *et al.*, 1999).

Today, consumer awareness towards maintaining health and keeping diseases at bay is blooming. Hence, a new health drink without synthetic additives might be fit for the purpose.

1.2 Nutritional values and medicinal properties of *Carissa carandas* fruits

Carissa carandas L., an evergreen thorny shrub (Parotta, 2001), belongs to the family of Apocynaceae (Pakrashi *et al.*, 1968), produces berry like fruits (Hasnain & Ali, 1990) and locally named 'karenda' or 'asam renda' in Malay. The fruits are oval, resembling large olives (Khory & Katrak, 1984), in clusters of 3 to 10 (Morton, 1987), 2.32cm to 2.48cm long and are about 1.79cm to 1.83cm in diameter (Khuzma, unpublished result). They have fairly thin but tough, purplish red skin which turns to dark purple or nearly black when ripe, smooth and glossy surface with 2 to 8 flat brown seeds. Freshly picked fruits can be kept at room temperature for only 3 to 4 days before it begins to shrivel (Morton, 1987). Different stages of *Carissa carandas* fruits maturity can be seen in **Plate 1.1**.

Pino (2004) studied the volatile flavour constituents of *Carissa carandas* fruits, and found that the major constituents in this fruit flavour were isoamyl alcohol (6.65ppm), isobutanol (6.06ppm) and β-caryophyllene (5.94ppm). The author indicated that the exotic flavour which characterized this fruit is because of the interaction of fruity-winey (isoamyl alcohol), floral-woody (β-caryophyllene), with acidic and pungent (fatty acids), contributing to the complexity of the fruit's flavour.

A lot of work has been done to explore and characterize the physical and chemical properties of *Carissa carandas* fruits. For instance, work done by Khuzma (unpublished result) is presented in **Table 1.1**.

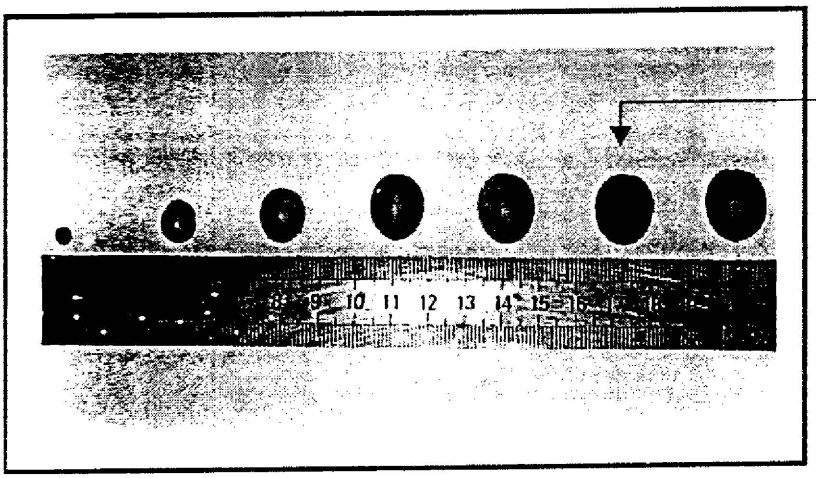


Plate 1.1 Different stages of *Carissa carandas* fruits maturity. Fruit that is indicated by arrow will be used in this study.

Table 1.1 Analysis on *Carissa carandas* fruits (Khuzma, unpublished result)

Juice (ml/100 units of fruits)	496.00
pH	2.69
Total insoluble solids (°Brix)	11.20
Pectin (%)	25.15
Total dietary fiber (%)	87.55
	(mg/100g) fruit
Potassium	185.72
Sodium	1.59
Calcium	67.28
Magnesium	11.22
Phosphorus	80.22
Iron	1.24
Zinc	1.11
Copper	0.25
	(mg/100g) fruit
Glucose	30.35
Glucose-6-phosphate	162.01
Fructose	41.51
Stachyose	0.71
Sucrose	2.90
	(mg/100g) fruit
Anthocyanin	624.49
Carotenoid	18.10

Based on the data on Table 1.1, 100 units of fruits contain about 496ml of juice having pH 2.69 and a Brix value of 11.20. The high acidity of the juice provides a longer shelf life and is responsible for astringency in taste (Hasnain & Ali, 1990). The amount of pectin and total dietary fiber found were 25.15% and 87.55% respectively. The fruit possesses appreciable amount of jelly grade pectin, hence a large number of processing factories in India, during the last decade have been built for making commercial jelly and a product by the name of 'Nakal cherry' which closely resembles canned cherry fruits (Mandal *et al.*, 1992). The mineral composition profile shows the

presence of five macrominerals i.e. potassium, sodium, calcium, magnesium and phosphorus, along with a trace amount of iron, zinc and copper. Potassium is the most abundantly-found mineral element in fruits and vegetables and usually exists between 60mg to 600mg/100g of fresh material (Hasnain & Ali, 1990). The potassium content of *Carissa carandas* fruits which is 185.72mg/100g shows a significant nutritive importance of the fruit. Different types of sugars i.e. glucose, fructose, stachyose, sucrose and glucose derivative, glucose-6-phosphate were found in the fruit. The large quantities of glucose, glucose-6-phosphate and fructose make these sugar the major sugars in *Carissa carandas* fruits as in strawberry. And of course, anthocyanin, the pigment that is responsible for the red colour of the fruit is present in a large quantity, apart from carotenoid. Meanwhile, ascorbic acid content has been reported by Morton (1987) as 9mg to 11mg/100g fruits. Proximate analysis done by Khuzma (unpublished result) however showed a slight difference with the values reported by Morton (1987) as can be seen in **Table 1.2**.

Table 1.2 Comparison of percentage values between data obtained by Morton (1987) and Khuzma (unpublished result) on proximate analysis of *Carissa carandas* fruits

Moisture	83.40	82.40
Ash	0.78	1.10
Protein	0.66	1.09
Fat	4.63	1.67
Fiber	1.81	2.74
Carbohydrate	0.94	9.00

Koehler *et al.* (1987) and Salunkhe & Kadam (1989) based on their findings, reported that species and soil affect the mineral composition of *Phaseolus vulgaris* L. Thus, the differences in the values reported by Morton (1987) and Khuzma (unpublished result) may be due to the soil variation, climatic factors, plant genotype and agronomic management.

Quantification of vitamin C, citric acid and sugar in *Carissa carandas* fruit at different stages of fruit maturity have been done by Loh (2003). The results obtained by the author are presented in **Table 1.3**.

Table 1.3 The vitamin C, citric acid and sugar contents of *Carissa carandas* fruits at different stages of maturity (Loh, 2003)

Stages of maturity	Vitamin C (mg/100g)	Citric acid (%, as mg/g juice)	Sugar (%, as mg/g)
Unripe	5.60	4.50	1.25
Half-ripe	5.00	2.71	0.53
Ripe	34.70	0.62	0.13
Rotten	30.20	0.77	0.23
Artificially-ripe*	32.70	2.08	0.16

* Artificially ripe – fruits were plucked at the half-ripe stage and kept at room temperature to ripen

From Table 1.3, it can be observed that the vitamin C content increased with maturity and the decreased of value suggesting the degradation of vitamin C in rotten fruits. Citric acid and sugar content somehow showed the same pattern which involved the decreased of values till the fully-ripe stage. Then, the values increased in rotten fruits. The artificially-ripe fruits were lack in the vitamin C content, but high in citric acid and sugar compared to the fruits that undergo the natural process of ripening.

The unripe fruit of *Carissa carandas* is used medicinally as an astringent, while the ripe fruit is taken as an antiscorbutic and remedy for biliousness (Morton, 1987). Wong (2003) had tested the effectiveness of *Carissa carandas* extracts as anticancer agent on the human ovarian carcinoma, Caov-3 cells. The results obtained suggested that the chloroform extract from the ripe fruits exhibited good anticancer activity with the effective concentration fifties (EC_{50}) value of 23.42µg/ml when assayed using the methylene blue assay (MBA). In addition to its medicinal uses, the sour unripe fruits are used for preparing pickles while the ripe fruits are used to prepare juices or carbonated drinks and in tarts, salads, puddings and jellies (Parotta, 2001).

Based on nutritional values profile, medicinal properties and unique flavour, *Carissa carandas* fruits do indeed have a high potential to be utilized as a health drink in order not to put the nutritional benefits to waste.

1.3 Anthocyanins as food colourants?

The appreciation of good quality food and drink is one of life's great pleasures. Colour plays a very important role in the acceptability of foods. Consumers first judge the quality of food product by its colour, and the food industry has used colourants for centuries to enhance or restore original appearance of foods or to ensure uniformity, as indicator of food quality. Colour is a vital constituent of foods because it is one of the first characteristics perceived by the senses and is used by consumers for the rapid identification and ultimate acceptance of foods (Noonan, 1972).

The safety of synthetic colourants has been questioned in the past years, leading to a reduction in the number of permitted colourants. Hence, interest in natural colourants has significantly increased as a consequence of both legislative action and consumer awareness to the use of synthetic additives in their foods.

Anthocyanins comprise a diverse group of intensely coloured pigments responsible for the appealing and often spectacular orange, red, purple and blue colours of many fruits, vegetables, flowers, leaves, roots and other plant storage organs. They are water soluble, which facilitates their incorporation into aqueous food systems (Markakis, 1992), and have been consumed for centuries without adverse effects (Giusti & Wrolstad, 2003). Examples of edible sources with anthocyanins that may impart desirable colour include radishes, red cabbage, carrots and purple sweet potatoes.

However, some limitations have restricted the use of natural colourants in food systems. As long as the plant tissue remains intact, the anthocyanins pigments remain stable, but their stability is strongly affected during processing and storage. The highest susceptibility is towards changes in pH, and the colour-expressing flavylium ion remains more or less stable at a very low pH and degrades very quickly, forming chalcones with increasing pH (Brouillard, 1982). Due to these transformation with pH variation, anthocyanins' application for food systems were typically sought in acidic food (pH<3), to assure a predominance of the flavylium cation. The rate of pigment degradation also increases with temperature, presence of light, hydrolysis to aglycones, use of sulfite or as a result of enzymatic or non-enzymatic browning (Markakis, 1974; Wesche-Ebeling & Montgomery, 1990).

Much work has been concentrated on the stability of anthocyanins due to the growing interest in widespread use of anthocyanins as natural food colourants. For example, Maccarone *et al.* (1985) studied the stabilization of anthocyanins in blood orange juice. They found that microwave pasteurization and addition of tartaric acid and glutathione improved the stability. They also found that complexation of anthocyanins with copigments provided the stability effect on anthocyanins. Thermal degradation of anthocyanins has been studied in black raspberry (Daravingas & Cain, 1968), Concord grape (Calvi & Francis, 1978), plum (Raynal & Moutounet, 1989) and sour cherry (Cemeroglu *et al.*, 1994). In a recent study by Krifli *et al.* (2000), the degradation of anthocyanins in blood orange juice during storage was investigated.

There is still a continuing effort to stabilize anthocyanins. Highly stable colourant systems may already be present in nature. However, they need to be identified and characterized for their phytochemical composition and stability attributes.

1.4 Anthocyanins as antioxidant

By definition, antioxidants are substances that, when present at low concentrations compared to those of an oxidizable substrate, significantly inhibit or delay the oxidation of that substrates (Halliwell, 1995a) by inhibiting the initial or propagation of oxidizing chain reactions (Bergman *et al.*, 2001). Oxidizable substrates include DNA, lipids, proteins and carbohydrates (Halliwell, 1995a). Antioxidants may help to protect the human body against damages caused by reactive oxygen species (ROS) (Halliwell, 1995b). Various ROS such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), H_2O_2 and hydroxyl radical ($^\bullet OH$) are generated as by-products during aerobic metabolism in cells (Gutteridge, 1994). These highly reactive species have the potential for bringing about extensive damages, including lipid peroxidation, DNA lesions and protein fragmentation within the cells of biological macromolecules. It is widely acknowledged that the accumulation of oxidative damages of intracellular macromolecules is an essential element in aging processes and in certain neurodegenerative diseases (Vaughan, 1997) such as Alzheimer's disease, multiple sclerosis, Parkinson's disease and Downs syndrome (Arts *et al.*, 2001).

Besides the colour attributes, interest in anthocyanins has intensified because of their possible health benefits. The biological activities of anthocyanin, such as antioxidative activity have been investigated and shown to have some beneficial effects in the treatment of diseases. For instance, there have been reports on the strong antioxidative activity exhibited by pelargonidin 3-*O*-beta-D-glucoside, cyanidin 3-*O*-beta-D-glucoside and delphinidin 3-*O*-beta-D-glucoside isolated from the *Phaseolus vulgaris* seed cot in a liposomal system (Tsuda *et al.*, 1996). Anthocyanins can also prevent the oxidation of ascorbic acid caused by metal ions through chelating the metal ions, and forming ascorbic(copigment)-metal-anthocyanin complex (Sarma *et al.*, 1997). Anthocyanins were found to have many times more activity than common

antioxidants such as ascorbate and they also found that cyanidin 3-glucoside is the most active antioxidant (Wang *et al.*, 1997). Noda *et al.* (1998) reported that the anthocyanin delphinidin 3-(*p*-coumaroylrutinoside)-5-glucoside, which was isolated as purple coloured crystals from eggplant skins, *Solanum melongena* L. can directly scavenge superoxide radical. Tsuda *et al.* (1999) suggested that cyanidin 3-*O*-beta-D-glucoside, a typical anthocyanin pigment, acts as a potent antioxidant *in vivo* when acute oxidative stress is encountered. The *in vitro* enzymatic and non-enzymatic polyunsaturated fatty acid peroxidation was significantly inhibited in a dose dependent manner by purified anthocyanin, a deep-red colour pigment from carrot cell cultures (Narayan *et al.*, 1999). Meanwhile, Espin *et al.* (2000) found that the anthocyanin extracts from black chokeberry, black-thorn and strawberry possess an antiradical capacity.

In recent years, many papers have been published on the *in vitro* antioxidant activity of anthocyanins and their other biological functions, as well as studies assessing the correlation between their antioxidant capacity and chemical structure. Overall, anthocyanins may represent a class of important antioxidants, as they are so common in foods. Being high in anthocyanin, *Carissa carandas* fruit is both a good colourant and potentially a good source of antioxidants.

1.5 Antidepressant study

Depression is recurrent, life threatening and among the most prevalent forms of mental illness (Gainotti *et al.*, 2001; Wong & Licinio, 2001; Nestler *et al.*, 2002a). It is a major disease affecting nearly 13% to 20% of the population (Licinio & Wong, 1999) and constitutes the second most common chronic condition in clinical practice, exceeded only by hypertension (Campos *et al.*, 2004). The symptoms of depression, such as

lowered mood and shortage of interest or pleasure, made the patients lose capabilities for work and logical communication, and even suicidal (Johnson *et al.*, 1992).

Various classes of compounds with different chemical structures have been found to have antidepressant activity. Tricyclic antidepressants, namely imipramine and imipramine-like compounds, have been widely used for the treatment of depression. A heterogeneous group includes, among other dugs, tetracyclic antidepressants such as mianserin. The compounds belonging to these pharmacological classes have been shown to block the neuronal uptake of serotonin and norepinephrine (Hollister, 1995; Frazer, 1997). Moreover, an original series of successful antidepressant agents has recently emerged and consist of drugs such as fluoxetine or sertraline which have been described as selective inhibitors of the reuptake of serotonin into nerve terminals. This series of compounds has been named selective serotonin reuptake inhibitors. Besides these three groups of compounds, other drugs including atypical antidepressants and monoamine oxidase inhibitors are also used in treating depression (Hollister, 1995; Frazer, 1997).

Despite recent progress achieved in the development of clinically relevant antidepressant drugs in recent years, the currently available antidepressant therapy is not yet totally effective and it is associated with many undesirable collateral effects (Whooley & Simon, 2000; Nestler *et al.*, 2002b). Autonomic, neurologic, cardiovascular, gastro-intestinal and metabolic adverse effects are common (Baldessarini, 1989; Hollister, 1995; Frazer, 1997). Changes in brain monoaminergic neurotransmission as well as the capacity of antidepressants to block muscarinic, adrenergic or histaminergic receptors can contribute to the broad range of side effects and/or toxicity (Baldessarini, 1989; Frazer, 1997). In addition, only 60% of patients are responsive to the treatment with the available antidepressants (Moller & Volz, 1996;

Gareri *et al.*, 2000). Thus, the search for new drugs for the control of the symptoms associated with depressive disorders is still desirable.

To the best of my knowledge, there is not a single beverage that gives antidepressant effect to those who drink it. For this reason, a health drink that has this additional value of antidepressant effect is desirable.

1.6 Toxicological study

Antinutritional and toxic components, such as oxalic acid, nitrate and erucic acid are present in many plants (Guil *et al.*, 1997). Several substances in plants express cytotoxic and genotoxic activities and show correlation with the incidence of tumors (Ames, 1983). Therefore, extensive toxicological studies *in vitro* and/or *in vivo* animal assays must be undertaken before the development of natural product for safety reason.

1.7 Objectives of study

The objectives of this study are as follows :

1. The major goal of this study is to enhance the stability of *Carissa carandas* anthocyanin in syrup by using selected copigments. My approach involves determining the effects of copigmentation on the stability of anthocyanin during storage and when exposed to light and high temperature through time.
2. Another purpose of this study is to investigate the level of antioxidative activity of *Carissa carandas* syrup and how it relates to the addition of copigments through 2 different antioxidant assays, namely linoleic acid peroxidation method and 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Their

antioxidative activity will be compared with the commercial antioxidant agent, butylated hydroxytoluene (BHT). The effect of sugar will be determined.

3. This study will also focus on the evaluation of *in vivo* antidepressant activity by samples of study in animal models of depression, the forced swimming and tail suspension test, in comparison with the effects of reference antidepressant, imipramine.
4. To take a closer look at the visible spectrum of *Carissa carandas* anthocyanin in syrup at the end of storage period and then, further with the characterization of any changes in colour and pigment composition of copigmented and non-copigmented syrup which may occur during the storage.
5. To determine the toxicological effect of copigmented *Carissa carandas* syrup using sheep erythrocytes in hemolytic assay and through brine shrimp lethality assay. The differences between both methods used to assay cytotoxicity will also be explored.

CHAPTER 2 LITERATURE REVIEW

2.1 Anthocyanins stability

2.1.1 Copigmentation effects on stability of anthocyanins

So far, anthocyanins have not been broadly used in foods and beverages, since they are not as stable as synthetic colourants. However, recent investigations (Mazza & Brouillard, 1990; Davies & Mazza, 1993; Boulton, 2001) have suggested that the molecular copigmentation of anthocyanins with other compounds is the main colour-stabilizing mechanism in plants. From a molecular point of view, copigmentation is a hydrophobic interaction between monomeric anthocyanins and other compounds known as copigments or copigmentation cofactors (Gutierrez *et al.*, 2005). Copigments are usually colourless substances which can form a coloured cluster with colourless forms of anthocyanins (Brouillard *et al.*, 1989; Baranac *et al.*, 1997a). Copigments can exert a strong stabilizing effect on the colour of anthocyanins (Brouillard & Dangles, 1994a). The copigmentation reaction in anthocyanins was first reported by Robinson & Robinson (1931), and subsequently studied in model solutions.

Darias-Martin *et al.* (2001) stated that anthocyanins are not naturally red, but show a red colour in acid solution, as in wine. At a typical red wine pH of 3.5, about 6% of free anthocyanins change into a red form. So, when certain substances, in this case, phenol, which is not a pigment are added, they may form a cluster (stack) with free colourless anthocyanins, increasing the colour of the wine. The copigmentation equilibrium could be written as:

Free anthocyanins + Copigmentation cofactors ↔ Copigmented anthocyanins

An increase in the concentration of cofactors leads to colour intensification, since it displaces colourless free anthocyanins in favour of coloured forms (Darias-Martin *et al.*, 2001). The anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium (flavylium) salts (Mazza & Brouillard, 1990). The structure of flavylium cation is shown in **Figure 2.1**.

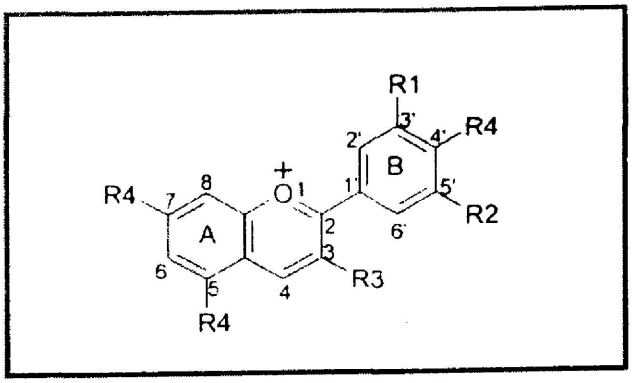


Figure 2.1 The flavylium cation. R1 and R2 are H, OH, or OCH_3; R3 and R4 are glycosyl or H (Kong *et al.*, 2003)

From Figure 2.1, it is clear that flavylium cation is electron-poor. Thus, this is where the copigments come in handy. Copigments have electron-rich π systems, which are able to associate with the comparatively electron-poor flavylium cation. This association provides protection from nucleophilic addition of water on the flavylium cation (Mazza & Brouillard, 1987). The attack by water converts the flavylium cation into colourless pseudobase, which consequently results in the loss of colour. The complexation of a copigment with an anthocyanin causes a hyperchromic effect (ΔA) and a bathochromic shift ($\Delta\lambda$) (Mazza & Miniati, 1993). The hyperchromic effect means an increase in colour intensity, while the bathochromic shift consists of a shift of the wavelength of maximum absorbance (Bakowska *et al.*, 2003). Moreover, Maccarone *et al.* (1987) reported that anthocyanin-copigment complexes are more resistant than free anthocyanins to chemical and photochemical degradations.

Copigments include a large variety of structurally unrelated compounds, such as flavonoid and non-flavonoid, phenols, amino acids and organic acids (Brouillard *et al.*, 1989). Various substances, such as quercetin 5'-sulphonic acid (Sweeny et al., 1981), chlorogenic acid (Brouillard *et al.*, 1991), rutin (Baranac *et al.*, 1996) and quercetin (Baranac *et al.*, 1997) have been identified as good copigments. Eiro & Heinonen (2001) studied the copigmentation reactions of pelargonidin 3-glucoside and cyanidin 3-glucoside, which are the main anthocyanins of strawberry juice. The copigments used were series of hydrocinnamic acids i.e. gallic acid, ferulic acid, caffeic acid, chlorogenic acid and rosmarinic acid. The authors found that the best copigment which showed the highest increase of λ_{max} were ferulic acid and rosmarinic acid for both anthocyanins. Meanwhile, Maccarone *et al.* (1985) studied the stabilization of anthocyanins in blood orange juice, *Citrus sinensis* L. and discovered that the complexation of anthocyanins with rutin and caffeic acid provided the stability effect to the anthocyanins in the juice. Using wine as a model, Darias-Martin *et al.* (2001) demonstrated that pre-fermentation addition of simple phenolics as copigment, for example catechin, could substantially improve wine colour and stability. Isoflavonoid extracts from red clover, *Trifolium pratense* leaves were found to enhance overall colour and stability of anthocyanin 3,5-diglucosides present in muscadine grape, *Vitis rotundifolia* juice and wine through intermolecular copigmentation reactions. Predominant isoflavonoids present in red clover included formononetin, biochanin A, and prunetin were the major polyphenolics identified to influence anthocyanin colour and stability (Talcott *et al.*, 2005). A limited number of studies have reported on anthocyanin copigmentation in actual food systems due to its inherent phytochemical complexity, yet, extensive work is reported using model systems with a diversity of anthocyanins and copigments (Talcott *et al.*, 2005). The incorporation of copigments also serves to enhance other functional properties of food, such as to reduce oxidation and increasing health-promoting benefits in products (Talcott *et al.*, 2005).

2.1.2 Previous studies on *Carissa carandas* anthocyanins stability and characteristics

Iyer & Dubash (1993) have studied and characterized the anthocyanin in *Carissa carandas* fruits. Their findings lead to the identification of cyanidin 3-rhamnoglucoside based on the R_f values[1] of *Carissa carandas* anthocyanin in three solvent systems i.e. B.A.W. (butanol: acetic acid: water - 4: 1: 5), formic (formic acid: hydrochloric acid (HCl): water - 5: 2: 3) and forestal (acetic acid: HCl: water - 30: 3: 10). The R_f values in B.A.W., formic and forestal were found to be 3500, 4500 and 7400 respectively and the R_f values in B.A.W. and forestal are in close agreement with those of cyanidin 3-rhamnoglucoside, as reported by Harborne (1958). The UV-visible spectrum gives the maximum values of anthocyanidin in *Carissa carandas* as 529.3 and 527.1 for the anthocyanin component. These values agreed well with those reported as maximum absorption for cyanidin (Harborne, 1958). The authors also discovered that the anthocyanin gave a bathochromic shift upon addition of 5% ethanolic aluminium chloride, thereby indicating the presence of organophenolic groups in their aglycone. The sugar moieties were identified by comparison with standard sugar and were found to be glucose and rhamnose. The structure of cyanidin 3-rhamnoglucoside as proposed by the authors is shown in **Figure 2.2**.

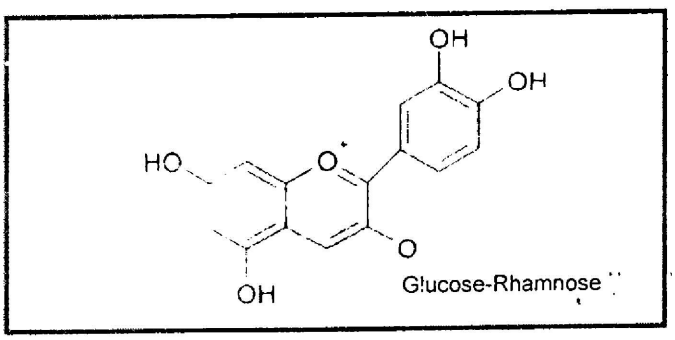


Figure 2.2 Cyanidin 3-rhamnoglucoside (Iyer & Dubash, 1993)

[1] **R_f value** is the distance a compound moves in chromatography relative to the solvent front. It is obtained by measuring the distance from the origin to the centre of the spot produced by the compound, the value then divided by the distance between the origin and the solvent front i.e. the distance the solvent travels.

Loh (2003) had separated the anthocyanin from the fruit juices of *Carissa carandas* at five stages of fruit development i.e. the unripe, half-ripe, ripe, rotten and artificially-ripe fruits by the high performance liquid chromatography (HPLC) at λ=520nm in a solvent system of methanol: acetic acid: water (71: 10: 19) and two dimensional paper chromatography in B.A.W. Based on the chromatographic data analysis, the author had identified the anthocyanins of *Carissa carandas* as cyanidin 3-glucoside, cyanidin 3-rhamnoside and pelargonidin 3-glucoside. Both cyanidin 3-glucoside and cyanidin 3-rhamnoside are present at the highest percentage in the rotten stage while the pelargonidin 3-glucoside content is increasing with fruits maturity.

The stability of *Carissa carandas* anthocyanin concentrate was studied by Iyer & Dubash (1993) in two model systems. One model system was designed to study the stability of anthocyanin in 12°Brix, sugar-citrate solution of pH 3.5, to which 10% of the anthocyanin colourant concentrate was added. This solution was stored at two different temperatures, 5°C and 30°C for a period of 20 days. The anthocyanin content was determined every alternate day. In the other model, yoghurt was prepared by acidifying milk with 2% lactic acid, to which 1%, 1.5% and 2% of anthocyanin colourant concentrates were added. The mixtures were stored at 5°C for seven days and the anthocyanin content was measured. The results of work done by Iyer & Dubash (1993) are presented in Tables 2.1, 2.2 and 2.3.

Table 2.1 Stability characteristics of *Carissa carandas* anthocyanins and ascorbic acid relative to processing treatments (Iyer & Dubash, 1993)

Carissa carandas material	Total anthocyanin (mg/100g fruits)	Ascorbic acid (mg/100ml fruit juice)
Fresh	21.55	105.00
Process 'A'*	15.90	54.95
Process 'B'^	17.38	65.94

* Bottled in 40°Brix sugar solution and autoclaved at 3lbs for 12 minutes

^ Bottled in 40°Brix sugar solution and immersed in boiling water for 15 minutes

Table 2.1 shows the stability characteristics of the anthocyanin and ascorbic acid present in *Carissa carandas* relative to the different processing treatments. The processing treatments resulted in loss of anthocyanins and ascorbic acids. Several factors, either individually or in combination, might have played an important role in this destruction. For instance, heat and heating time are the major factors responsible for anthocyanin destruction (Adams & Ongley, 1972). It was observed that, when a product was exposed to a high temperature in a short time as a part of processing treatment, it had more anthocyanin retention as compared to that in the products kept at a lower temperature for a longer period of time. Iyer & Dubash (1993) revealed that *Carissa carandas* anthocyanins are relatively stable to the processing treatments applied, as the degradation was in the range of 19% to 26% which was much lower than that of 63% to 78% in case of plum anthocyanin under the same treatment.

Table 2.2 Stability characteristics of *Carissa carandas* anthocyanins colourant concentrate in sugar-citrate solution at two different storage temperatures (Iyer & Dubash, 1993)

Days	Anthocyanin content (mg/100ml)	
	5°C	30°C
0	0.51	0.51
2	0.50	0.44
4	0.50	0.43
6	0.48	0.40
8	0.47	0.39
10	0.46	0.38
12	0.45	0.38
14	0.45	0.37
16	0.44	0.36
18	0.44	0.35
20	0.43	0.34

Table 2.3 Comparison of stability characteristics of *Carissa carandas* anthocyanins at different percentage of colourant concentrate in yoghurt during storage (Iyer & Dubash, 1993)

Colourant concentrate (%)	Anthocyanin content (mg/100ml) on days			
	0	2	4	7
1.00	0.46	0.44	0.43	0.40
1.50	0.92	0.87	0.84	0.79
2.00	1.29	1.21	1.18	1.11

The results obtained by Iyer & Dubash (1993) on the stability of *Carissa carandas* anthocyanins in both model systems as presented in **Table 2.2** and **Table 2.3** indicate a progressive loss in anthocyanin content, which accelerated when stored at a higher temperature which is 30°C. The increase in anthocyanin colourant concentration in yoghurt made no significant difference, as pigment retention at all concentrations was almost the same. The authors reported that no visible microbial growth could be seen at the end of the stipulated period, though no pasteurization treatment was applied. Based on their findings, the authors summed up that *Carissa carandas* anthocyanin could be used as a colourant in food products but required mild processing treatments, and could preferably be stored under low temperatures condition.

To the best of my knowledge, results reported by Iyer & Dubash (1993) was the only published material on *Carissa carandas* anthocyanin stability characteristics. Therefore, no comparison with other study can be made due to this scenario.

2.2 Antioxidant study

2.2.1 Lipid peroxidation

Lipid peroxidation has been the subject of extensive studies for several decades, and its mechanisms, dynamics and products are now fairly well established. It was first studied in relation to the oxidative deterioration of foods (Niki *et al.*, 2005). Dorman *et al.* (1995) and Farhoosh (2005) defined lipid peroxidation as an *in vitro*/*in vivo* chain

reaction that produces highly reactive toxic secondary products that cause damage to cell membranes and biological compounds leading to aging and degenerative diseases. Lipid peroxidation has been described to cause gradual changes in membrane structure, ultimately leading to the loss of membrane function and integrity (Halliwell & Gutteridge, 1985).

Lipids are oxidized by four distinct mechanisms; enzymatic oxidation, non-enzymatic, free radical-mediated oxidation and non-radical oxidation. Each oxidation mechanism yields specific products. The free radical-mediated peroxidation of lipids has received a great deal of attention in connection with oxidative stress[2] *in vivo*. Researchers have also focused their attention on lipid peroxidation by non-enzymatic and non-radical mechanisms. Singlet oxygen and ozone are examples of molecules that induce such oxidation. Both polyunsaturated fatty acids (PUFA) and cholesterol are oxidized by enzymatic and non-enzymatic pathways (Niki *et al.*, 2005).

Aside from lipids, DNA and protein are also major cellular components and are prone to oxidative attack. DNA damage is often measured as single or double-stranded breaks and chromosomal aberrations (Aruoma, 2003).

2.2.1.1 Inhibition of lipid peroxidation

As mentioned earlier, lipid peroxidation has been implicated in various disease and aging, including atherosclerosis, cataract, rheumatoid arthritis and neurodegenerative disorders. Consequently, the role of antioxidants has received extensive attention.

[2] Imbalance between generation of free radicals and antioxidant defences

Antioxidant defenses may be divided into four categories i.e. prevention of the formation of active oxidants, repair of damage, excretion of toxic oxidation products, and adaptive responses. Roberfroid & Calderon (1995) divided antioxidants into three categories, according to their functions i.e. preventive antioxidants, which reduce the formation of radicals and ROS, radical scavenging antioxidants which inhibit chain initiation and break chain propagation and antioxidants which involves in repair and de novo mechanisms. The inhibition of enzymatic lipid oxidation may be achieved by inhibition of either the activation or reaction of an enzyme. Free radical-mediated lipid peroxidation may be inhibited by the inhibition of chain initiation and chain propagation and/or acceleration of chain termination. Lipid peroxidation induced by singlet oxygen may be inhibited by the inhibition of its formation by, for example, quenching of ultraviolet light and the singlet oxygen itself (Niki *et al.*, 2005).

Numerous types of antioxidants with various activities were identified in our diets and the candidate of phenolic antioxidants in foods includes anthocyanins, flavonoids, chalcones, hydroxybenzoic and hydroxycinnamic acids (Hertog *et al.*, 1993). Most phenolic antioxidants (PPH) functions as terminators of free radicals and may also chelate metal ions that are capable of catalyzing lipid peroxidation. They interferes the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals (RO•) as indicated in the equation below:

$$RO\bullet + PPH \rightarrow PP\bullet$$

The phenoxy radical (PP•) intermediates are relatively stable and act as terminators of the propagation route by reacting with other free radicals (Ferguson, 2001).

$$RO\bullet + PP\bullet \rightarrow ROPP$$

The antioxidant activity *in vivo* is determined by several factors, such as reactivity towards radicals, fate of antioxidant-derived radicals, absorption, distribution, localization and mobility of antioxidant, and interaction with other antioxidants (Niki & Noguchi, 2000).

2.2.1.2 Biological role of lipid peroxidation products

It has been known that lipid peroxidation gives complex products including hydroperoxides, cleavage products such as aldehydes and polymeric materials, and that these products exert cytotoxic and genotoxic effects (Esterbauer, 1993). Niki *et al.* (2005) reported that lipid peroxidation products and modified proteins have been found in human atherosclerotic lesions, although their pathological significance, such as cause and consequences has not yet been fully elucidated. Meanwhile, the cholesterol oxidation products, commonly referred to as oxysterols were found to be involved in the regulation of gene expression, cholesterol metabolism and homeostasis (Nishimura *et al.*, 2005). Furthermore, recently, the role of lipid peroxidation products as signaling messengers has received a great deal of attention (Poli *et al.*, 2004). It has been found that cyclopentenone prostaglandins induce detoxification of enzymes (Kawamoto *et al.*, 2000), and exert a complex array of neurodegenerative and neuroprotective effects (Musiek *et al.*, 2005).

Cells exhibit a broad spectrum of responses to oxidative stress, depending on the stress type and level encountered. Oxidative stress exceeding the antioxidant capacity level may induce oxidative damage, but low-level stress may enhance the defense capacity. Such an adaptive response has been observed in several instances, particularly in low-dose irradiation (Mathers *et al.*, 2004).

2.2.2 Previous studies on the antioxidative activities of *Carissa carandas*

Siti Rafidah (2003) has investigated the antioxidative activities of various extracts from different organs of *Carissa carandas* including stems, leaves and fruits. The extraction was carried out using three different methods. The first method involved the use of three different polarity solvents (hexane, chloroform and 80% methanol) in soxhlet apparatus and this procedure was applied to all the three organs tested. The second method is by immersing the unripe and ripe fruits in the mixture solution of 80% methanol and 1% HCl, and the final method involved squeezing both the unripe and ripe fruits to yield the juices. The ferric thiocyanate (FTC), thiobarbituric acid (TBA) and 2-thiobarbituric acid direct value assays were employed to evaluate the antioxidative activities. The results obtained from the FTC and TBA assays showed that all the extracts provided positive effects and the antioxidative activity exceeding that of the commercial antioxidant, α-tocopherol. However, in 2-thiobarbituric acid direct value assay, only 80% methanol extract of all organs and chloroform extract of the unripe fruits exhibited the antioxidant properties. Chloroform and 80% methanol extracts of the unripe fruits showed the highest activity comparable with BHT, the synthetic antioxidant which is widely used in the food industry, and therefore, both of the extracts were considered as the best extracts.

Meanwhile, Wong (2004) had compared the antioxidative activities of the leaves and the ripe fruits of *Carissa carandas* with *Oxalis barrelieri* extracts, through FTC and TBA assays. From the results obtained, the author found that the chloroform extract of *Carissa carandas* ripe fruits showed the highest antioxidative activities and in fact, the activity exhibited by the extract is even better than the two positive controls consisting of BHT and α-tocopherol used in both assays.

Consequently, Khoo (2004) had attempted to fractionate the antioxidant bioactive components in *Carissa carandas* fruit extracts. Fruits from two different stages of maturity were tested. The unripe and half-ripe fruit extracts were fractionated with paper chromatography in B.A.W. solvent system. The methanol extract of the unripe fruit yields five fractions, while the chloroform extract of the same age fruit lacks one fraction from the methanol extract. The juice from the half-ripe fruit provided five fractions. Similar to the other two researchers mentioned earlier, Khoo (2004) also used the FTC and TBA assays to evaluate the antioxidative activities of the fractions obtained. Results showed that the first fraction from both chloroform and methanol extracts of the unripe fruits were the best fraction as antioxidant agent. These fractions have the R_f values of 4 and 8 respectively, in B.A.W. solvent system. The chloroform and methanol extracts were then fractionated by using five solvents with different polarity i.e. hexane, ethyl acetate, mixture of ethyl acetate and 15% acetic acid, chloroform with a few drops of ammonia, and butanol. The fractions obtained from each solvent were tested with the FTC and TBA assays. Both chloroform and methanol extracts that were fractionated by chloroform with a few drops of ammonia exhibited the best antioxidative activities comparable to BHT. To further the study, the author tested the most potential fractions for antioxidant agent with terpene test. The results revealed that the chloroform extract fractionated with chloroform contained both monoterpene and diterpene while the methanol extract which was also fractionated with chloroform contained only monoterpene.

Based on the literature studies, on the whole, I have deduced that the fruits of *Carissa carandas* have been proven to contain antioxidative properties. As such, it can be further exploited as a commercial antioxidant agent in the form of a health drink.

2.3 Animal models of behaviour for evaluation of antidepressant activity

Stressful events are considered as an important factor in the development of central nervous system disorders such as depression (Brown, 1993), and a single experience of a stressful event enhances the vulnerability to stress-related disorders (Koolhaas *et al.*, 1997). On the basis of clinical association of depressive episodes and stressful life events, many drug activities assess stress-precipitated behaviours. The two most widely used animal models for antidepressant screening are the forced swimming and tail suspension tests in mice (Sousa *et al.*, 2004). The forced swimming test and tail suspension test are non-escapable stressful situations, quite sensitive and relatively specific to all major classes of antidepressant drugs including tricyclic, serotonin-specific reuptake inhibitors, monoamine oxidase inhibitors and atypicals (Porsolt *et al.*, 1977; Steru *et al.*, 1985).

2.3.1 Forced swimming test (FST)

The FST, originally introduced by (Porsolt *et al.*, 1977b), has been extensively used for several decades to screen for new drugs with potential antidepressant activity (Healy *et al.*, 1999; Wong *et al.*, 2000). The paradigm consists of a pre-test session, in which a mice is placed into a cylinder filled with water for 15 minutes and a test session will take place after 24 hours later, in which the mice is replaced in the same tank for five minutes. The test is based on the observation that mice, following initial escape-oriented movements, develop an immobile floating posture in the water cylinder. When they are replaced in the testing apparatus 24 hours later, they resume this posture quickly. This posture was interpreted by Porsolt *et al.* (1977a) as reflecting the animal's state of despair that was elicited by the inescapable situation learned during the first session. The total amount of time that the animal demonstrates this behaviour is then measured (Gersner *et al.*, 2005).

2.3.2 Tail suspension test (TST)

The TST, a well-validated antidepressant screening test (Porsolt *et al.*, 1987; Steru *et al.*, 1987) paradigm hangs mice by its tail for six minutes. A typical response in this paradigm is struggling alternating with passive immobility. The duration of immobility is accumulated throughout the six minutes period. This duration of immobility has been the principal measure in the TST and as in FST. This immobility is interpreted as a measure of 'behavioural despair' (Liu *et al.*, 2003).

It is well demonstrated that drugs with antidepressant activity reduce the time during which the animals remain immobile (Porsolt *et al.*, 1977; Borsini & Meli, 1988).

2.4 Toxicological determinations

There are a variety of studies investigating the toxic and biochemical effects of food and beverage constituents on human cells and rodent organisms. However, although the necessity to evaluate the safety of plant extracts and natural products are mounting, there is currently a tendency to limit the use of laboratory animals in toxicological tests (Yajes, 1997). Because of this particular reason, and the brine shrimp (*Artemia salina* L.) is a crustacean whose larvae are sensitive to a variety of substances, the brine shrimp bioassay can be useful as a quick and simple test for predicting toxicity. Due to its commercial availability, *Artemia salina* is widely used in toxicological applications and research (Lagarto-Parra *et al.*, 2001). Some authors perform the toxicity tests with different cell lines and combine the cytotoxicities found to deduce conclusion (Schweikl & Schmalz, 1996). Recent investigations have focused on measuring specialized cell synthesis or damages to predict cytotoxicity (Schweikl *et al.*, 1998). For instance, in order to evaluate cytotoxicity and verify whether the observed cytotoxicty is related to membrane disruption, plant extracts or natural

CHAPTER 3

MATERIALS

AND METHODS

CHAPTER 3 MATERIALS AND METHODS

3.1 Preparation of *Carissa carandas* concentrated syrup and addition of copigments

Materials for preparation of syrup (56°Brix) are as follows:

1kg of *Carissa carandas* ripe fruits
(collected from Cangkat Minden, Pulau Pinang)
1kg of sugar
1L of distilled water

Carissa carandas ripe fruits were washed, deseeded and then blended with distilled water. The pulps were removed with muslin cloth. Sugar was then added to the juice (pH 2.8) obtained and heated until the sugar was totally dissolved. Syrup prepared (pH 3.16) was rapidly cooled in an ice bath.

Ten different copigments as listed bellow were selected in this study based on previous experiments on colour stabilization of anthocyanins done by other researchers.

1. quercetin
2. naringin
3. rutin
4. (+)-catechin
5. L-tartaric acid
6. caffeic acid
7. aluminium chloride
8. iron (II) sulphate
9. iron (II) chloride
10. gum arabic

All the copigments listed were purchased from Sigma-Aldrich Co., USA except for gum arabic which was kindly provided by a friend from Universiti Putra Malaysia. Copigments were added to the syrup at a concentration of 0.2mg/ml, followed by

microwave (Sanyo Super Showerwave, 900W) pasteurization for four minutes, T_{max}=92°C (Maccarone *et al.*, 1987). Bottles of syrups were quickly cooled, capped and ready to be used in stability studies.

3.2 Measurements of colour and stability

The following stability studies were done to determine the effects of copigmentation on the stability of anthocyanins in *Carissa carandas* syrup. All experiments were repeated in triplicate.

3.2.1 Storage study

This experiment was performed according to the method described by Maccarone *et al.* (1987) with a slight modification. Pasteurized samples of *Carissa carandas* syrups with and without copigments (20ml) in screw-capped bottles were stored at room temperature (23±1°C) in the dark for 90 days. At five days interval, the anthocyanins content was measured spectrophotometrically (Thermo Spectronic Genesys 20), by monitoring the absorbance of the samples at 535nm after dilution with distilled water.

3.2.2 Thermal stability

Thermal stability study was done according to the method reported by Cevallos-Casals & Cisneros-Zevallos (2004). Copigmented samples (10ml) were placed inside capped glass vials covered with aluminium foil sealed with parafilm and immersed in a water bath at 99±1°C for 0, 30, 60, 90 and 120 minutes. Then, the absorbance of samples was monitored at 535nm after dilution with distilled water. Non-copigmented syrup inside capped vials covered with aluminium foil sealed with parafilm and stored at room temperature was used as a negative control.

3.2.3 Light stability

This experiment was performed with copigmented syrups inside screw-capped vials sealed with parafilm and exposed to white fluorescent light for seven days. The absorbance of samples at 535nm was monitored each day. Non-copigmented syrup inside screw-capped vials sealed with parafilm and stored at room temperature in the dark was served as a negative control (Cevallos-Casals & Cisneros-Zevallos, 2004).

3.2.4 UV-Visible spectrophotometric analysis

Absorption spectrum of the copigmented samples which gave the best stabilization effects in storage study, compared to non-copigmented syrup were recorded in the visible wavelength ranging from 400nm to 600nm. These spectra were measured using Cary 50 Con-UV spectrophotometer.

3.2.5 Colorimetric measurements

Minolta spectrophotometer CM-3500d (Osaka, Japan) was employed for colour measurements. The colour measured was expressed by the CIE L^* a^* b^* colour space values using Spectramagic software version 2003 (Minolta). Copigmented syrups that showed the best stabilization effects underwent colour analysis. Samples' (56°Brix, pH 3.16) colour parameters (L^*, a^*, b^* and h) were measured in triplicate. Measurements were done every two weeks for 70 days for L^*, a^* and b^*, while the h value of samples were determined at the end of the storage period.

3.3 Antioxidative activities study

3.3.1 2,2'-di-phenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

Two sets of experiments were carried out for evaluation of radical scavenging activity by the DPPH assay. One set of experiment tested samples of copigmented and non-copigmented syrups of 56°Brix and another experiment tested the same samples after dilution to 9.8°Brix.

The procedure used is an adaptation of those previously described by Brand-Williams *et al.* (1995). Ethanolic DPPH (Sigma Chemical Co., USA) (300μM) was used in the reaction mixture. Test samples (50μl) were combined with 150μl DPPH solution in a 96 well micro-titer plate. Distilled water was used as a negative control. The reaction mixtures were incubated for 30 minutes at 37°C and the change in absorbance at 515nm was measured using microplate reader (V_{max} kinetic). The radical scavenging activity by the samples was calculated using the following formula:

Radical scavenging activity (%) = [100 - (AS/AC) x 100]

AS: absorbance of DPPH radical in the presence of sample

AC: absorbance of DPPH radical without sample

3.3.2 Ferric thiocyanate (FTC) assay

The antioxidative activity analysis using ferric thiocyanate was performed according to the method reported by Osawa & Namiki (1981) with a slight modification. Two sets of experiments as in the DPPH assay were tested. Samples (2ml) added to 2ml 99.5% ethanol (v/v) were mixed with linoleic acid (Sigma Chemical Co., USA) (2.51% v/v) in 99.5% (Systerm) (w/v) ethanol (4.1ml), 0.05M phosphate buffer pH 7.0 (consisting of

di-potassium hydrogen phosphate, R&M Chemicals and potassium *di*-hydrogen phosphate, Fisher Scientific, UK (8ml) and distilled water (3.9ml). The screw-capped vials were kept in the dark at 40°C. On the 10th day of incubation, 0.1ml of each sample solutions were added to 9.7ml of 75% (v/v) ethanol (drum) and 0.1ml of 30% (w/v) ammonium thiocyanate (Bendosen Laboratory Chemicals) and 0.1ml of 20mM ferrous chloride (Systerm) in 35% (v/v) hydrochloric acid (R&M Chemicals). Precisely three minutes after the addition of ferrous chloride to the reaction mixture, the absorbance of the resulting red colour was measured at 500nm.

3.3.3 Thiobarbituric acid (TBA) assay

The TBA assay was conducted according to the combined method of Kikuzaki & Nakatani (1993) and Ottolenghi (1959). On day 11 of incubation, 1ml of samples from the FTC assay were added to trichloroacetic acid (Fisher Scientific, UK) (2ml) and thiobarbituric acid (Sigma Chemical Co., USA) solution (2ml). This mixture was then placed in a boiling water bath at 100°C for ten minutes. After cooling, samples were centrifuged at 3000rpm for 20 minutes and absorbance of the supernatant was then measured at 532nm.

3.4 Evaluation of antidepressant activity

3.4.1 Animals

Experiments were carried out on male mice weighing 24g to 30g at the time of testing. All mice were housed at room temperature of 23±1°C in standard laboratory conditions, including free access to water and food. Each experimental group consisted of six randomly chosen mice. Mice were only used once during the testing period.

3.4.2 Substances and drug administration

The animals were divided into 15 groups (control and experimental) of six animals each. Two groups of mice received *Carissa carandas* ripe and unripe fruit juices. Ten samples of concentrated syrups with different copigments and syrup without copigment were diluted with distilled water to a concentration of 9.8°Brix, and were administered to 11 groups of mice. Tricyclic antidepressant drug, imipramine was also dissolved in distilled water, served as a positive control and was administered to another group of mice. The last group received distilled water only. All substances were administered intraperitoneally at a dosage of 0.01ml/kg (body weight) in male mice 60 minutes prior to testing.

3.4.3 Forced swimming test (FST)

The FST test was performed over a period of two days i.e. a day for the pre-swimming session and a day for the test session, following the procedure described by Porsolt *et al.* (1978). In the pre-swimming session, the mice were individually placed for 15 minutes in glass cylinders filled with fresh water (24±1°C) up to 15cm deep. Then, the mice were removed, dried with towel and returned to their cages. After 24 hours, the mice underwent the test session. In the test session, after 60 minutes of treatment, the mice were again placed in cylinders filled with water, and the duration of immobility was recorded for five minutes. Duration of immobility is defined as the absence of active, escape-oriented behaviours, such as swimming, jumping, rearing, sniffing or diving (Karolewicz & Paul, 2001). The mice were considered immobile when it remained floating in the water, without struggling, making only very slight movements necessary to keep its head above the water (Sousa *et al.*, 2004).

3.4.4 Tail suspension test (TST)

TST test was similar to that described by Steru *et al.* (1985). The mice were suspended on the edge of a shelf 58cm below the table top by adhesive tape, placed approximately 1cm from the tip of the tail. They were allowed to hang for six minutes, and the duration of immobility was recorded. The mice were considered immobile only when they hung passively and completely motionless. Test session was performed 60 minutes after the administration of substances and drug.

3.5 Toxicity tests

3.5.1 Brine shrimp

3.5.1.1 Hatching procedure

Brine shrimp eggs (*Artemia salina* L.) were hatched in artificial seawater prepared by dissolving 15g of sea salt (Fisheries Research Institute, Batu Maung) in 500ml of distilled water. After 24 hours of incubation at room temperature under light exposure, the larvae (nauplii) were collected with Pasteur pipette.

3.5.1.2 Brine shrimp lethality assay

This assay was performed according to Meyer *et al.* (1982) with slight modifications. One ml of ready-to-drink (9.8°Brix) copigmented and non-copigmented syrups, along with *Carissa carandas* ripe fruit juice were introduced in glass vials. Next, 100µl of suspension containing about ten nauplii was added into each vial and incubated for 24 hours. Each different sample was prepared in triplicate. The number of survivors after six hours of incubation were recorded for determining acute toxicity while for chronic case, the number of surviving nauplii at the end of the incubation period (24 hours) were counted. Nauplii were considered dead if they did not exhibit any internal or

external movement during ten seconds of observation (Barahona & Sanchez-Fortun, 1999).

3.5.2 Hemolytic assay

The method described by He *et al.* (1994) was also employed to evaluate cellular toxicity of copigmented syrups. A total volume of 0.8ml diluted copigmented and non-copigmented syrups (9.8°Brix) were placed in an eppendorf tube. A negative control tube containing phosphate buffered saline and a positive control tube containing distilled water were also included in the analysis. Fresh sheep erythrocytes were added to each tube, to give a final volume of 1ml. These solutions were then incubated at 37°C for 30 minutes, followed by centrifugation for five minutes. The degree of hemolysis was determined by measuring the optical density of the supernatant at 405nm.

3.6 Statistical analysis

Data were expressed as means ± S.E.M. Where applicable, One-way and Two-way analysis of variance, ANOVA followed by Fisher LSD for statistical evaluation of significant differences between sample groups using Sigmastat 3.11 demo version. A probability (P value) level of 0.001 was set as the threshold for significance.

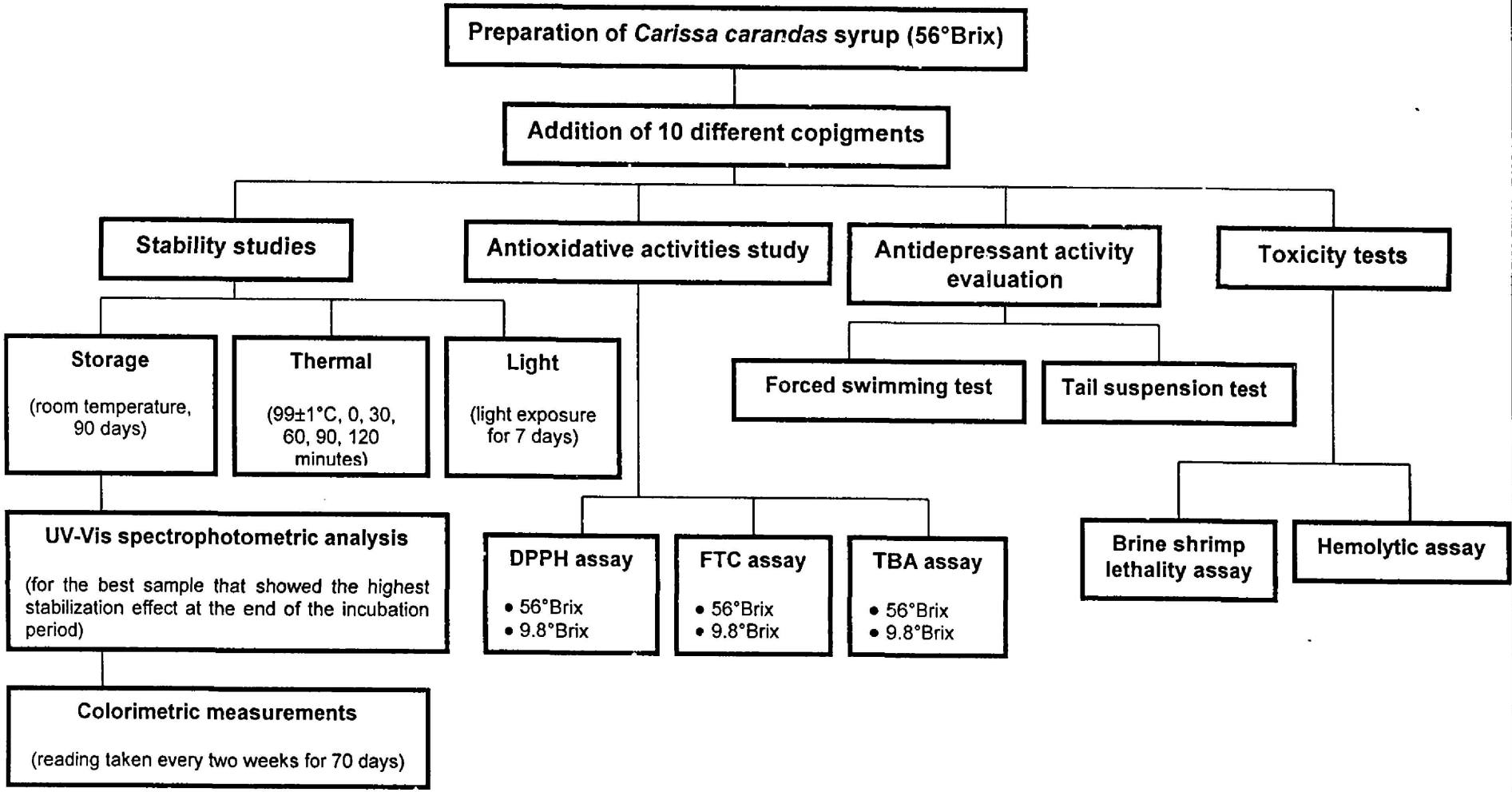


Figure 3.1 An overview of tests series done in this study on the whole

CHAPTER 4

RESULTS

CHAPTER 4 RESULTS

4.1 Effects of copigmentation on colour and stability of anthocyanins in *Carissa carandas* syrups

4.1.1 Storage study

Figure 4.1 illustrates the effect of copigmentation on stabilization of anthocyanins in *Carissa carandas* concentrated syrups during storage period. The absorbance values were measured at five days interval up to 90 days of storage. Degradation process took place in samples of study, resulting in the constant decrease of absorbance values through time. However, an increase of stability effect was observed in syrups with each of the copigments investigated compared to the non-copigmented syrup. The above statement is deduced based on the significant differences ($P<0.001$) between copigmented samples and non-copigmented syrup evaluated by Two-way ANOVA and Fisher LSD. The order of stabilization effect was indicated by superscript alphabets, as shown in the legend. Samples followed by greater superscript alphabets exhibited moderate to weak stabilization effect. Thus, based on statistical analysis, the stabilization effect decreased in the order of:

caffeic acid > rutin > iron (II) sulphate ≥ quercetin ≥ gum arabic > iron (II) chloride ≥ aluminium chloride > L-tartaric acid > (+)-catechin ≥ naringin

Caffeic acid showed a remarkable anthocyanins stabilization effect in syrup during storage, while naringin exhibited the least. The differences of absorbance values within days of incubation were also taken into account. In Figure 4.1, on the x axis, days 30 and 35 were followed by the same superscript alphabet, meaning that there were no significant difference ($P<0.001$) between the absorbance values measured on both days for all samples tested.

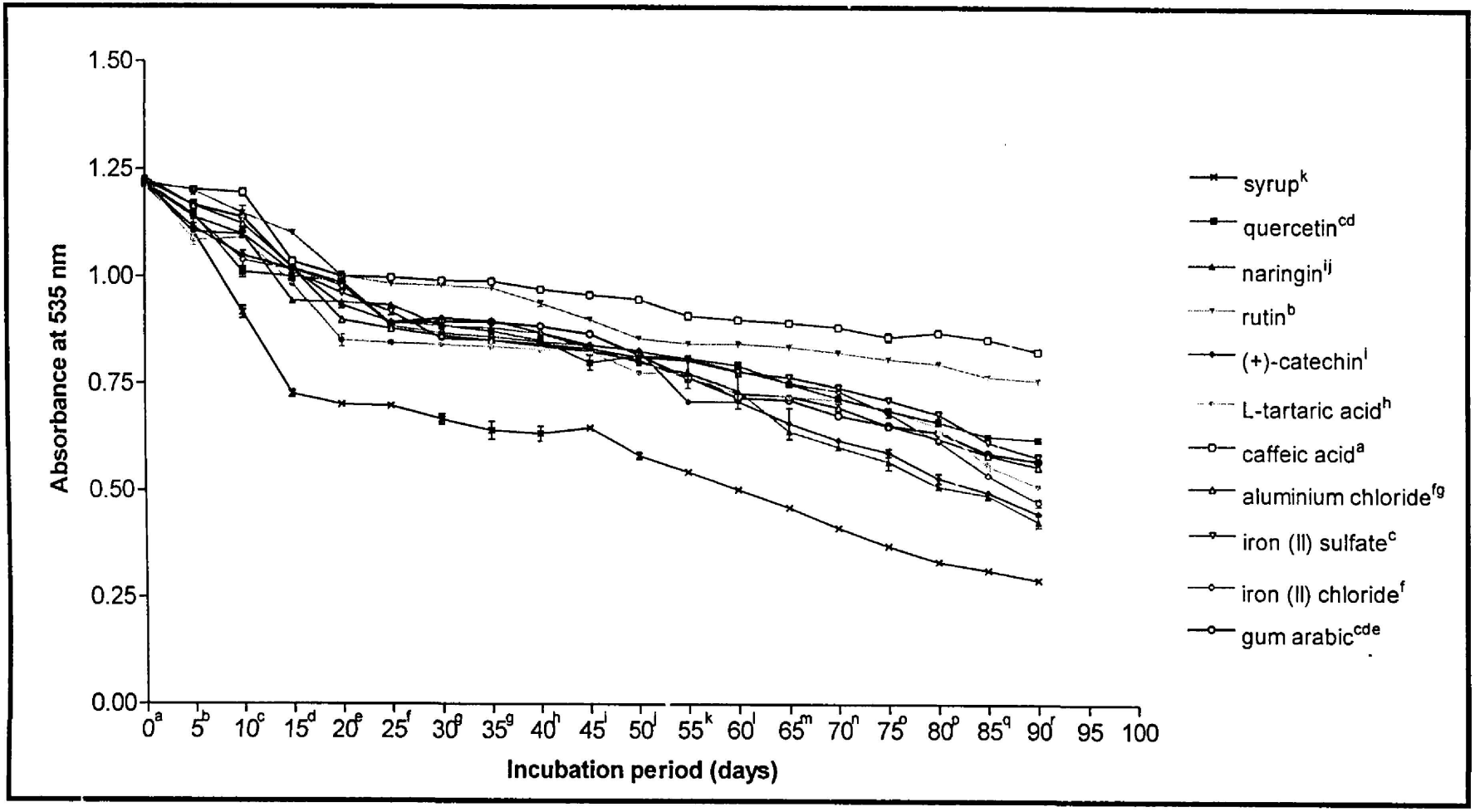


Figure 4.1 Degradation of anthocyanins in *Carissa carandas* syrup, with or without copigment during storage. Samples of study were stored at room temperature, protected from light. Absorbance values represents the mean ± S.E.M. (n=3). Syrup and copigments with the same alphabet (a, b, c) as shown in the legend and days with the same alphabet (a, b, c) are not significantly different ($P<0.001$). Statistical differences were determined by using Two-way ANOVA followed by Fisher LSD ($\alpha=0.05$).

4.1.2 Thermal stability

The effect of exposing samples of study to 99±1°C for 120 minutes was studied according to Cevallos-Casals & Cisneros-Zevallos (2004). Absorbance values were measured at determined time intervals. As expected, higher temperature and longer time of exposure promote higher anthocyanins degradations (**Figure 4.2**). Referring to statistical analysis done, all copigmented syrups showed significant difference ($P<0.001$) with non-copigmented syrup which act as a positive control, suggesting that copigments added did stabilize anthocyanins in *Carissa carandas* syrups. In other words, copigmented syrups degrade slower than the non-copigmented syrup when exposed to a high temperature. As can be seen in the legend, samples followed by greater superscript alphabets showed weaker stabilization effect. Thus, the stabilization effect decreased in the order of:

caffeic acid > rutin > aluminium chloride > iron (II) chloride ≥ gum arabic > (+)-catechin ≥ iron (II) sulphate ≥ L-tartaric acid > naringin > quercetin

The absorbance values measured at all time intervals are significantly different ($P<0.001$) for all samples tested.

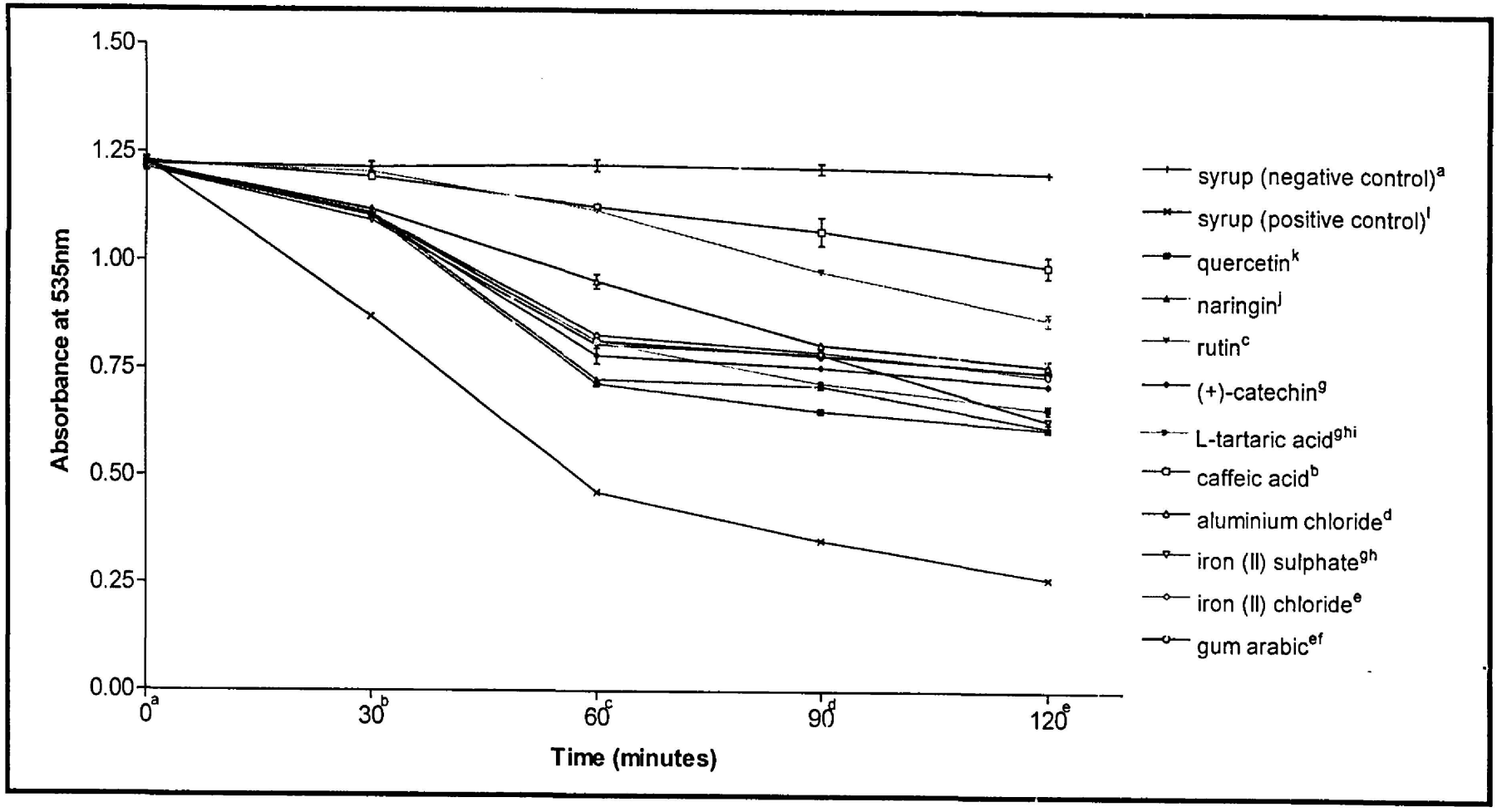


Figure 4.2 Degradation of anthocyanins in *Carissa carandas* syrup, with or without copigment when exposed to a high temperature. Samples of study were exposed to 99±1°C for 120 minutes. Absorbance values represents the mean ± S.E.M. (n=3). Syrup and copigments with the same alphabet (a, b, c) as shown in the legend and days with the same alphabet (a, b, c) are not significantly different ($P<0.001$). Statistical differences were determined by using Two-way ANOVA followed by Fisher LSD ($\alpha=0.05$).

4.1.3 Light stability

Another vital factor affecting anthocyanins degradation is exposure to light. From **Figure 4.3**, it is clear that anthocyanins content in all samples i.e. copigmented and non-copigmented samples degrades when exposed to white fluorescent light for seven days, but somehow, copigmented syrups degrade slower compared to syrup without copigment. Similar to previous stability tests done in this study, this phenomenon is definitely due to the anthocyanins stabilization by copigments. The legend in Figure 4.3 showed samples of study with superscript alphabets. The greater the superscript alphabet, the weaker the stabilization effect exhibited by samples. So, the descending order of anthocyanins stability by copigments can be written as:

caffeic acid > rutin > iron (II) chloride ≥ gum arabic > iron (II) sulphate ≥ quercetin ≥ aluminium chloride ≥ naringin > L-tartaric acid ≥ (+)-catechin

It is crystal clear that both caffeic acid and rutin constantly occupied the first and second highest rank in each order of stabilization effects. Consequently, both samples were chosen as subjects in UV-Visible spectrophotometric and colorimetric analysis.

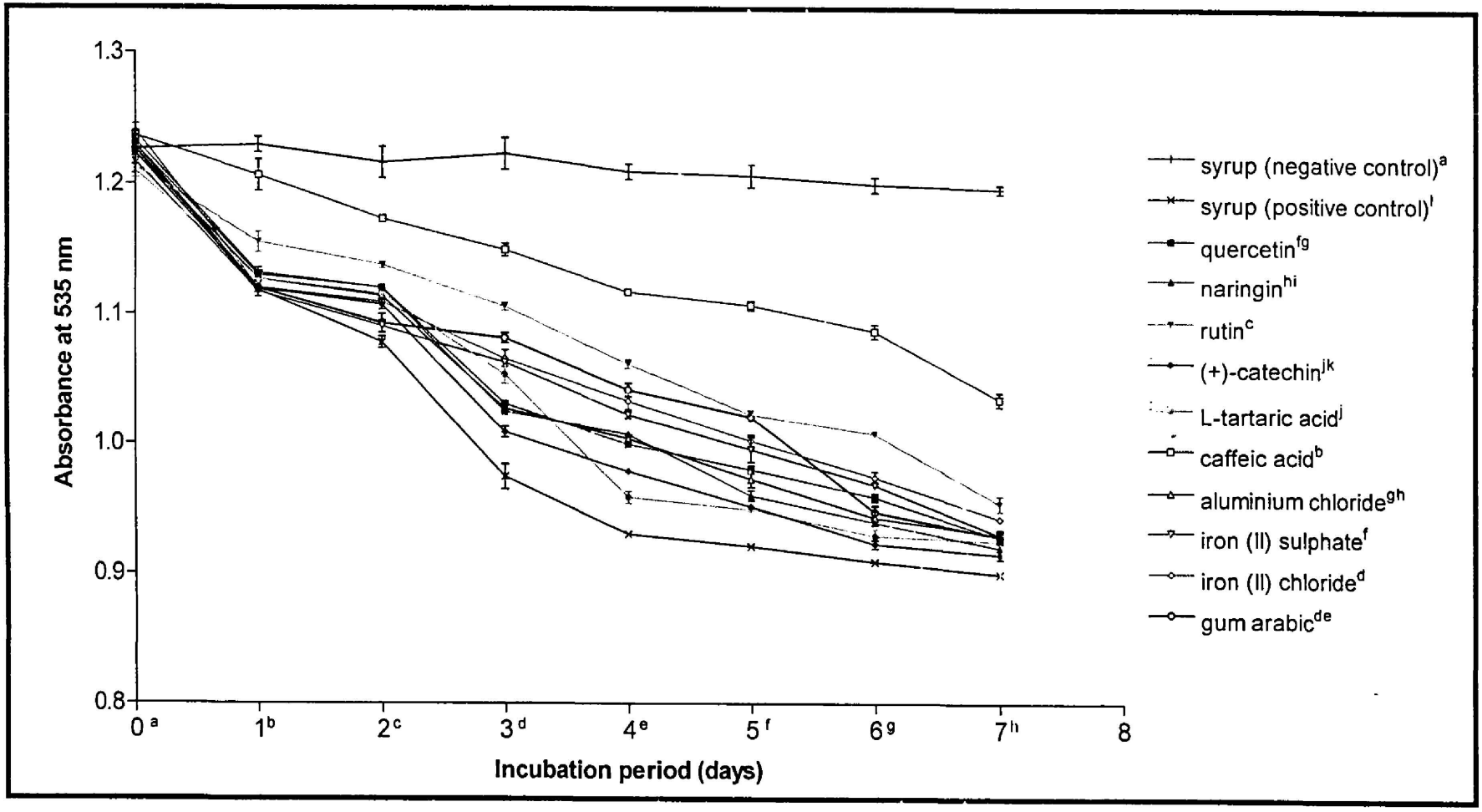


Figure 4.3 Effect of fluorescent light exposure on degradation of anthocyanins in *Carissa carandas* syrup, with or without copigment. Absorbance values represents the mean ± S.E.M (*n*=3). Syrup and copigments with the same letter (a, b, c) as shown in the legend and days with the same letter (a, b, c) are not significantly different (P<0.001). Statistical differences were determined by using Two Way ANOVA followed by Fisher LSD (α=0.05).

4.1.4 Spectral analysis

Colour stability was described on the basis of absorbance changes measured at anthocyanins' λ_{max} (Fossen *et al.*, 1998), thus, a comparison between non-copigmented, caffeic acid copigmented and rutin copigmented syrups' anthocyanins stability after 90 days of storage at room temperature is provided in **Figure 4.4**. The measurements were performed at wavelength ranges between 400nm and 600nm. Figure 4.4 marked the maximum absorption wavelength as 519nm, and this value is in close agreement with the maximum absorption of pelargonidin as reported by Harborne (1998). As mentioned in the earlier chapter, Loh (2003) had discovered pelargonidin 3-glucoside in *Carissa carandas* fruits and its content increased proportionately with fruit maturity. Based on the visible absorption spectrum of pelargonidin 3-glucoside, it was verified that both caffeic acid and rutin promoted a slight increase in the maximum absorption wavelength (bathochromic effect, $\Delta\lambda$) and absorbance (hyperchromic effect, ΔA), with caffeic acid exhibited a greater effect compared to rutin. These shifts simultaneously characterized an intermolecular copigmentation reaction (Asen *et al.*, 1972; Mazza & Brouillard, 1990; Davies & Mazza, 1993) occured in syrups.

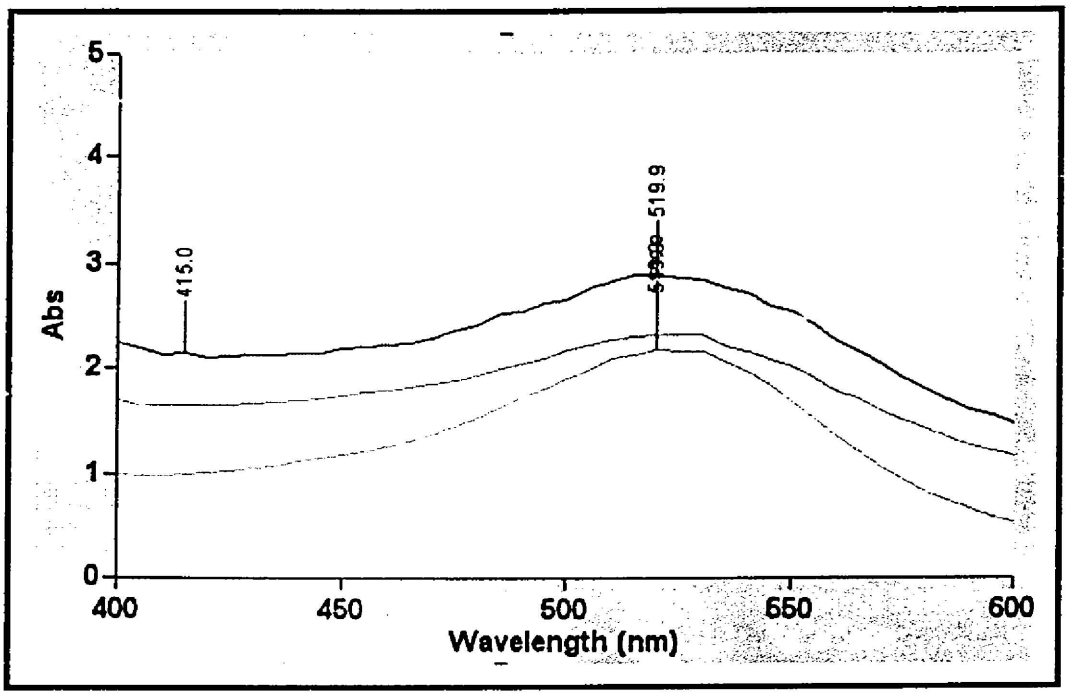


Figure 4.4 Visible maximum absorption spectrum of pelargonidin 3-glucoside in *Carissa carandas* syrups after 90 days of storage at room temperature.

4.1.5 Visual colour changes

A colorimeter (Minolta Spectrophotometer CM-3500d) was employed for colour measurement. The colour measured was expressed by the CIE (1976) L^* a^* b^* (CIELAB) colour space values (Judd & Wyszecki, 1975; Bilmeyer & Saltzmann, 1981; Hunter & Harold, 1987). Each colour in the CIELAB colour space has a unique location defined by its Cartesian coordinates with respect to the axis L^* a^* and b^* where L^* is the degree of lightness, a^* is the degree of redness and greenness and b^* is the degree of yellowness and blueness.

The results of colour changes occurred in samples of study during 70 days of storage are presented in Tables 4.1a, 4.1b and 4.1c. Data are expressed as means ± S.E.M. From the Two-way ANOVA analysis, all samples i.e. non-copigmented syrup, caffeic acid copigmented syrup and rutin copigmented syrup were significantly different ($P<0.001$) at all parameters (L^* a^* b^*) measured. Accordingly, measurements of colorimetric parameters on each interval also appeared to differ significantly ($P<0.001$). **Table 4.1a** is focusing on the changes of L^* parameter in syrups. Comparisons of values obtained were made within columns. For instance, on day one of incubation period, samples that provided the L^* values followed by greater superscript alphabets were darker. Thus, the greater the superscript alphabet, the darker the sample on the particular days measurements were recorded. From Table 4.1a, it can be noted that caffeic acid copigmented syrup was the darkest of the three samples at every interval. One of the possible factors contributing to this scenario is probably because caffeic acid retained the anthocyanins colour the most in syrup during storage. Caffeic acid was followed closely by rutin. Non-copigmented syrup's colour was the palest of all. Meanwhile, it can be seen that the L^* values increase over time and this is true to all samples investigated.

Table 4.1b marked the changes of a^* occuring in syrups during storage. Similar approach was applied to present the analyzed data. Values followed by the same superscript alphabet in the same column are not significantly different ($P<0.001$) statistically. The greater the superscript alphabet, the more syrup colour shifted towards red tonalities. In contrast with Table 4.1a, caffeic acid copigmented syrup exhibited the greatest values in each column. In other words, the colour of caffeic acid copigmented syrup was the most red at the end of the incubation period. The second greatest values were provided by rutin copigmented syrup and the least mean colour changes were showed by non-copigmented syrup. As in Table 4.1a, a^* values were constantly increased with time.

Additionally, changes of b^* parameter in samples of study is presented in **Table 4.1c**. Non-copigmented syrup's b^* values increased rapidly during storage and this condition is interpreted as the colour of syrup had moved towards yellower tonalities. The least changes in b^* values were illustrated by caffeic acid copigmented syrup.

Taken together, the increase in CIE L^* a^* b^* values were the characteristic changes in *Carissa carandas* syrups during storage. The a^*, b^* chromaticity diagram (**Figure 4.5**) is inserted for reference.

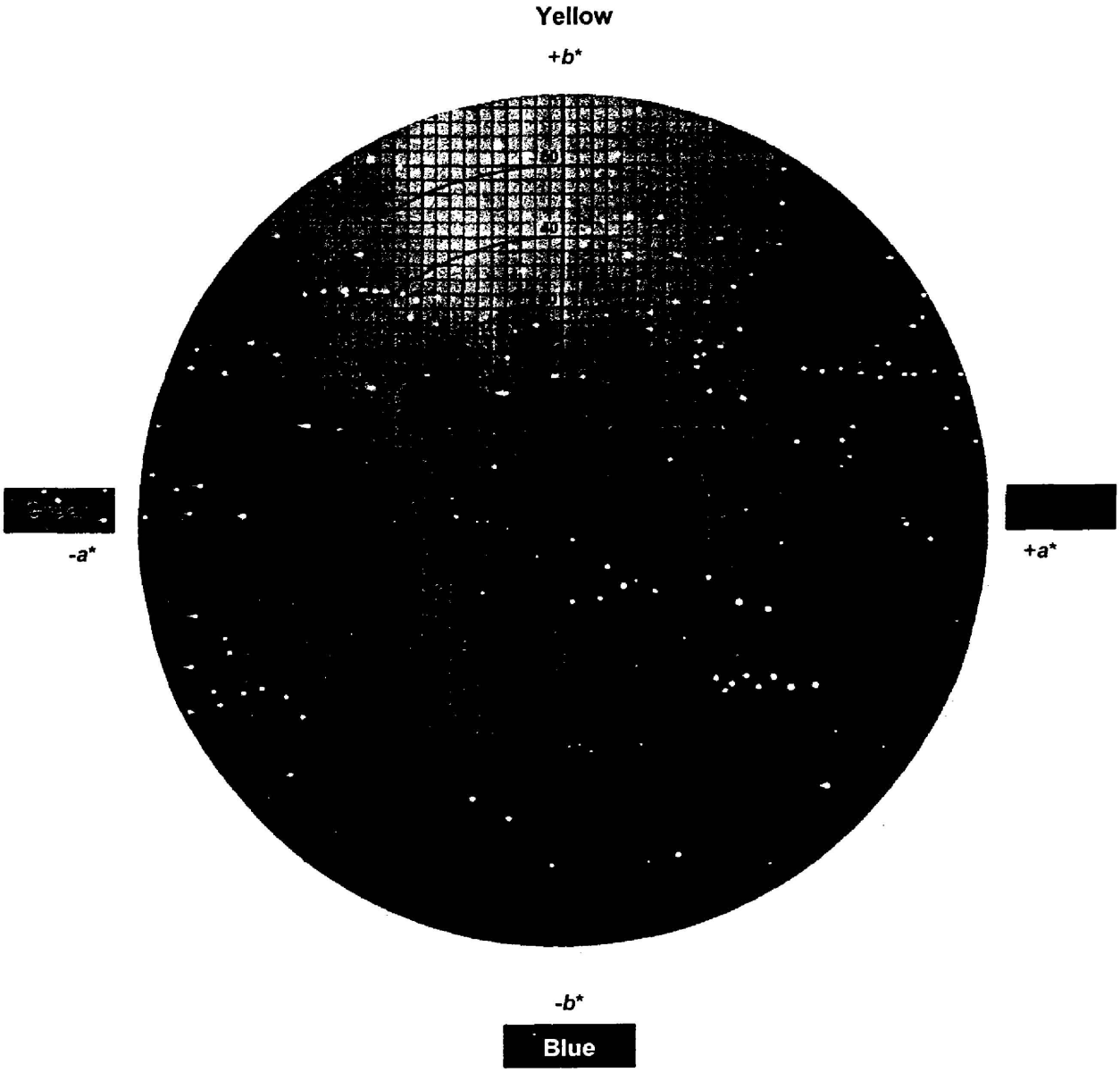


Figure 4.5 The *a**, *b** chromaticity diagram

Table 4.1a Changes in L^* (lightness) parameter measured in samples of study during storage period

Samples of study	Incubation period (days)					
	1[a]	14[b]	28[c]	42[d]	56[e]	70[f]
Syrup[a^]	49.73 ± 0.02[a]	50.04 ± 0.00[a]	68.54 ± 0.01[a]	70.69 ± 0.01[a]	71.24 ± 0.01[a]	71.72 ± 0.02[a]
Caffeic acid[b]	49.24 ± 0.01[c]	49.65 ± 0.01[c]	67.30 ± 0.01[c]	70.44 ± 0.01[c]	70.72 ± 0.01[c]	70.80 ± 0.01[c]
Rutin[c]	49.61 ± 0.00[b]	49.76 ± 0.01[b]	68.32 ± 0.01[b]	70.58 ± 0.00[b]	70.81 ± 0.01[b]	71.07 ± 0.01[b]

^ non-copigmented

Values represents the mean ± S.E.M. (n=3). Samples of study and days followed with the same alphabet (a, b, c) are not significantly different (P<0.001) statistically. The L^* values followed with the same alphabet (a, b, c) **in the same column** are not significantly different (P<0.001). Statistical differences were determined by using Two-way ANOVA and Fisher LSD (α=0.05).

Table 4.1b Changes in *a** (redness-greenness) parameter measured in samples of study during storage period

Syrup[a^]	23.71 ± 0.01[b]	24.00 ± 0.01[c]	27.14 ± 0.02[b]	27.08 ± 0.00[c]	27.31 ± 0.01[c]	27.54 ± 0.01[c]
Caffeic acid[b]	23.98 ± 0.01[a]	24.48 ± 0.01[a]	27.54 ± 0.00[a]	27.93 ± 0.01[a]	27.94 ± 0.01[a]	28.07 ± 0.01[a]
Rutin[c]	23.72 ± 0.01[b]	24.04 ± 0.00[b]	27.54 ± 0.00[a]	27.62 ± 0.00[b]	27.63 ± 0.01[b]	28.02 ± 0.01[b]

^ non-copigmented

Values represents the mean ± S.E.M. (*n*=3). Samples of study and days followed with the same alphabet (a, b, c) are not significantly different (P<0.001) statistically. The *a** values followed with the same alphabet (a, b, c) **in the same column** are not significantly different (P<0.001). Statistical differences were determined by using Two-way ANOVA and Fisher LSD (α=0.05).

Table 4.1c Changes in *b** (blueness-yellowness) parameter occurred in samples of study during storage period

Syrup[a^]	-4.01 ± 0.01[a]	-3.25 ± 0.01[a]	0.05 ± 0.02[a]	0.18 ± 0.01[a]	0.45 ± 0.01[a]	0.68 ± 0.00[a]
Caffeic acid[b]	-4.53 ± 0.00[c]	-4.26 ± 0.01[c]	-0.32 ± 0.01[c]	-0.17 ± 0.02[c]	-0.04 ± 0.02[c]	0.15 ± 0.01[c]
Rutin[c]	-4.31 ± 0.00[b]	-4.19 ± 0.01[b]	-0.07 ± 0.01[b]	-0.05 ± 0.00[b]	0.04 ± 0.01[b]	0.19 ± 0.01[b]

^ non-copigmented

Values represents the mean ± S.E.M. (n=3). Samples of study and days followed with the same alphabet (a, b, c) are not significantly different (P<0.001) statistically. The *b** values followed with the same alphabet (a, b, c) **in the same column** are not significantly different (P<0.001). Statistical differences were determined by using Two-way ANOVA and Fisher LSD (α=0.05).

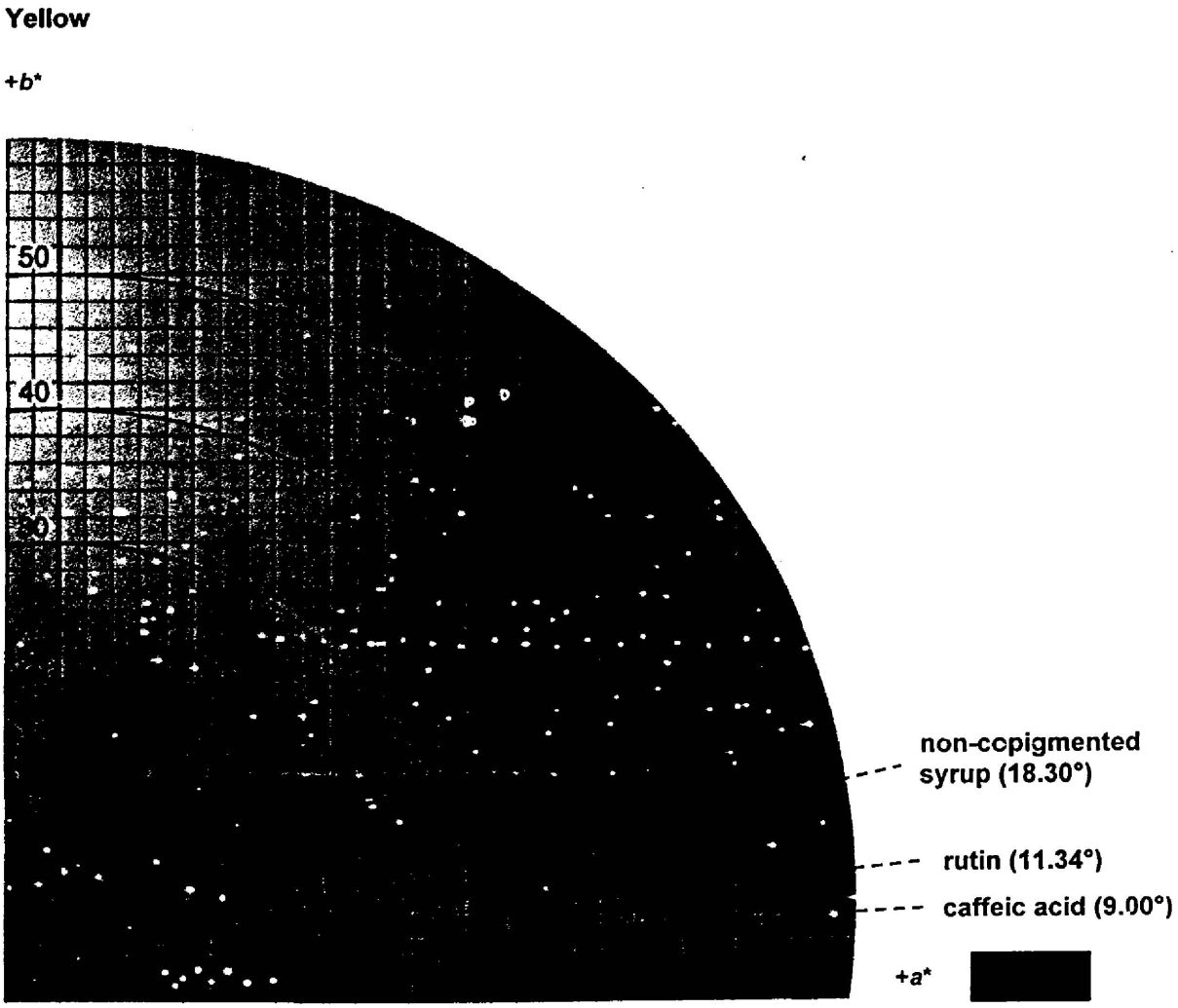


Figure 4.6 Portion of *a**, *b** chromaticity diagram. The values in degrees represent the *h* values of the non-copigmented syrup, rutin copigmented syrup and caffeic acid copigmented syrup at the end of 70 days storage period.

Subsequently, the *h* (hue) values of non-copigmented syrup, caffeic acid and rutin copigmented syrups were recorded at the end of the 70 days storage period and results obtained were illustrated in **Figure 4.6**. This figure is a portion of the *a**, *b** chromaticity diagram of Figure 4.5. Hue angle (*h*) is expressed in degrees and starts at the +*a**, 0° would be +*a** (red), 90° would be +*b** (yellow), 180° would be -*a** (green) and 270° would be -*b** (blue). As displayed in Figure 4.6, non-copigmented syrup provided the highest *h* value (18.30°), followed by rutin (11.34°) and the lowest value is provided by the caffeic acid copigmented syrup (9.00°). If we take a closer look at the figure, the *h* value of non-copigmented syrup was the closest to the +*b** axis if

compared to the other two. Consequently, the colour is more yellow while syrup with caffeic acid as copigment marked the *h* value which is the closest to the $+a^*$ axis and therefore, the colour is more red compared with the other two samples. In fact, it was the most red of them all. This is probably because the copigmentation reaction that took place between caffeic acid and anthocyanins in *Carissa carandas* syrup successfully stabilized the anthocyanins, and as a result, the degradation process was very much slower in comparison with syrup without copigment and rutin copigmented syrup during storage. All three samples showed significant difference ($P<0.001$) according to One-way ANOVA and Fisher LSD.

4.2 Antioxidative activities of *Carissa carandas* syrups

4.2.1 Free radical scavenging activity

DPPH radical is a stable radical with a maximum absorption at 515nm and can readily undergo reduction by an antioxidants. Because of the ease and convenience of this reaction, DPPH now has a widespread use in the free radical scavenging activity assessment (Brand-Williams *et al.*, 1995; Sanchez-Moreno *et al.*, 1998). The DPPH radical scavenging activity of syrup samples are summarized in Figures 4.7a and 4.7b. Two sets of experiments were carried out in order to evaluate the effect of sugar on the antioxidant capacity of samples. In Figures 4.7a and 4.7b, the bars labelled with greater alphabets illustrate lower percentage of radical scavenging activity.

Figure 4.7a showed that caffeic acid copigmented syrup is an excellent DPPH radical scavenger with 43.46% of radical scavenging activity, followed by (+)-catechin (35.70%), iron (II) sulphate (33.99%), aluminium chloride (32.22%), L-tartaric acid (28.08%) and rutin (26.62%). On the other hand, quercetin (19.12%), naringin (17.82%), iron (II) chloride (20.93%) and gum arabic (15.23%) copigmented syrups

showed no significant difference ($P<0.001$) with syrup without the addition of copigment (16.52%).

Meanwhile, **Figure 4.7b** presented the percentage of radical scavenging activity by 6x diluted syrups (9.8°Brix). Again, caffeic acid copigmented syrup proved to be the best radical scavenger (59.47%) among the other copigmented samples and was followed closely by (+)-catechin (54.28%). Diluted non-copigmented syrup's radical scavenging activity has shot up to 47.97%, tailed by quercetin (42.63%) and rutin (42.21%). Naringin, iron (II) sulphate and iron (II) chloride with 38.88%, 41.53% and 37.88% respectively were not significantly different ($P<0.001$) statistically. Thus, all three samples possessed radical scavenging power at the same strength. The least activity were shown by L-tartaric acid (35.50%), aluminium chloride (35.15%) and gum arabic (35.91%) copigmented syrups. It is interesting to note that all diluted syrups marked an increased in the percentage of radical scavenging activity compared to concentrated syrups (56°Brix).

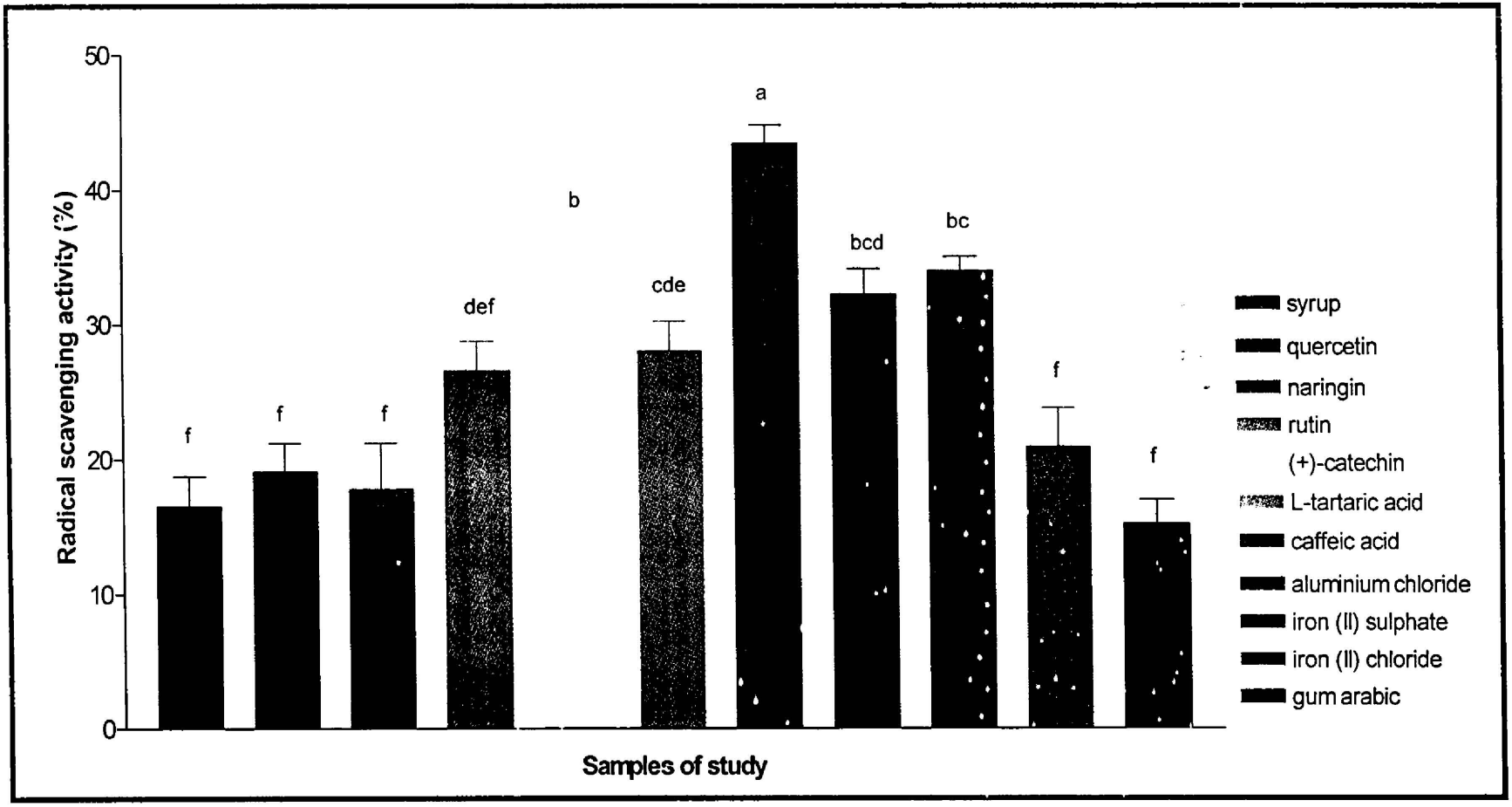


Figure 4.7a DPPH radical scavenging activity (%) of samples of study. Values with the same alphabet (a, b, c) are not significantly different ($P<0.001$) between samples. Statistical differences were determined by using One-way ANOVA followed by Fisher LSD ($\alpha=0.05$).

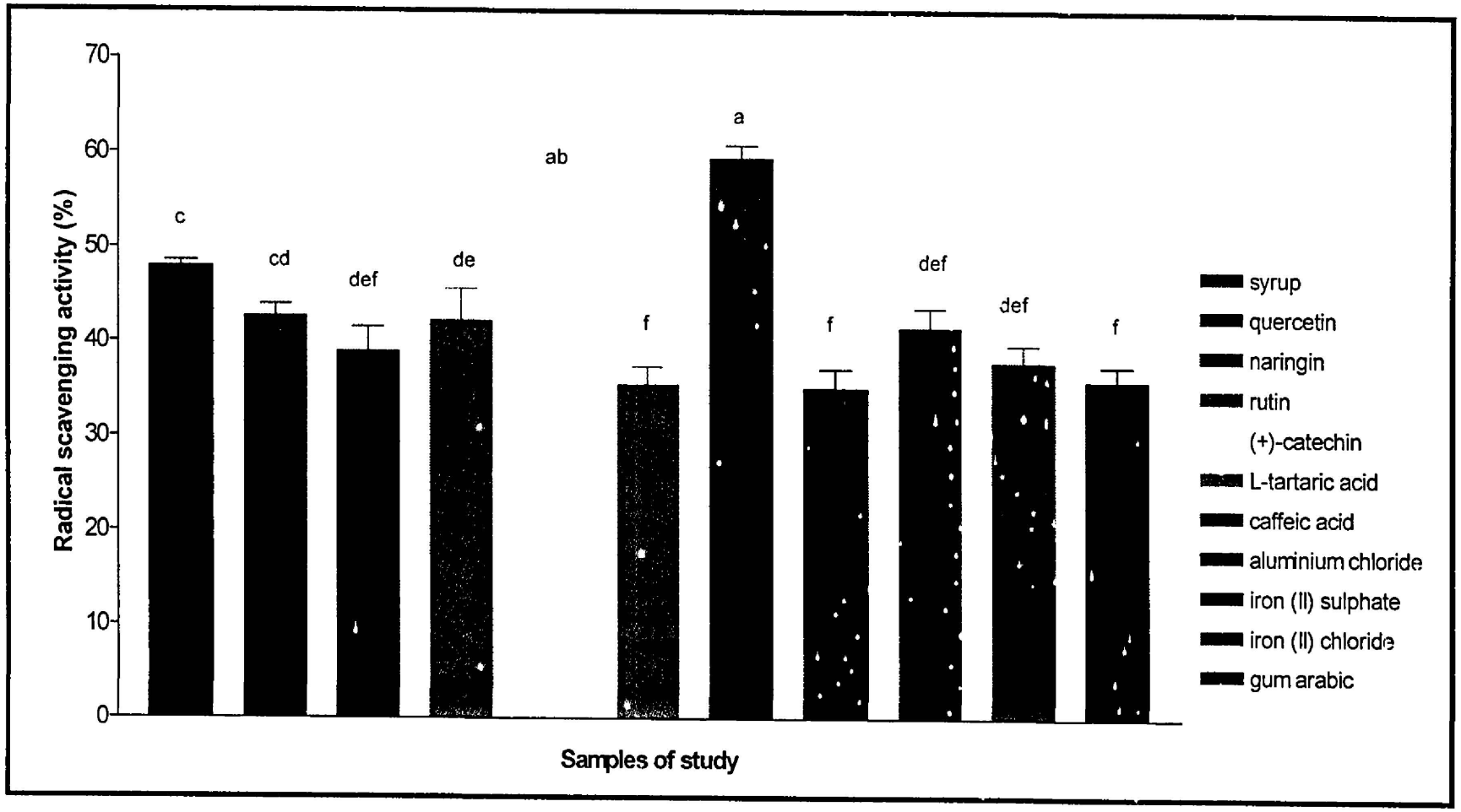


Figure 4.7b DPPH radical scavenging activity (%) of samples of study (6x dilution). Values with the same alphabet (a, b, c) are not significantly different ($P<0.001$) between samples. Statistical differences were determined by using One-way ANOVA followed by Fisher LSD ($\alpha=0.05$).

4.2.2 Inhibition of lipid peroxidation

4.2.2.1 Ferric thiocyanate (FTC) assay

The FTC assay was used to measure the peroxide level during the initial stage of lipid peroxidation. Low absorbance values would indicate high levels of antioxidative activity (Zin *et al.*, 2002). Similar to the previous DPPH radical scavenging assay, two sets of experiments were done to determine the influence of sugar on the antioxidative activity of samples. The results obtained from the FTC assay are presented in Figures 4.8a and 4.8b. In both figures, bars were labelled with alphabets based on the least significant differences evaluated between samples of study. However, it should be noted that, in Figures 4.8a and 4.8b, the greater the alphabet, the stronger the antioxidative activity displayed by samples.

As shown in **Figure 4.8a**, rutin copigmented syrup exhibited the best antioxidative activity in accordance to its lowest absorbance value measured in this assay. The rest of the samples' antioxidative capacity decreased in the order of:

gum arabic > caffeic acid ≥ (+)-catechin ≥ naringin ≥ quercetin > iron (II) chloride > BHT ≥ aluminium chloride ≥ L-tartaric acid ≥ iron (II) sulphate > syrup

BHT, a synthetic antioxidant exhibiting 100% inhibition against autoxidation of linoleic acid (Vimala *et al.*, 1999) was used as a reference sample. According to the above order, all copigmented syrups showed a comparable antioxidative activity with BHT. In fact, rutin, gum arabic, caffeic acid, (+)-catechin, naringin, quercetin and iron (II) chloride exhibited far greater activity compared with BHT.

Figure 4.8b summarized the antioxidative activity of 6x diluted syrups (9.8°Brix) measured by the FTC assay. From the figure, it is clear that all samples showed a decrease in absorbance values compared to concentrated syrups (56°Brix). Based on statistical analysis, the antioxidative activity of samples decreased in the order of:

caffeic acid > rutin > (+)-catechin > iron (II) chloride ≥ aluminium chloride > gum arabic > L-tartaric acid ≥ naringin ≥ iron (II) sulphate ≥ quercetin ≥ BHT > syrup

Surprisingly, all copigmented samples possess better antioxidative activity compared to the well known synthetic antioxidant, BHT.

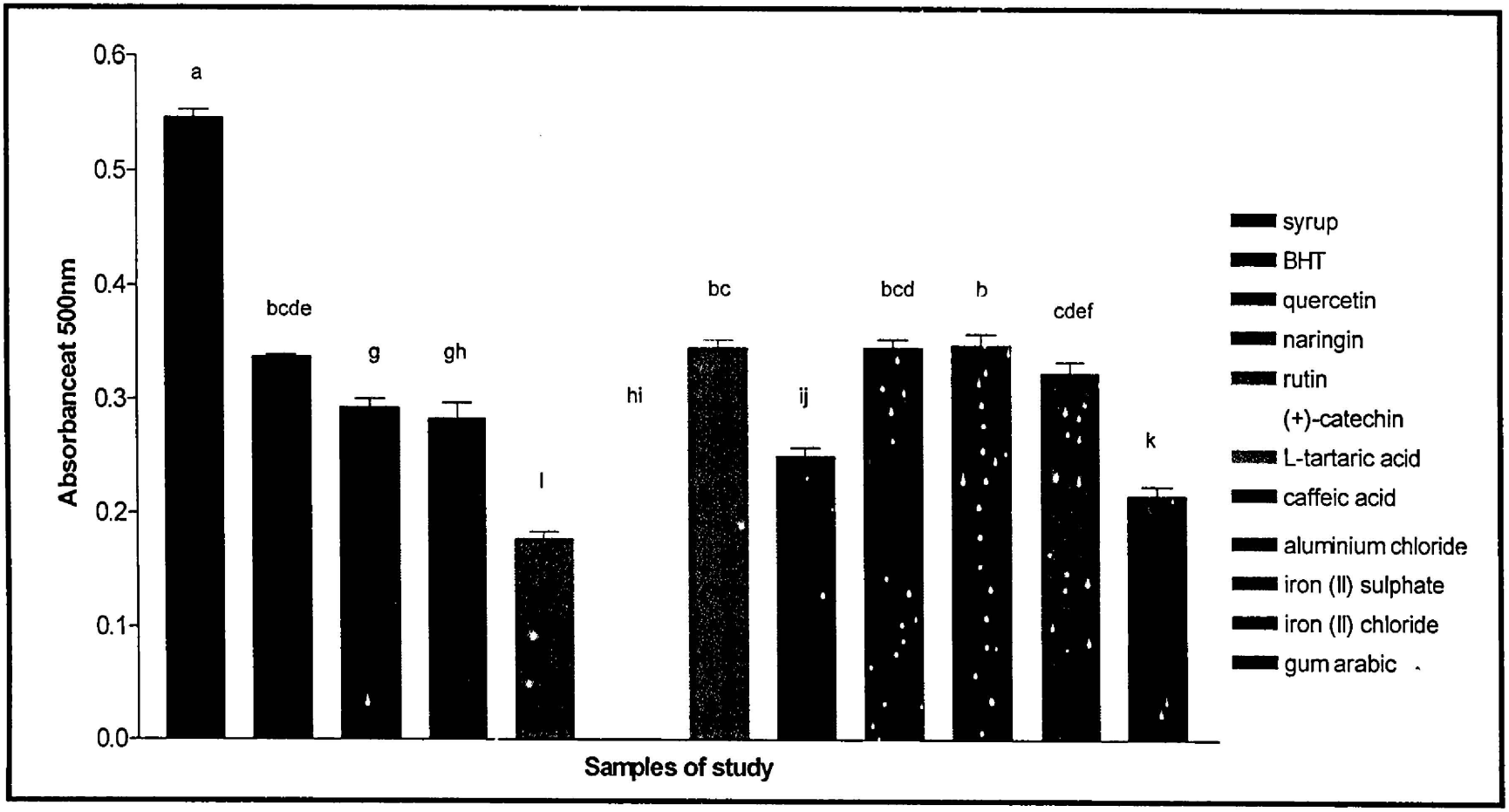


Figure 4.8a Antioxidative activity of samples of study as measured by the FTC assay on the 10th day of incubation period at 40°C in the dark. Absorbance values represents the mean ± S.E.M. (*n*=5). Values with the same alphabet (a, b, c) are not significantly different (P<0.001) between samples. Statistical differences were determined by using One-way ANOVA followed by Fisher LSD (α=0.05).

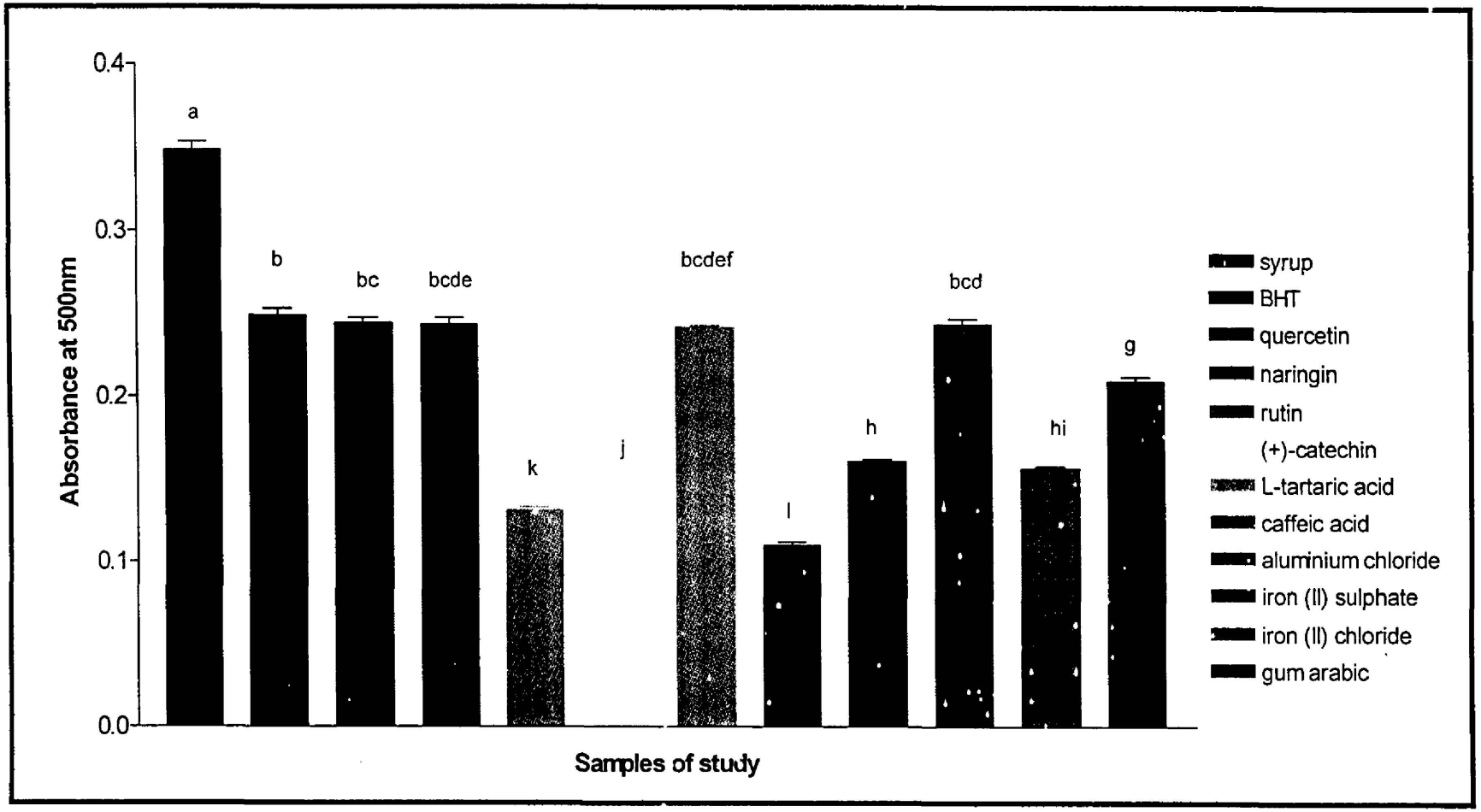


Figure 4.8b Antioxidative activity of samples of study (6x dilution) as measured by the FTC assay on the 10th day of incubation period at 40°C in the dark. Absorbance values represents the mean ± S.E.M. (n=5). Values with the same alphabet (a, b, c) are not significantly different (P<0.001) between samples. Statistical differences were determined by using One-way ANOVA followed by Fisher LSD.

4.2.2.2 Thiobarbituric acid (TBA) assay

Unlike the FTC assay, which is related to the peroxide formation in the initial stage of lipid peroxidation, the TBA assay measures the amount of malondialdehyde (MDA) produced after the decomposition of the lipid peroxide during the oxidation process (Lee *et al.*, 2004). Figures 4.9a and 4.9b summarized the antioxidative activity of samples of study measured by the TBA assay. The bars that are labelled with a greater alphabet indicate a stronger antioxidative capacity.

In **Figure 4.9a**, the descending order of antioxidative activity as measured in this assay can be written as:

rutin = (+)-catechin > gum arabic ≥ caffeic acid ≥ iron (II) sulphate ≥ quercetin ≥ naringin ≥ BHT ≥ aluminium chloride > L-tartaric acid ≥ iron (II) chloride > syrup

Both rutin and (+)-catechin are not significantly different ($P<0.001$) and are an excellent antioxidant agents. All copigmented syrups showed good antioxidative activity comparable with BHT.

Meanwhile, **Figure 4.9b** illustrated the antioxidative activity of 9.8°Brix syrups (6x dilution) as evaluated by the TBA assay. The antioxidative capacity decreased in the order of:

rutin = (+)-catechin = caffeic acid > iron (II) chloride ≥ aluminium chloride ≥ gum arabic > BHT ≥ naringin ≥ L-tartaric acid ≥ quercetin ≥ iron (II) sulphate > syrup

The results of the TBA assay agreed well and showed more or less the same pattern with the FTC assay. The decrease of absorbance can be noted in all samples tested. Corresponding to this scenario, sugar definitely affected the antioxidative activity of syrups, resulting in the decrease of activity observed in samples of study.

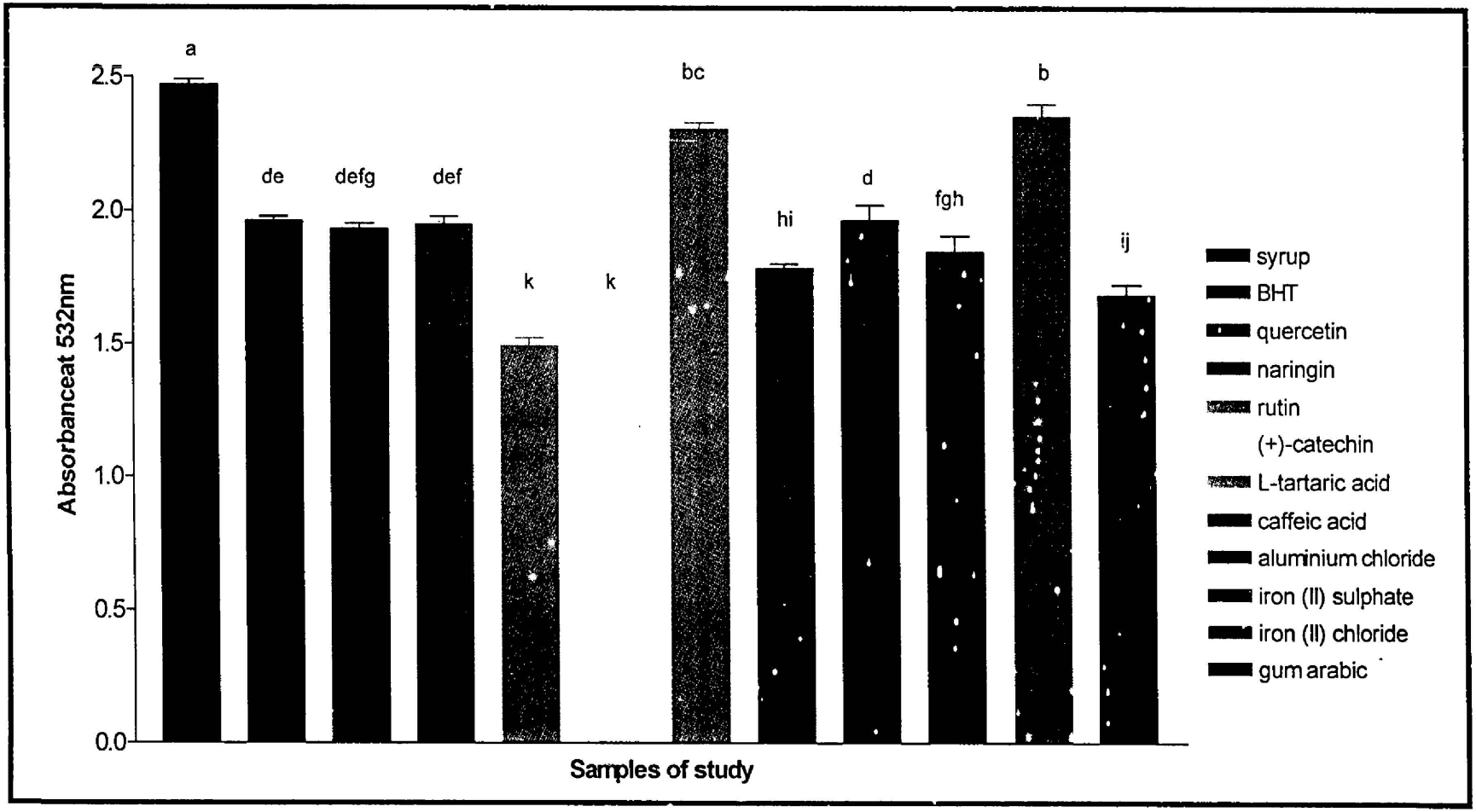


Figure 4.9a Antioxidative activity of samples of study as measured by the TBA assay on the final day of incubation period at 40°C in the dark. Absorbance values represents the mean ± S.E.M. (n=5). Values with the same alphabet (a, b, c) are not significantly different ($P<0.001$) between samples. Statistical differences were determined by using One-way ANOVA followed by Fisher LSD (α=0.05).

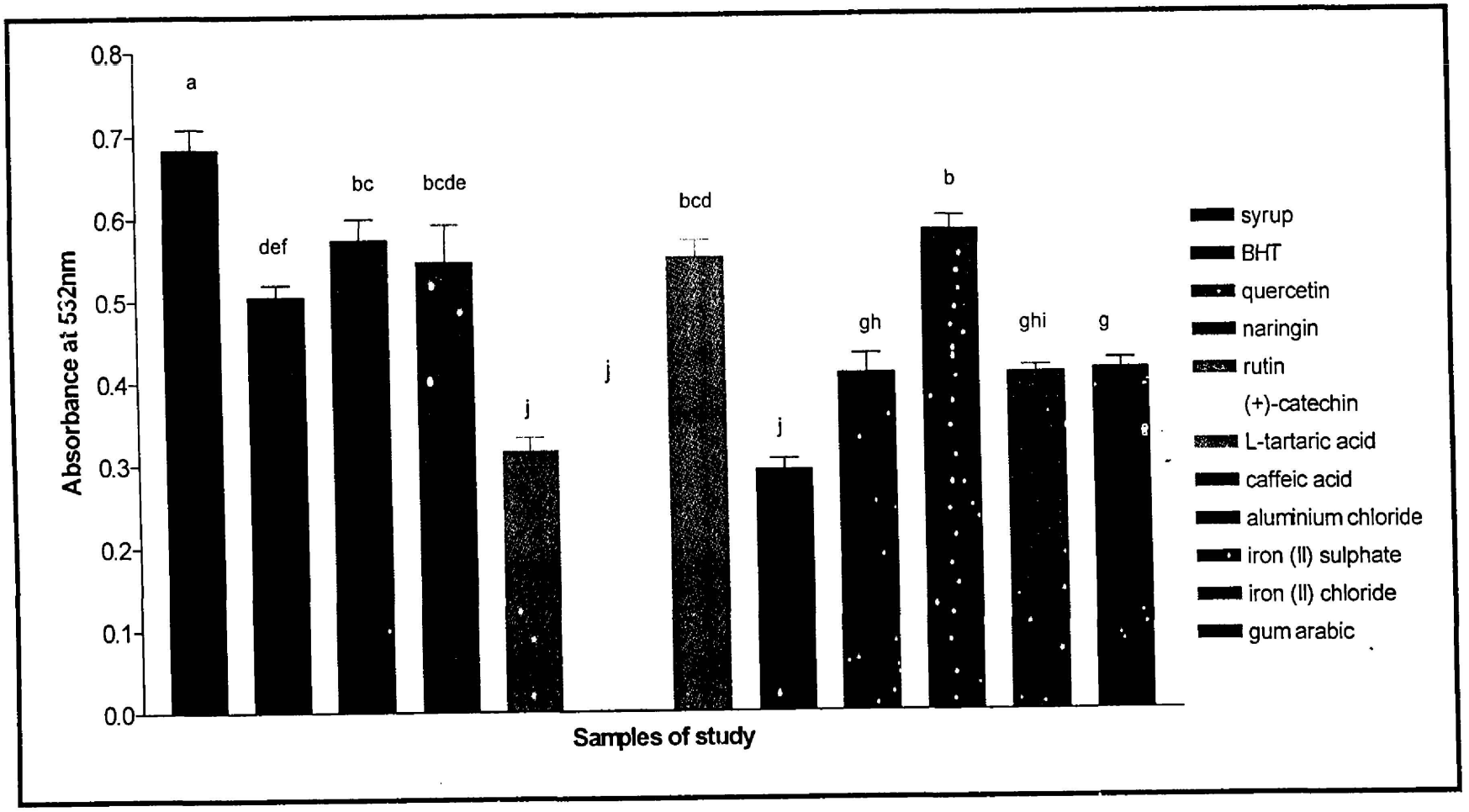


Figure 4.9b Antioxidative activity of samples of study (6x dilution) as measured by the TBA assay on the final day of incubation period at 40°C in the dark. Absorbance values represents the mean ± S.E.M. (n=5). Values with the same alphabet (a, b, c) are not significantly different ($P<0.001$) between samples. Statistical differences were determined by using One-way ANOVA followed by Fisher LSD ($\alpha=0.05$).

4.3 Antidepressant effect of *Carissa carandas* syrups and juices

4.3.1 The forced swimming test (FST)

Effects of intraperitoneal administration of the ripe and unripe fruit's juices, non-copigmented and copigmented syrups of *Carissa carandas* along with distilled water and imipramine that acts as a negative and positive control respectively are shown in **Figure 4.10**. The immobility time was recorded when mice remained floating in the water with the absence of active, escape-oriented behaviours, such as swimming, jumping, rearing, sniffing or diving (Yan *et al.*, 2004). **Plate 4.1** illustrated the escape-oriented behaviour and immobile posture captured in this study. The results depicted in this Figure 4.10 demonstrated that all test samples significantly ($P<0.001$) shortened the immobility time in comparison to the negative control group, revealing that all samples possess an antidepressant effect in this animal model of behaviour. Same as before, bars labelled with greater alphabet showed more intense antidepressant effect when tested in mice. So, the descending order of antidepressant effect that can be derived from the One-way ANOVA and the least significant differences between samples groups can be written as:

imipramine > unripe fruit's juice = caffeic acid ≥ ripe fruit's juice > rutin > (+)-catechin ≥ quercetin ≥ naringin > gum arabic ≥ syrup > L-tartaric acid > iron (II) chloride > iron (II) sulphate ≥ aluminium chloride

The tricyclic antidepressant agent, imipramine, caused the most reduction in the duration of immobility and this effect is classically recognized as the anti-immobility effect of the antidepressant (Guilhermano *et al.*, 2004). It can be observed that there was no significant difference between the unripe fruit's juice and caffeic acid copigmented syrup on the immobility time and it is interesting to note that the antidepressant effect showed by both samples were comparable with that of

imipramine, the reference antidepressant. In contrast, aluminium chloride copigmented syrup displayed the least reduction of immobility time and therefore illustrated the weakest antidepressant activity. Based on the above order, it seems like the addition of L-tartaric acid, iron (II) chloride, iron (II) sulphate and aluminium chloride did not improve the antidepressant activity possess by the *Carissa carandas* syrups.

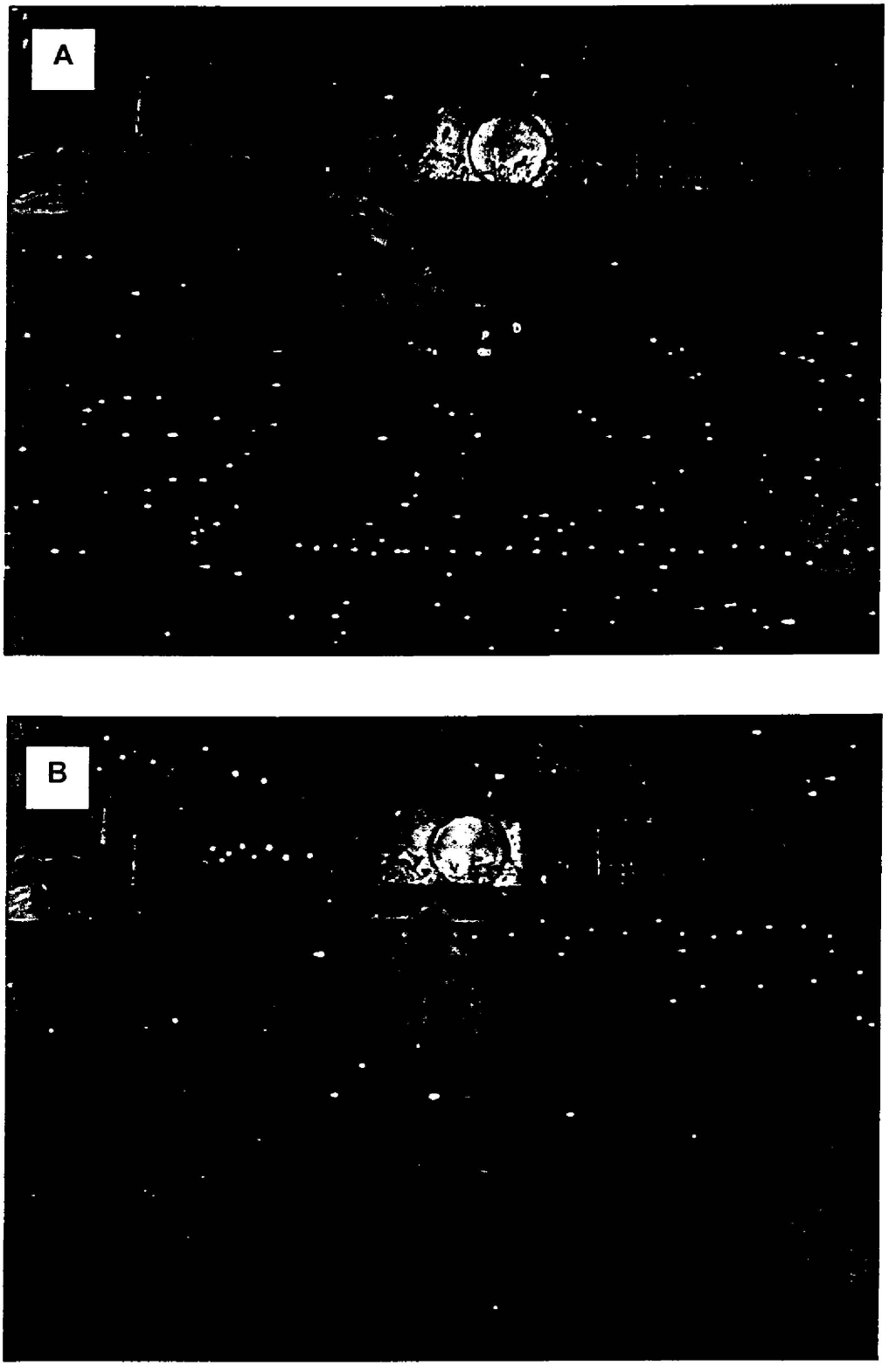


Plate 4.1 The escape-oriented (A) and immobile (B) postures captured in the FST test

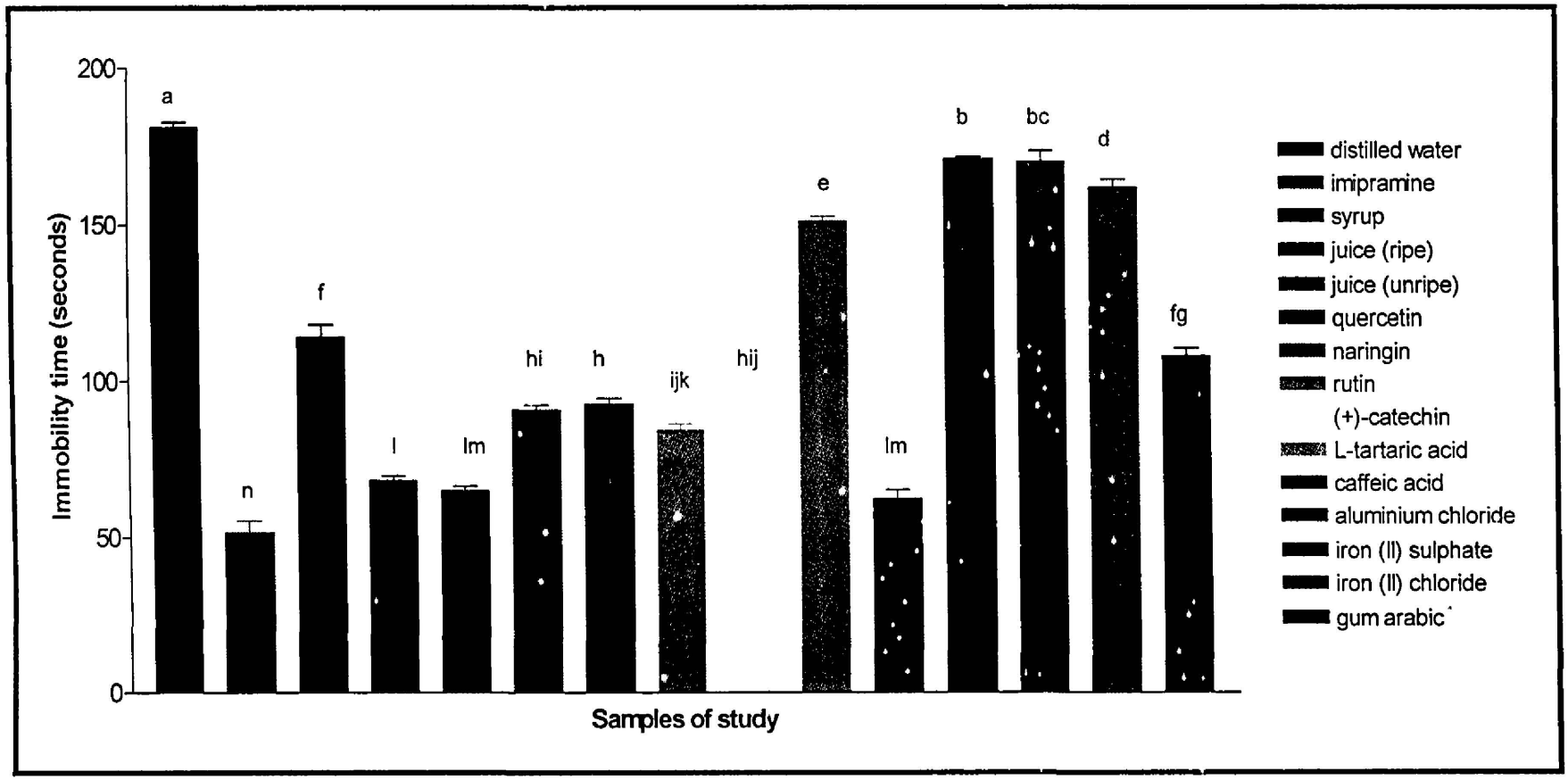


Figure 4.10 Effect of treatment with non-copigmented, copigmented syrups, ripe and unripe fruit juices, imipramine and vehicle (i.p.) on the FST in mice. Each column represents the mean ± S.E.M. of six animals. Statistical differences were determined using One-way ANOVA followed by Fisher LSD (α=0.05). Values with the same alphabet (a,b,c) are not significantly different ($P<0.001$) between samples.

4.3.2 The tail suspension test (TST)

The results of the TST were given in **Figure 4.11**. From the figure, it can be seen that all samples significantly ($P<0.001$) reduced the duration of immobility compared to the negative control group and this scenario further proven the hypothesis that *Carissa carandas* syrups and juices may possess an antidepressant activity. As implied in all previous tests, bars were labelled with alphabets to indicate the least significant differences between samples and the greater the alphabets, the longer the reduction of immobility time and the stronger the antidepressant activity possessed by samples of study. According to the One-way ANOVA and Fisher LSD tests conducted on data obtained, the antidepressant activity as evaluated by the TST test can be written as:

imipramine > unripe fruit's juice ≥ caffeic acid ≥ ripe fruit's juice ≥ rutin > L-tartaric acid ≥ syrup ≥ (+)-catechin ≥ quercetin > iron (II) sulphate ≥ gum arabic > iron (II) chloride ≥ naringin ≥ aluminium chloride

Likewise, imipramine significantly ($P<0.001$) reduced the longest duration of immobility time in this model of behaviour. Meanwhile, the ripe and unripe fruit's juice, caffeic acid and rutin copigmented syrups showed more or less the same intensity in antidepressant activity in this model and with that of in the FST test. And this time, (+)-catechin, gum arabic and naringin joined the same group of copigments that did not improve the antidepressant effect when added in *Carissa carandas* syrup. Aluminium chloride copigmented syrup still occupy the same place in the order of activity as in the FST test.

Plate 4.2 displayed the escape-oriented and immobile postures taken in this study. Immobility time was recorded when the mice hung passively and completely motionless.

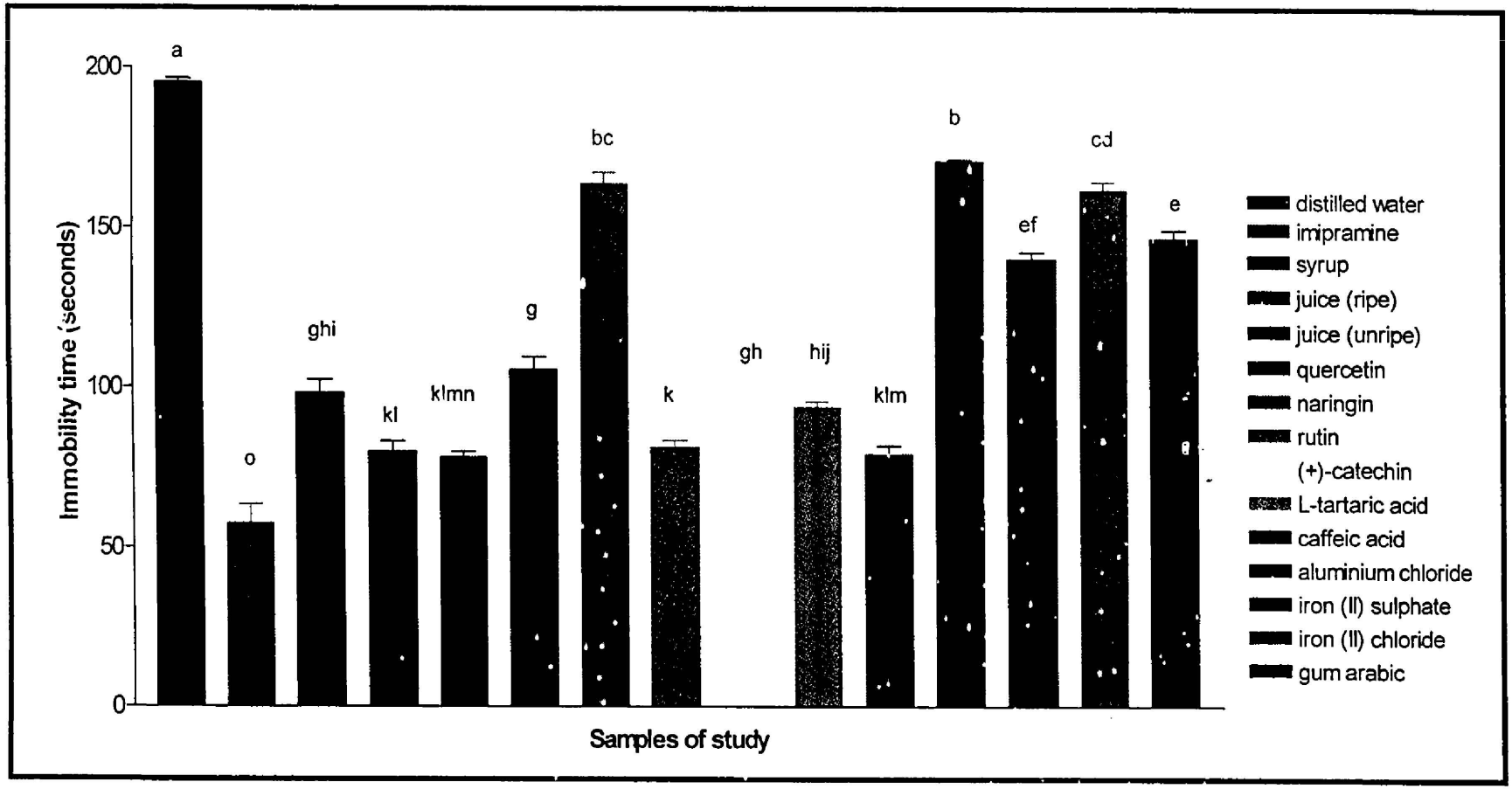


Figure 4.11 Effect of treatment with non-copigmented, copigmented syrups, ripe and unripe fruit juices, imipramine and vehicle (i.p.) on the TST in mice. Each column represents the mean ± S.E.M. of six animals. Statistical differences were determined using One-way ANOVA followed by Fisher LSD (α=0.05). Values with the same alphabet (a,b,c) are not significantly different ($P<0.001$) between samples.

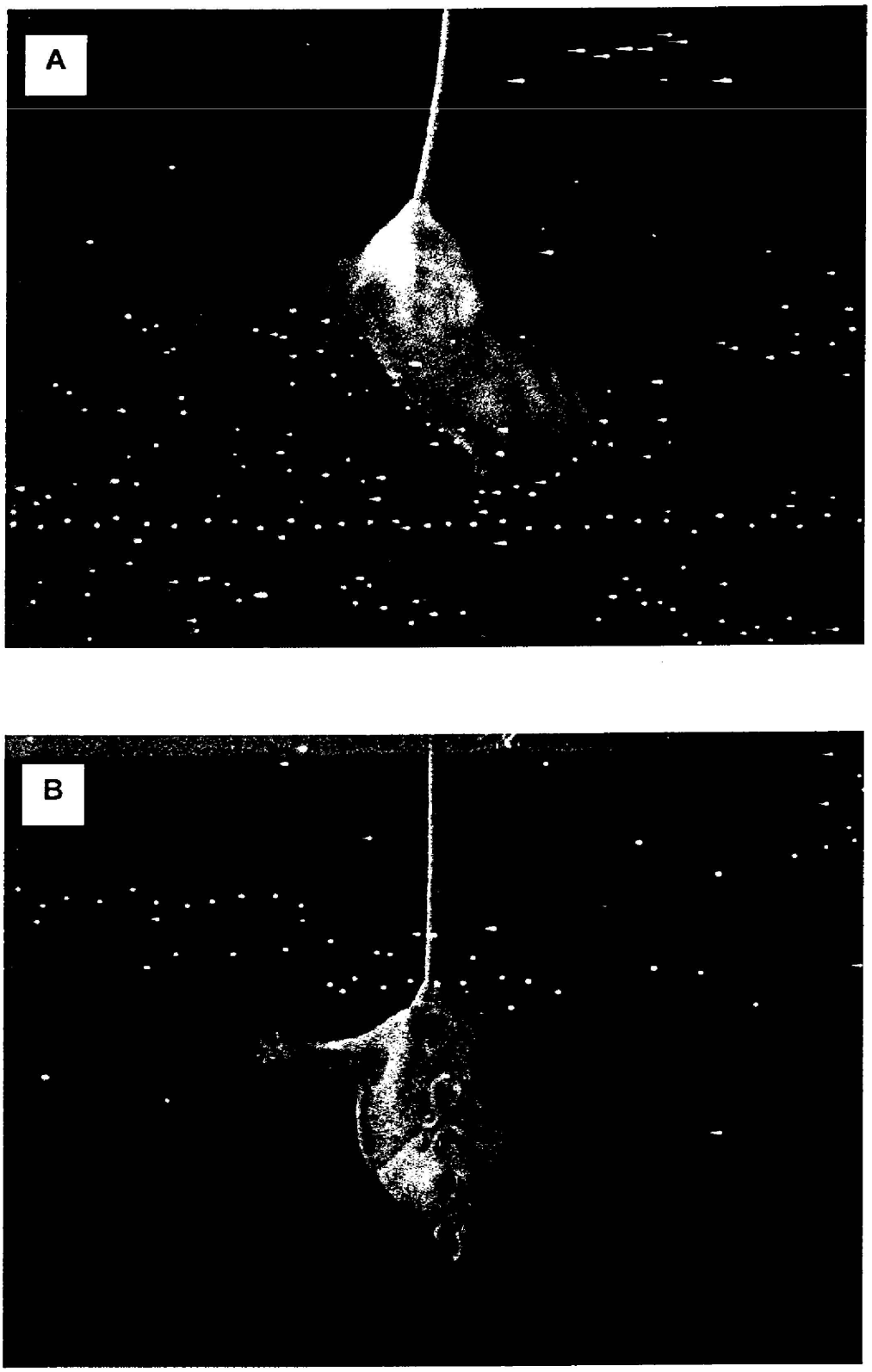


Plate 4.2 The escape-oriented (A) and immobile (B) postures captured in the TST test

4.4 Cytotoxicity of *Carissa carandas* syrups

4.4.1 Brine shrimp lethality assay

Sadly, the outcome of this assay was the most unfortunate result obtained in this study. At 30 minutes after nauplii were transferred into glass vials containing test substances, 100% death occurred in all vials with one exception, ten out of ten nauplii had survived and lasted for 24 hours in the vial containing distilled water as a negative control. The experiment was even repeated twice, but the outcome was still the same. So, consequently, the acute and chronic toxicity of syrup cannot be further determined in this assay.

4.4.2 Hemolytic assay

Although the cytotoxicity evaluation using the brine shrimp leads to a negative finding, the cytotoxicity of *Carissa carandas* syrup have been successfully studied in hemolytic assay based on the lysis of sheep erythrocytes and the result obtained is presented in **Table 4.2**. As shown by Table 4.2, at 405nm, hemolysis of the erythrocytes was not observed in any test samples i.e. non-copigmented and copigmented syrups. Meanwhile, positive control of distilled water exhibited intense hemolysis, whereas, as it should be, no hemolysis occurred in phosphate buffered saline, the negative control.

Table 4.2 Cytotoxicity of *Carissa carandas* syrup against fresh sheep erythrocytes

non-copigmented syrup	-
quercetin	-
naringin	-
rutin	-
(+)-catechin	-
L-tartaric acid	-
caffeic acid	-
aluminium chloride	-
iron (II) sulphate	-
iron (II) chloride	-
gum arabic	-
phosphate buffered saline (negative control)	-
distilled water (positive control)	+

- no hemolysis occurred + hemolysis occured

CHAPTER 5

DISCUSSION

CHAPTER 5 DISCUSSION

5.1 The dependence of anthocyanins stability in *Carissa carandas* syrups on added copigments

Copigmentation is a molecular interaction that occurs between anthocyanins and copigments (Goto & Kodo, 1991; Gonzalez *et al.*, 2001) and is regarded today as one of the significant factors of structure stabilization (Boulton, 2001). Copigments' basic role is to protect the coloured flavylium cation from nucleophilic attack of water molecule (Mazza & Brouillard, 1990). Red coloured flavylium cation is the primary, pH dependent, anthocyanins equilibrium structure, from which secondary structure are derived either by proton transfer to produce bluish quinoidal bases and corresponding ionized quinoidal bases, or by hydration, to form colourless carbinol pseudobases, which can tautomerise to colourless chalcones (Brouillard & Dangles, 1994b). Tertiary structure can result from molecular associations of primary or secondary forms, either with themselves (self association) or with other molecule in a solution (intermolecular copigmentation) (Bridle & Timberlake, 1997). In acidic or neutral media, the four anthocyanins structures i.e. the flavylium cation, quinoidal base, carbinol pseudobase and chalcone exist in equilibrium (Hrazdina, 1974).

It has been my focus of interest to stabilize anthocyanins in *Carissa carandas* fruits sugar drink model since its present in a remarkably large quantity, 624.49mg/100g fruits (Khuzma, unpublished result). As we know now, there is abundant existing evidence from literature studies which proved the copigmentation reaction as the saviour to anthocyanin stability problem, thus, in this study, I employed ten copigments to do their job and improved *Carissa carandas* anthocyanins stability. Whether or not copigmentation reaction successfully took place in syrup solution and efficiently stabilize anthocyanins, were determined in three different sets of experiments i.e.

storage, thermal and light stability studies. However, before I go in depth on the effects of copigmentation on stability of copigmented syrups, it is vital to note that the microwave pasteurization process applied to all samples do play a role in stabilizing anthocyanins in terms of deactivation of fruit enzymes to avoid fermentation. The presence of fruit enzymes are capable of splitting the glycosidic linkage in anthocyanins, resulting the less stable aglycones and rendering pigments more susceptible to oxidation (Chichester & McFeeters, 1970). Marchese (1995) reported the impact of pasteurization on juice discolouration, and suggested mild pasteurization, less than 80°C, to minimize the degradation of anthocyanins. Moreover, Maccarone *et al.* (1985) studied the stabilization of anthocyanins in blood orange (*Citrus sinensis*) juice at 95°C and found that pasteurization by microwaves did not change the initial colour intensity of the orange juice and somewhat improved the stability. In this present study, syrups were pasteurized at maximum temperature of 92°C, following the method described by Maccarone *et al.* (1987).

Based on observation supported by statistical evidence, in this study, it is crystal clear that the addition of copigments did stabilize *Carissa carandas* anthocyanins in syrup during storage, thermal treatment and light exposure. Caffeic acid added syrup proved to have the most stabilize anthocyanins after 90 days of storage, followed by rutin copigmented syrup. Interestingly, Maccarone *et al.* (1985) who had attempted to stabilize the anthocyanins in blood orange juice also found that the complexation of anthocyanins with rutin and caffeic acid provided the highest stability during the 112 days of storage. Sweeny *et al.* (1981) reported that the improved stabilization of anthocyanin-copigment complex is ascribed to the reduced availability of anthocyanins to undergo nucleophilic reactions with water or other agents, because of the probable formation of more stable intermolecular complexes. A stable complexes of anthocyanin-rutin and anthocyanin-caffeic acid as suggested by Maccarone *et al.* (1985) is presented in **Figure 5.1**.

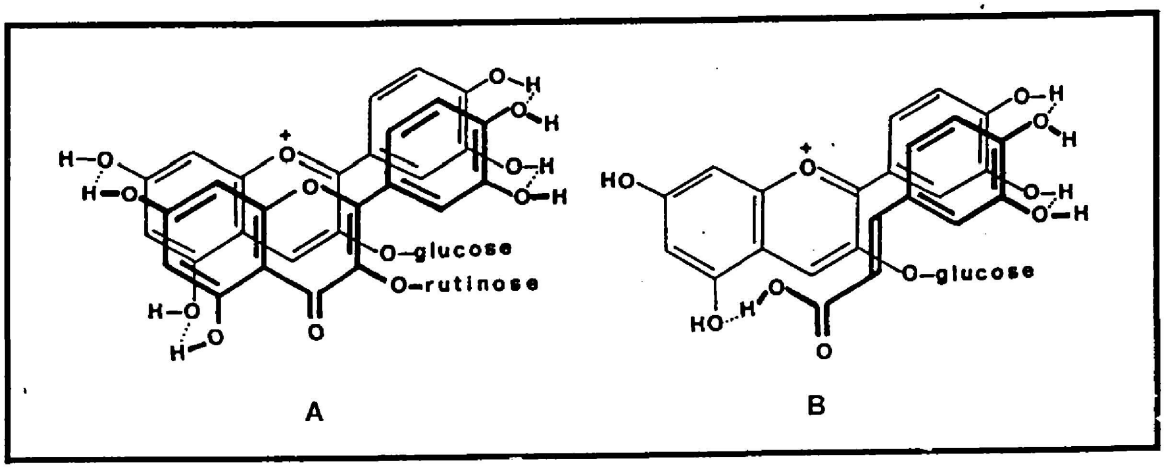


Figure 5.1 Anthocyanin-Rutin complex (A), Anthocyanin-Caffeic acid complex (B) (Maccarone *et al.*, 1985)

Furthermore, Darias-Martin *et al.* (2001) found that the pre-fermentation addition of caffeic acid at 120mg/ml, resulted in 60% enhancement in wine colour after fermentation. Hence, caffeic acid appears to contribute to young wine colour, as its addition before fermentation enhanced colour extraction. Phenolic compounds form intermolecular complexes with anthocyanins by hydrogen bonding and charge transfer (Osawa, 1982). Dangles *et al.* (1993) carried out a copigmentation experiments which involved the addition of alkaline solution of rutin to an acidic solution of malvin. They observed that, when enough rutin was added, the copigmentation reaction was able to overcome the colour fading, probably due to the slowing down of hydration process, which promoted changes of flavylium cation to colourless hemiacetal. The hydration rate of anthocyanins to colourless carbinols and chalcones may be lowered by the increased hydrophobic interactions associated with the formation of anthocyanin-copigment complexes (Brouillard, 1982), so that most of the pigments are present as coloured flavylium salts and quinoidal bases (Maccarone *et al.*, 1987).

Anthocyanins become paler on heating, because the equilibrium between the four anthocyanins species shifts towards the colourless carbinol pseudobase and chalcone

forms. Under perfect conditions, the original colour should be regained on cooling if sufficient time, probably a few hours is allowed for reconversion of the chalcone. However, in practice, in the presence of oxygen, some pigment browning or degradation usually occurs and this is most noticeable in the cooling cycle (Bridle & Timberlake, 1997). In this study, samples were heated at a high temperature of 99±1°C. This temperature was chosen to reflect a severe heat treatment during thermal food processing operations, for example, blanching, pasteurization, cooking, etc.

To my pleasure, all copigmented syrups showed a significant increase in stabilization of anthocyanins compared to non-copigmented syrup, with caffeic acid and rutin copigmented syrups displayed the highest stability effect towards severe heat treatment. This was in agreement with previous studies where malvidin 3,5-diglucoside copigmented with various flavonoids had greater thermal stability (Baranac *et al.*, 1996, 1997a, 1997b, 1997c). Talcott *et al.* (2005) also discovered that the stability of copigmented Muscadine grape (*Vitis rotundifolia*) juice with isoflavonoid extracts from red clover (*Trifolium pratense*) had greater thermal stability than non-copigmented control at varying copigment ratios. The authors suggested that the protective effects of copigments were attributed to either increased antioxidant protection or to the physical associations between anthocyanins and copigments that served to protect the anthocyanins from the damaging effects of heat.

Morais *et al.* (2002) reported that the presence of light did not exert a significant impact on the amount of pigments. In contrast to this statement, in this study, light significantly influenced anthocyanins degradation in *Carissa carandas* syrups and this finding is in accordance to result obtained by Gris *et al.* (2005) who discovered that the presence of light significantly interfered in anthocyanins' $t_{1/2}$ values, thus influencing the stability of these anthocyanins from Cabernet Sauvignon grape extracts in model systems. On

the other hand, Cevallos-Casals & Cisneros-Zevallos (2004) admitted that the heat treatment is more severe in reducing anthocyanins' $t_{1/2}$ values in comparison to light exposure. Fortunately, in this study, all copigmented syrups significantly marked an improved stabilization effect compared to syrup without addition of copigment when exposed to light and again, caffeic acid and rutin copigmented syrups exhibited the best stabilization effect.

The bathochromic shift, referred to a shift of the wavelength of maximum absorbance, is evidence of the formation of either inter- or intramolecular complexes by anthocyanins (Figueiredo *et al.*, 1999). In addition, Mazza & Brouillard (1990) reported that the magnitude of copigmentation effect is also estimated from the increase in absorbance at the visible λ_{max} (hyperchromic effect) besides the extent of the bathochromic shift of visible λ_{max}. The complex formation of copigments and anthocyanins which resulting in an increase in both absorbance and in a bathochromic shift is based mainly on hydrogen bond formation between the carbonyl group of anthocyanins anhydrobase and aromatic hydroxyl groups of the complex-forming copigments. The larger the number of hydroxyl groups in copigments molecule, the stronger the complex formation (Chen & Hrazdina, 1981). In this study, both quercetin and (+)-catechin have the most hydroxyl groups present (refer to **Appendix A**), however, the stabilization effects displayed is poor. Meanwhile, Salas *et al.* (2004) suggested that the hyperchromic effect may be due to a displacement of the hydration equilibrium towards the flavylium form whereas the bathochromic shift can be attributed to an increased proportion of quinoidal bases in the pigment-copigment complexes.

In present study, the spectrophotometric result revealed that caffeic acid and rutin proved to promote a slight increase in the maximum absorption wavelength (bathochromic effect) and absorbance (hyperchromic effect) of pelargonidin 3-glucoside in *Carissa carandas* syrups suggesting copigmentation. The magnitude of

both bathochromic and hyperchromic effects were greater in caffeic acid copigmented syrup in comparison with rutin copigmented syrup. Therefore, it can be concluded that the stabilization of anthocyanins was greater in caffeic acid added syrup. Gris *et al.* (2005) evaluated the caffeic acid copigmentation of anthocyanins from Cabernet Sauvignon grape extracts in model and yogurt systems as mentioned earlier, discovered that caffeic acid promoted an increase in maximum absorption wavelength and absorbance. So, the addition of caffeic acid significantly increased the stability of anthocyanins in both model systems.

The precision and the objectivity of the colour characterization of some colourants (Malien-Aubert *et al.*, 2001), model anthocyanins, wine solutions (Heredia *et al.*, 1998) and fruit juices (Cheynier *et al.*, 1990; MacDougall, 2000) was improved, thanks to the introduction of several colour meter systems i.e. CIEXYZ, CiELUV and CIELAB. As pigment qualities were best described by the use of spectral colorimetric assessment, best objectivity was achieved by the use of the homogeneous CIELAB colour space (Prodanov *et al.*, 2005). Seghi *et al.* (1986) reported that colour measurement using a colorimeter provides consistent colour evaluation, even some anthocyanins extract manufacturers use tristimulous colorimetric parameters for assessing quality control of their products (Prodanov *et al.*, 2005). Colorimeters often report colour using the CIELAB colour system, which is a method developed in 1978 by the Commision Internationale de l' E'clairage for characterizing colour based on human perception. It designates colour according to three spatial coordinates, L^*, a^* and b^*, where L^* represents the brightness (value) of a shade, a^* represents the amount of red-green colour and b^* represents the amount of yellow-blue colour. L^* coordinates are located along a vertical axis that ranges from a value of 0 (blackest) to 100 (whitest). The a^* and b^* are two chromatic components, with values varying from -120 to +120 and the coordinates revolve on axis around L^*. As a^* becomes more positive in value, the colour is more red; as a^* becomes more negative in value, the colour becomes more

green. And as b^* becomes more positive in value, the colour becomes more yellow; as b^* becomes more negative in value, the colour becomes more blue. Absolute measurements can be made in L^*, a^* and b^* coordinates (Haselton *et al.*, 2005). CIELAB colour space is device-independent, creating consistent colours regardless of the device used to acquire the image (Briones & Aguilera, 2005).

Analysis of any changes in colour of caffeic acid and rutin copigmented syrups in comparison with non-copigmented syrup during 70 days of storage were carried out in present study. Test samples were actually the same samples that had been stored for 90 days in storage stability study. The increased in L^*, a^* and b^* values were the characteristic changes in *Carissa carandas* syrups during storage and this is true to all three samples evaluated. The L^* values were constantly increased probably due to constant conversion of the flavylium cations to colourless carbinols and chalcones in anthocyanins degradation process. Hence, samples become lighter with time. This finding is in contradiction with what reported by Gonnet (1999), who claimed that the hyperchromic effect of copigmentation always caused a decreasing lightness of solutions. Although hyperchromic effect was observed in the visible specrum, the L^* values of samples were steadily increased in this study. As for the increased in a^* and b^* values observed, for the copigmented samples, it may relate to the copigmentation reaction that occurred in these samples. Additions of copigments somewhat helped to shift the equilibrium of the four anthocyanins species towards red coloured flavylium cations, and this may explained the increased of a^* and b^* values measured in samples during storage. Caffeic acid which showed an excellent performance in stabilizing *Carissa carandas* anthocyanins marked the lowest increased in L^*, highest increased in a^* and lowest increased in b^* parameter compared to the other two samples. So, it can be deduced that the flavylium cation change to carbinol pseudobase and chalcone at the slowest rate in caffeic acid copigmented syrup while at the same time, with the help of caffeic acid, the equilibrium shifted towards red

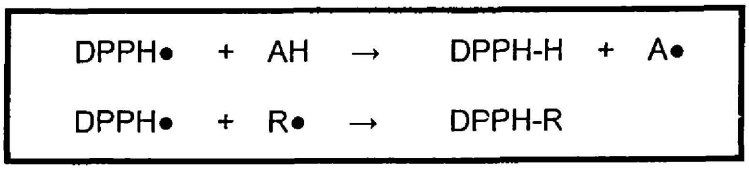
coloured flavylium cation instead of the three other forms i.e. bluish quinoidal base, colourless carbinol and chalcone. The most qualitative parameter is the *h* which specifies the exact colour of substances. *h* is a function of a^* and b^* [h = arc tg (b^*/a^*)] (Prodanov *et al.*, 2005) and can be written as h_{ab}. Caffeic acid and rutin copigmented syrups, along with non-copigmented syrup *h* values were measured at the end of 70 days storage period and as a result, the most prominent copigment, caffeic acid *h* value which is 9.00°, was the closest to the $+a^*$ axis. This indicates that colour of caffeic acid copigmented syrup was the most red among the two other samples, in conjunction with the conversion of colourless carbinols and chalcones to flavylium cations.

As a whole, the addition of copigments significantly stabilize anthocyanins, namely, pelargonidin 3-glucoside in *Carissa carandas* syrup with caffeic acid conferred the best stabilization effect.

5.2 Antioxidative activities of *Carissa carandas* syrups and the role of copigments on improving the activities

The DPPH assay is a non-enzymatic method currently used to provide basic information on the reactivity of compounds to scavenge free radicals (Braca *et al.*, 2003). The method is based on the reduction of alcoholic DPPH• solutions in the presence of a hydrogen donating antioxidant (Kulisic *et al.*, 2004). Brand-Williams *et al.* (1995) described the method involving the use of free radical, DPPH•, as where an antioxidant is allowed to react with the stable radical in a methanol solution. The reduction in the concentration of the DPPH• is followed by monitoring the decrease in its absorbance at a characteristic wavelength during the reaction. In its radical form, DPPH• absorbs at 515nm, appearing in a deep violet colour. Upon reduction by an antioxidant (AH) or a radical species (R•), the absorption vanishes and the resulting

decolourization is stoichiometric with respect to the degree of reduction. The remaining DPPH•, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant (Blois, 1958).



DPPH• will undergo changes into a stable compound which is yellow in colour upon reduction with an antioxidant (Bondent *et al.*, 1997) and the more rapid the absorbance decreases, the more potent the antioxidant activity of the compound in terms of hydrogen donating ability (Von Gadow *et al.*, 1997).

In this present study, all 56°Brix samples except for four i.e. quercetin, naringin, iron (II) chloride and gum arabic copigmented syrups showed a significant increased in radical scavenging activity compared to non-copigmented syrup. On the other hand, for 9.8°Brix samples, only caffeic acid and (+)-catechin addition in *Carissa carandas* syrups did improved the radical scavenging activity of syrups. Moreover, caffeic acid and (+)-catechin copigmented syrups proved to be an excellent radical scavenger in both concentration (caffeic acid: 56°Brix - 35.70%, 9.8°Brix - 59.47%; (+)-catechin: 56°Brix - 35.70%, 9.8°Brix - 54.28%). Caffeic acid, being common in many plants and often present in our diet, is a strong radical scavenger with a stoichiometry of five to six reduced DPPH• molecules per molecule of antioxidant (Brand-Williams *et al.*, 1995), slightly superior to rosmarinic acid (Cuvelier *et al.*, 1992; Kulisic *et al.*, 2004) and has been reported to exert strong inhibitory activity on xanthine oxidase (Chan *et al.*, 1995). Meanwhile, much of the antioxidant potential of teas are ascribed to catechin-derivatives which are able to effectively reduce Fremy radical (Gardner *et al.*, 1997). For instance, (+)-catechin and its associated gallates reduce between 2.6 and 4.3 radicals/molecule and therefore, are more effective antioxidants than vitamin C and are

similar in effectiveness to Trolox, a water soluble analogue of vitamin E (Gardner *et al.*, 1997). The addition of both excellent radical scavengers, caffeic acid and (+)-catechin to *Carissa carandas* syrups work wonders to improve its radical scavenging capacity.

Interestingly, all diluted syrups showed an increased in the percentage of radical scavenging activity compared to concentrated syrup. This finding is in close agreement with Ferguson (2001) who reported that free radical scavenging activity decreases with the presence of sugar moiety. Siddhuraju *et al.* (2002) also shared the same view on this issue. Based on their study on *Cassia fistula* L. extracts, the authors concluded that the presence of considerable quantities of sugars such as glucose, fructose, reducing disaccharides and sucrose, might decreased and deactivated the antioxidative potential of compounds present in the extracts.

The determination of the antioxidative activity using the DPPH free radical scavenging assay seems to barely represent the biological situation, because the DPPH• is an exogenous radical (Chen *et al.*, 2006). However, this method has been widely accepted as a tool for estimating the free radical scavenging activity of potential antioxidants (Jin & Chen, 1998; Kim *et al.*, 2002; Leong & Shui, 2002; Fenglin *et al.*, 2004).

Many different methods have been used to measure the resistance of lipid to oxidation when in the presence of potential antioxidants and these tests are generally performed in either a lipid or emulsion medium. Inhibition of linoleic acid autoxidation assay systems represents inhibition of lipid peroxidation (Vimala *et al.*, 1999). Autoxidation refer to a slow, radical process which proceeds via a chain reaction including induction, propagation and termination steps. During the induction period, alkyl radicals are formed which undergo reaction with oxygen molecules to from hydroperoxides and peroxide radicals during propagation phase. Termination proceeds via association of

two radicals to form a stable adduct (Brand-Williams *et al.*, 1995). Frankel (1993) suggested that linoleic acid is not an appropriate lipid to test antioxidants since its behaviour would be significantly different than that in foods composed mainly of triglycerides. Yet, linoleic acid is commonly used to evaluate natural antioxidants.

Oxidation of linoleic acid generates linoleic acid hydroperoxides, which leads to many secondary oxidation products (Hua-Ming *et al.*, 1996). In the FTC assay, the oxidized products, namely, linoleic acid hydroproxides react with ferrous chloride to form ferric chloride. Ferric chloride will then react with ammonium thiocyanate to form ferric thiocyanate of blood-red colour which was measured spectrophotometrically at 500nm. After the incubation period reached its maximum, the formation of peroxides is stagnated, due to non-availability of linoleic acid. Also, the intermediate products may be converted to stable end-products. The non-availability of hydroperoxides results in the retardation of oxidation of ferrous chloride. Hence, the absorbance does not increase after the maximum day of incubation period (Jayaprakasha *et al.*, 2006). In the presence of antioxidants, oxidation of linoleic acid will be slow. Thus, the colour development due to the formation of thiocyanate will be slow. Or it can be put this way, high absorbance is an indication of high concentration of formed peroxides. Therefore, low absorbance indicates high antioxidant activity. From my previous study (Siti Rafidah, 2003), on the antioxidative activity of *Carissa carandas* extracts using the FTC assay, reaction between the oxidized products, ferrous chloride and ammonium thiocyanate that took place in samples reached its maximum on the 10^{th} day of incubation period. Thus, in this study, the absorbance of test samples were measured only on the 10^{th} day of incubation period instead of monitoring the absorbance values since day one of incubation.

Addition of antioxidant is a method for increasing the shelf life of foods (Zainol *et al.*, 2003). Synthetic antioxidants have restricted use in food as various studies have

shown then to be carcinogenic (Madavi & Salunkhe, 1995) and this is especially true for BHT. It is interesting to note that in this study, the addition of copigments successfully improved the antioxidative capacity of *Carissa carandas* syrups comparable, in fact, greater compared to BHT in both concentrated (56°Brix) and diluted (9.8°Brix) samples, with rutin and caffeic acid acts as the best antioxidant agents.

During linoleic acid oxidation process, peroxides are gradually decomposed to lower molecular weight compounds. One such compound is malondialdehyde (MDA), which is measured by the TBA assay on the final day of the incubation period, which is one day after the control reached maximum (Zin *et al.*, 2002). What actually happened in this assay is, when test samples were heated at a high temperature (100°C) with TBA at a low pH (provided by the trichloroacetic acid), peroxides were rapidly decompose to give MDA which then combines with TBA to form a pink $(TBA)_2MDA$ adduct (Aruoma, 2003). MDA is a very unstable compound causing mutagenic and cytotoxic events (Zin *et al.*, 2002). The coloured product was monitored at 532nm. Measuring the occurance of MDA spectrophotometrically with TBA is the common assay used for determining the rate extent of lipid peroxidation (Czinner *et al.*, 2001).

Similar to FTC assay, all copigmented samples exhibited stronger antioxidative activity compared to syrup without copigment in the TBA assay and it does feel good to state that all copigmented samples showed comparable activity to that syrup added with BHT. Rutin and (+)-catechin played as the best antioxidant agent in 56°Brix syrups while caffeic acid joined the duo in 9.8°Brix set of experiment. Zhang *et al.* (2001) reported that rutin, purified compound from hawthorn fruits (*Crataegus sp.*) was found to be protective to human low density lipoproteins (LDL) and same goes to (+)-catechin which was discovered by Meyer *et al.* (1998) as the most potent antioxidant according to the LDL oxidation assay. Dimitric-Markovic *et al.* (2003a, 2003b) evaluated the

antioxidant properties of the copigmentation complexes of malvidin 3,5-diglucoside and caffeic acid. Their results confirmed that caffeic acid has the capacity to copigment this anthocyanin and that the antioxidant activity of the complex anthocyanin-caffeic acid was greater than the antioxidant activity of the anthocyanin alone. The radical scavenging potential and antioxidant properties of caffeic acid in terms of its ability to increase the resistance of LDL to cholesterol oxidation were reported (Castelluccio *et al.*, 1995; Marinova & Yanishlieva, 2003; Sroka & Cisowski, 2003). Results obtained are in accordance with the FTC assay. Arts *et al.* (2001) studied the antioxidative capacity of quercetin, rutin and (+)-catechin in blood plasma using trolox equivalent antioxidant capacity (TEAC) assay. The authors observed that the antioxidative capacity of plasma is enhanced after the addition of quercetin, but the increase is much smaller than the antioxidant capacity that quercetin itself has. Same thing happened in present study. Quercetin which is well known as an excellent antioxidant agent, reported to inhibit the oxidative modification of LDL by macrophages (De Whalley *et al.*, 1990) and have been proved to protect LDL against the glucose-induced lipid peroxidation implicated in the increased prevalence of atherosclerosis in diabetic patients (Vedavanam *et al.*, 1999), however, did not show good antioxidative activities in DPPH, FTC and TBA assays when added in syrup. Thus, it can be conclude that, besides the antioxidative capacity of the compound itself, the matrix in which the antioxidant agent has to execute its job is also important in influencing the activity.

Coincidently, both caffeic acid and rutin marked the best stabilization effect on *Carissa carandas* anthocyanin, namely, pelargonidin 3-glucoside in syrup. If the strong antioxidative activity was provided by the anthocyanins, so, it may not be a coincidence at all! Excellently stabilized anthocyanins definitely displayed better activity compared to the unstabilized or moderately stabilized anthocyanins. (Duhard *et al.*, 1997) suggested that sugars and their degradation products tend to accelerate the degradation of anthocyanins, though certain sugars have been proven to increase

anthocyanins colour intensity (Lewis *et al.*, 1995). So, this may relate to the influence of sugar on the antioxidative activity of *Carissa carandas* syrup in DPPH, FTC and TBA assays. Based on observation, in this study, sugar tends to decreased the antioxidative capacity of samples and this scenario occurred probably because anthocyanins in 56°Brix syrups degrades at a faster rate compared to anthocyanins in 9.8°Brix samples. And if it is true that the antioxidative activity is majorly provided by the anthocyanins, then the above hypothesis is acceptable.

The FTC and TBA assays are non-specific, meanwhile, the DPPH free radical scavenging assay is mechanism-based (Ahmad *et al.*, 2005). These antioxidant assays suggest that both caffeic acid and (+)-catechin copigmented syrups are excellent radical scavengers and able to inhibit lipid peroxidation while rutin copigmented syrup inhibit oxidation through a mechanism other than radical scavenging.

While the DPPH, FTC and TBA are *in vitro* antioxidant assays, are chemically-based and the behaviour in this system might not reflect the *in vivo* behaviour, still, these assays may serve as a reasonable preliminary indicator of antioxidant potential.

5.3 Antidepressant effect of *Carissa carandas* syrups and juices in animal models of behaviour

The forced swimming and tail suspension test were two behavioural tests in mice and accepted stress models of depression (Luo *et al.*, 2000) that predicted the clinical efficacy of many types of antidepressant treatments (Butterweck *et al.*, 1998). In FST test, mice are forced to swim in a restricted space from which there is no escape, and will, after periods agitation, cease attempts to escape and become immobile. The characteristic behaviour scored in this test is termed as immobility, reflecting

behavioural despair as seen in human depression (Porsolt *et al.*, 1977). While in the TST test, mice are suspended by their tail and exhibit struggling or escape-like behaviours, followed by immobility (Steru *et al.*, 1985). The longer the duration of immobility, the higher the levels of behavioural despair and vice versa (Oliveira *et al.*, 2004). Antidepressant drugs somehow modify the balance between activity and immobility in both FST and TST paradigms in favour of activity and at the expense of energy (Karolewicz & Paul, 2001).

The FST consists of twice placing the animal into tank filled with water with 24 hours interval (a pre-test session and a subsequent test). The exposure to the previous swimming session reduces the latency to immobility and enlarges the time spent in immobile posture during the overall period of the subsequent test (Porsolt *et al.*, 1977). The immobility posture relates to the development of passive behaviour that disengages the animal from active forms of coping with stressful stimulus (Cryan *et al.*, 2002). Noguchi *et al.* (1992) reported that the FST increased the brain content of histamine and histamine turnover, while Perez-Garcia *et al.* (1999) suggested that the H_1 and H_3 receptor antagonists reduced the duration of immobility in the FST. Furthermore, previous studies using FST test have suggested that either the activation of α_1-adrenoreceptors or the inhibition of nitric oxide production may also be involved in the expression of antidepressant effect (Harkin *et al.*, 1999; Yildiz *et al.*, 2000). The implication of dopamine pathways in the pathogenesis of depression was suggested by various studies (Brown & Gershon, 1993). This role of dopamine is commonly based on the hypothesis that noradrenaline and/or serotonin and/or the dopamine systems could be deficient in depression (Renard *et al.*, 2004). Interestingly, according to Renard *et al.* (2003), the FST provoked a significant increase of the concentration of dopamine during the test. In contrary to this, Renard *et al.* (2004), based on their study, concluded that a high concentration of dopamine in the whole brain could be a limiting factor for the antidepressant effect.

In agreement to literature studies, in the FST test that has been carried out in this study, mice adopted an immobility posture between struggling to escape during test and the duration of immobility was recorded for five minutes. The antidepressant effect of test samples were compared to the tricyclic antidepressant, imipramine, which acts as an inhibitor of serotonin and norepinephrine reuptake transporters (Sun & Alkon, 2003). Imipramine can be referred as first-generation antidepressant (Becker *et al.*, 2004). While distilled water was used as negative control. The result revealed that all test samples showed a potential antidepressant effect with caffeic acid copigmented syrup and the unripe fruit's juice as the best antidepressant agent. The duo was followed closely by the ripe fruit's juice. Addition of quercetin, naringin, rutin, (+)-catechin and caffeic acid to syrups significantly improved the activity. Whereas, in the TST test, more or less, a similar order of activity was achieved. The unripe fruit's juice, caffeic acid copigmented syrup and the ripe fruit's juice still dominating the three best places in the order of activity after imipramine. However, in the TST test, only three copigments i.e. caffeic acid, rutin and L-tartaric acid worked to improve the antidepressant activity of syrups.

Takeda *et al.* (2002) have investigated the antidepressant effect of caffeic acid in mice by using the FST test and found that caffeic acid produced antidepressant activity via some mechanism (s) other than the inhibition of monoamine transporters and monoamine oxidase. Unfortunately, the detailed mechanisms involved in the antidepressant property of caffeic acid are not yet clear. However, existing evidence have revealed that caffeic acid can activate the α_1-adrenoreceptor system (Cheng & Liu, 2000) and inhibit the production and release of nitric oxide (Soliman & Mazzio, 1998; Yokozawa & Chen, 2000). It has been suggested that these systems in the brain may contribute to stress and depression. It is also possible that the antidepressant effect may involve the direct modulation of a second messenger system since it has recently been reported that caffeic acid inhibits both protein kinase A and protein

kinase C activity *in vitro* (Nardini *et al.*, 2000). Increased evidence suggests that the therapeutic effects of existing antidepressants are associated with adaptive changes in post-receptor signaling (Popoli *et al.*, 2000). For instance, chronic administration of various types of monoamine reuptake inhibitors that exhibited therapeutic activity clinically decreased the activity of protein kinase A or protein kinase C (Mann *et al.*, 1995). Although it is not known whether the previously reported *in vitro* inhibition of the activity of protein kinase A and C by caffeic acid could play a significant role in the modulation of cell function *in vivo*, these inhibitory effect of caffeic acid might be involved in the excellent antidepressant effect found in present study.

Although the relationship between the immobility posture and depression remains controversional (Gardier & Bourin, 2001), it is well demonstrated that drugs with antidepressant activity able to reduce the time which animals remain immobile (Borsini & Meli, 1988). Thus, the animal models are indeed indispensable in searching for new antidepressants and for clarifying pathophysiology that underlies depression.

5.4 Toxicity of *Carissa carandas* syrups against the brine shrimp and sheep erythrocytes

Artemia salina, like other zooplankton is a significant food source for many fish and aquatic invertebrates (Sorgeloos, 1980). It has gained popularity as a test organism because of its ease of culture, short generation time, cosmopolitan distribution and the commercial availability of its dormant eggs (cysts) (Barahona & Sanchez-Fortun, 1999). Since test animals hatching from cysts are of similar age, genotype and physiological condition, test variability is greatly reduced (Vanhaeke *et al.*, 1981; Persoone *et al.*, 1989). *Artemia salina* has the same purine metabolism as that of mammalian cells and the DNA-dependent RNA polymerases are also similar to the mammalian type (McLaughlin, 1991; Solis *et al.*, 1993). Consequently, it have been

used as in the brine shrimp lethality assay, a simple bench top bioassay which known to yield good results. Additionally, in the brine shrimp lethality assay, no food was provided to the nauplii, such that they were starving during the experimental time, so, the sensitiveness to toxic substances was enhanced (Pelka *et al.*, 2000).

Somehow, in the present study, none of the nauplii could survived and lasted for more than 30 minutes after they were placed in sample solutions and this scenario was observed in all samples tested. The phenomenon occurred probably because the test samples were extremely toxic to the nauplii! However, I do not want to jump to this conclusion just yet, so, the hemolytic assay was employed to further determine the toxicity of *Carissa carandas* syrups.

According to Aki & Yamamoto (1991), the erythrocyte membrane is a dynamic structure that can dictate significant changes in its interaction with drugs or other substances. Hence, this trait has been exploited to verify the toxicity effect of test compounds and in this case, to evaluate the toxicity effect of syrups. There is a direct correlation between cytotoxic effect and hemolysis. Costa-Latufo *et al.* (2002) put it as, if a test substance is cytotoxic, it might produce membrane instability and eventually causing hemolysis.

In this study, the sheep erythrocytes were used in hemolytic assay as described by He *et al.* (1994) and Ahmad *et al.* (1998). Based on literature studies, apart from sheep, other researchers used different sources to obtain erythrocytes, for instance, mice (Costa-Latufo *et al.*, 2002; Prokof'eva *et al.*, 2004; Costa-Latufo *et al.*, 2005), rainbow trout (*Oncorhynchus mykis*) (Kempton *et al.*, 2002), human (Costa-Latufo *et al.*, 2002) and pig (Diaz *et al.*, 2004). The occurance of hemolytic activity was determined by the absorbance of released hemoglobin measured at 405nm. Result obtained suggested that the non-copigmented and copigmented syrups did not induce lysis and cell

membrane damage, therefore, did not exert toxicity effect against erythrocytes. This finding is absolutely, totally in contrast with result obtained in the brine shrimp lethality assay and in contradiction with work done by Costa-Latufo *et al.* (2005) who carried out both of these assays and found that the results from both assays are following the same trend i.e. the extracts tested did not possess any activity against nauplii or against mice erythrocytes. Hence, these results suggested that the cytotoxicity effect displayed by samples of study towards the brine shrimp nauplii were probably caused by other mechanism (s) and not related to membrane instability.

CONCLUSION

AND

RECOMMENDATION

FOR FUTURE

RESEARCH

CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

In the present work, *Carissa carandas* syrups (56°Brix), with or without the additions of copigments i.e. quercetin, naringin, rutin, (+)-catechin, L-tartaric acid, caffeic acid, aluminium chloride, iron (II) sulphate, iron (II) chloride and gum arabic were examined for its anthocyanins stability during 90 days of storage, when exposed to a high temperature and in the presence of light. Based on observations supported by statistical analysis, copigmentation reaction seems to alter the stability of anthocyanins and this study strongly confirmed that all investigated copigments enhance the anthocyanins stability in syrups in all three conditions evaluated. Caffeic acid clearly appears to contribute to the highest anthocyanins stability which resistant to a high temperature, light exposure and maintained anthocyanins' colour the most for extended periods of time. Furthermore, caffeic acid conferred the greatest increase in the maximum absorption wavelength (bathochromic effect, $\Delta\lambda$) and absorbance (hyperchromic effect, ΔA) in the absorption spectrum of pelargonidin 3-glucoside, which characterized an intermolecular copigmentation reaction in *Carissa carandas* syrup. Increasing effect of lightness, coupled with increasing effects of red and yellow tonalities were clarified in the non-copigmented and copigmented syrups. Taken together, copigmentation reaction showed efficacy in present attempt to stabilize anthocyanins, but, microwave pasteurization also did contribute. However, it should be kept in mind that anthocyanins stability may also be influenced by other fruits' components, especially the interaction of ascorbic acid with anthocyanins, and subsequent mutual degradation has been reported in various fruit juice model systems including strawberry and blackcurrant products (Skrede *et al.*, 1992). The interaction of ascorbic acid with anthocyanins results in the degradation of both and a decrease in colour and nutritional quality of products (Markakis, 1992). Thus, further investigations are necessary to evaluate the effects of ascorbic acid which proved present in *Carissa carandas* fruits on the anthocyanins stability. All in all, the successful attempt to

stabilize anthocyanins in this present work may afford some insight into methods to improve anthocyanins stability and consequently will open a new window of opportunities for the use of anthocyanins as natural food colourants in various food applications.

Diets high in food with natural antioxidant substances will supply the body with the essential antioxidant components needed to supplement the immune system and prevent chronic degenerative diseases. The present results provide evidence that all copigmented syrups exhibit excellent to moderate antioxidative properties expressed either by their capacity to scavenge DPPH• or by the inhibition of lipid peroxidation. The antioxidative activity displayed by copigmented syrups are comparable, or in some cases, even better than BHT copigmented syrup. The remarkable activity may be provided by the stabilized anthocyanins. In addition, all added copigments confirmed to improve the antioxidative potential of syrups. Caffeic acid, rutin and (+)-catechin copigmented syrups proved to be a great antioxidant agent, where (+)-catechin copigmented syrup act as an excellent radical scavenger, while rutin copigmented syrup is effective to inhibit lipid peroxidation, whereas caffeic acid copigmented syrup is good in both actions. It is concluded that the presence of sugars results in the decreased of the antioxidative capacity of syrups. Further work especially *in vivo* studies are needed for better understanding on the mechanisms of actions of these highly potential antioxidant agents.

Moreover, this study is clearly demonstrated that the non-copigmented and copigmented syrups, including both the unripe and ripe fruits' juices were significantly reduced the duration of immobility in the FST and TST tests conducted on mice. In contrast with the antioxidant assays, not all added copigments effective to improve the antidepressant effect of syrups. In the FST test, caffeic acid, rutin, (+)-catechin, quercetin, naringin and gum arabic worked to improve the activity, whereas in the TST

test, only the addition of caffeic acid, rutin and L-tartaric acid seems to improve the antidepressant effect compared to the non-copigmented syrup. The unripe fruit's juice and caffeic acid copigmented syrup stands up as the best antidepressant agents and the activity is comparable to the reference antidepressant drug, imipramine. As mentioned, this study was conducted on male subjects. In this regard, there is still very few experimental evidence on the stress behaviour which linked to depression on female subjects, although in human, the depressive disorders affect more females than males (Morley-Fletcher *et al.*, 2004). This issue should be considered for future studies.

In this study, the non-copigmented and copigmented syrups seems to be cytotoxic against the brine shrimp nauplii and in contrary, evidently non-cytotoxic to sheep erythrocytes. The observed effects reflect the non-specific nature of cytotoxicity displayed by samples of study. Toxicity tests on different test organisms and assays using different cell lines could be employs to further verify the cytotoxicty of *Carissa carandas* syrups.

Taken all results in the present study into account, *Carissa carandas* fruits are evident to be useful and beneficial from the health point of view and definitely fit to market as a health drink. It certainly merits further research as not to let the nutritional benefits and therapeutic effects to waste.

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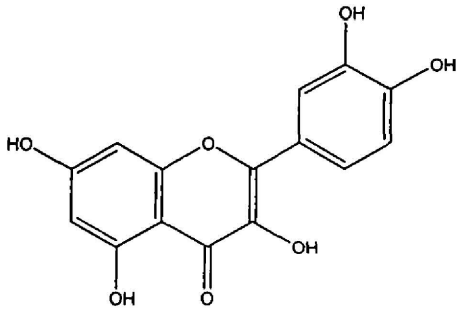
APPENDIX

Appendix A

Chemical structures of copigments



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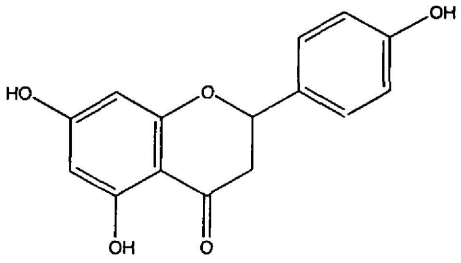

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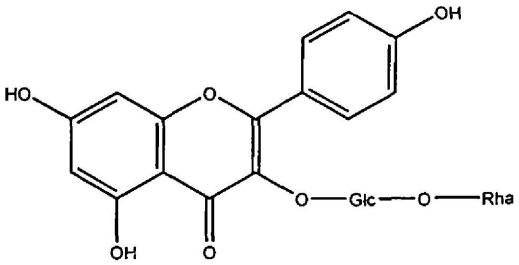

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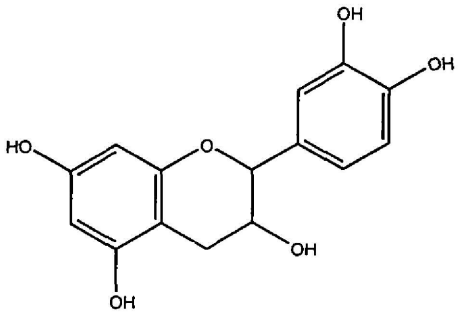
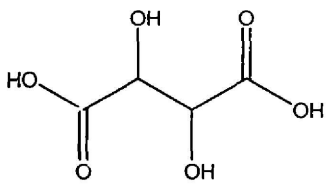
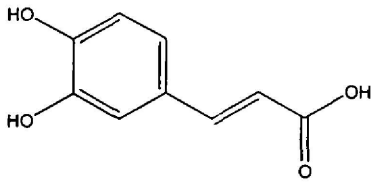


(+)-catechin



L-tartaric acid



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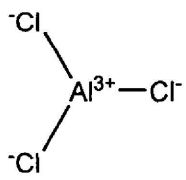
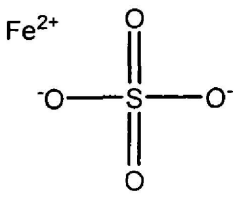


aluminium chloride



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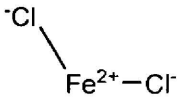

iron (II) chloride

PUBLICATIONS