The correlation between the antioxidant properties of the *Carissa carandas* extracts with their flavonoids occurrences

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The antioxidant activities of various extracts from different organs of *Carissa carandas* were screened for by using Ferric thiocyanate (FTC) and Thiobarbituric acid (TBA) methods. All the extracts showed strong antioxidant activities as compared to BHT (a commercial antioxidant) towards the autooxidation of linoleic acids. The FTC method measures the amount of peroxide produced during the initial stage of lipid oxidation. The lowest optical density value was indicated by the chloroform extract of the unripe fruit, followed by the 80% methanolic extract of the stem and the half-ripe fruit juice. The later stage of lipid oxidation, where peroxide composes to form carbonyl compound are measured using TBA method. The optical density value of the 80% methanolic extract of unripe fruit and the half ripe fruit juice are the lowest after 10 days of incubation. Flavonoid compounds from the half-ripe fruit juice were isolated and characterized as the extract performed the best activity in both methods. The identification of major flavonoid content was made based on the co-chromatography comparison with authentic markers, rf values, colours, ultra violet and mass spectrum analyses. The major compound in the extract was tentative identified as Apigenin 6-*C*-rhamnosil-7-*O*-rhamnoside. After 4 hours hydrolysis treatment, Apigenin 6-*C*-rhamnoside and rhamnose were identified. The UV spectrum analysis of this compound revealed the absence of NaOAc shift indicating that the 7-hydroxyl was substituted by the rhamnose. The minor components found in the extract

are Pelargonidin 3-*O*-glucoside, Chrysoeriol 7-*O*-glycoside and Quercetin 3-*O*-methy-7-*O*-glucoside.

INTRODUCTION

Much interest has been focused on the use of anti-free radical or antioxidant supplements as a form of protection against various diseases. Nutrients such as flavonoids, beta-carotene, vitamins C and E and zinc have the ability to neutralise the damaging effects of free radicals. Each of these nutrients can block the conversion of free radicals into damaging chemical compounds within the body, preventing oxidative damage to biomolecules such as proteins, lipids and DNA (Grossman *et. al.*, 2001). Among the sources of antioxidants are fruits, fruit juices, vegetables and legumes.

Several reputed antioxidants compounds, which belong to flavonoids group, are quercetin, rutin, kaempferol, anthocyanin, cathecin, fisetin and daidzein. Meanwhile, tocopherol is a commercial natural antioxidant, which belong to the terpenoid group and can be obtained from the plant lipid.

Carissa carandas is native to India and has been cultivated in a limited way in the tropical, subtropical and Mediterranean regions. It produces abundant berries through out the year in the monsoon tropical climate especially in the northern part of Malaysia. *Carissa carandas* is a perennial plant and very easily maintained hardy shrub. Pruning of the plant seemed to enhance the production of berries. The sour unripe fruit is reputed for its aphrodisiac, appetiser, antipyretic and astringent properties, and is used in the treatment of diarrhoea and intermittent fever (Jayaweera, 1981). The ripe fruit is used to treat mouth ulcer, sore throat and skin disorders. The root is used in healing stomach disorders and as an antihelmintic (Burkill, 1935). In this study, we examined and compared the antioxidant activities of various extracts of *Carissa carandas* and characterised their flavonoids and terpenoids contents.

MATERIAL AND METHOD

Plant extract

30g of the fresh stems, leaves and fruits samples were dried in the drying cabinet (Protech, Malaysia). The materials were then successively extracted in the soxhlet apparatus using *n*-hexane, followed by chloroform and 80% methanol. The extracts were then filtered using filter paper and concentrated in the rotary evaporator (Eyela, U.K.). The concentrated extracts were then evaporate to dryness in a fume cupboard

Ferric thiocyanate (FTC) method

The method of Kikuzaki and Nakatani (1993) was slightly modified. A screw-cap vial containing a mixture of 4mg (4ml) of sample (final concentration 0.02%) in 99.5% ethanol. 4.1 ml of 2.5% linoleic acid (Sigma, USA) in 99.5% ethanol. 8.0 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water was placed in an incubator (WTB Binder, Germany) at 40°C in the dark. To 0.1 ml of this mixture, 9.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% ammonium thiocyanate (Sigma, USA) were added. Three minutes after addition of 0.1 ml of 2X10-2 M ferrous chloride (Sigma, USA) in 3.5% hydrochloric acid to the reaction mixture, the absorbance was measured at 500 nm. This step was repeated every 24 h until the control reached its maximum absorbance value.

Thiobarbituric (TBA) method

Two ml of 20% trichloroacetic acid (Fisher, UK) and 2 ml of 0.67% TBA (Fisher, UK) solution were added to 2.0 ml from the mixture (containing sample) prepared in the FTC method (Ottolenghi, 1959). This mixture was kept in a water bath (100°C) for 10 min and after cooling to room temperature, it was centrifuged at 3000 rpm for 20 min. Antioxidant activity was based on the absorbance of the supernatant at 532 nm at one day after the final day of the FTC assay.

Purification and identification of flavonoid compounds

For purification the extracts will be applied as a streak on Whatman no 3MM paper and run in solvent BAW (*n*-butanol-acetic acid-water 4:1:5) overnight. The bands will be cut out and eluted in 80% methanol overnight. The eluates will be concentrated and again streaked and rerun on the Whatman no 3MM paper in the solvent 15% acetic acid. Then the separated band of phenolic compounds will be cut out and eluted in 80% methanol. To test the purity, the compounds will be spotted on small TLC plates and rerun with the following solvents: BAW, 15% acetic acid, water and phenol.

Acid hydrolysis

This method involved the acid hydrolysis treatment where the
flavonoid glycosides were separated to flavonoid aglycones and sugars.
The aglycones were identified

Identification of the sugars

Sugars occurred in the aqueous residues of the acid hydrolysed samples. The sugars were then redissolved with a few drops of water and were identified by chromatographic comparison with an authentic sugar mixture, in solvents TPBW (toluene: pyridine: butanol: water = 1: 3: 5: 3). After 48 hours, the chromatograms were developed and dried in the fume cupboard. To visualise the sugar, the dried chromatograms were dipped in aniline hydrogen phthalate solution and were heated in an oven for 10-15 minutes at 100°C. The papers were viewed under UV light to detect the clear sugar spots.

HPLC with photo-diode array detector

using a gradient method and a 3.9 x 300 mm reverse phase column with C18 phenyl packing material to confirm the identification

UV-visible spectrophotometry

with shirt reagents was used to determine the position of hydroxylation, methylation and glycosilation in flavonoid nucleus.

Statistical analysis

The results are the means ± SEM of triplicates from 2-4 independent experiments. Statistical values ($p<0.05$) were determined by one-way ANOVA using Graphpad Prism version 3.0

Figure 1. Antioxidant activity of *Carissa carandas* extracts using FTC method

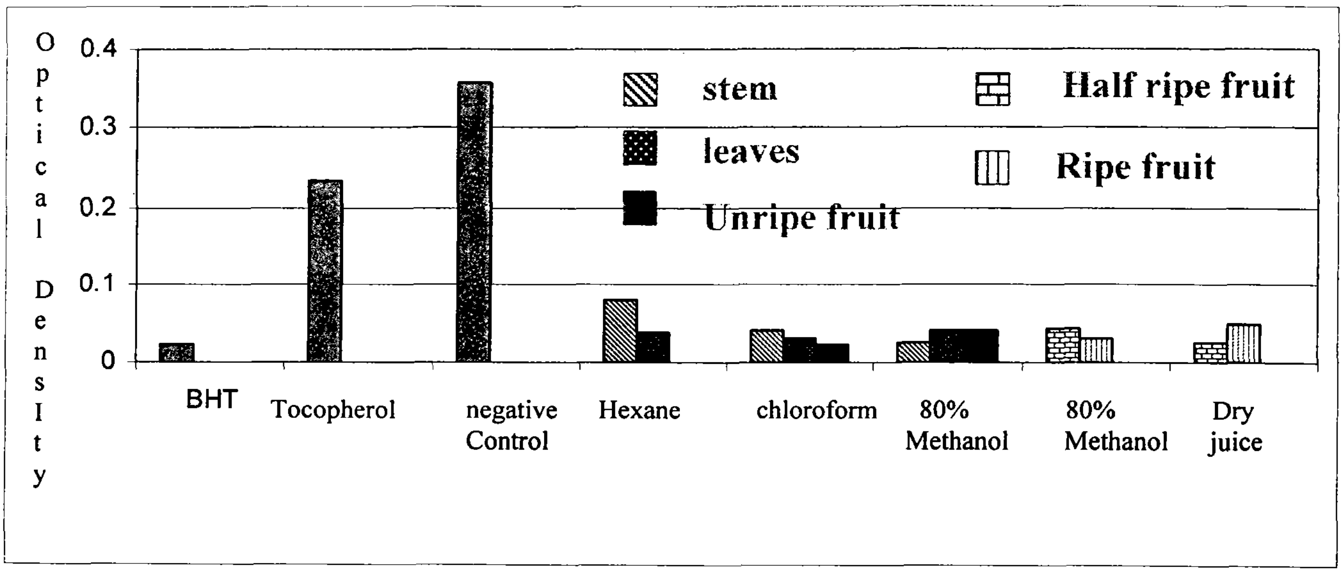
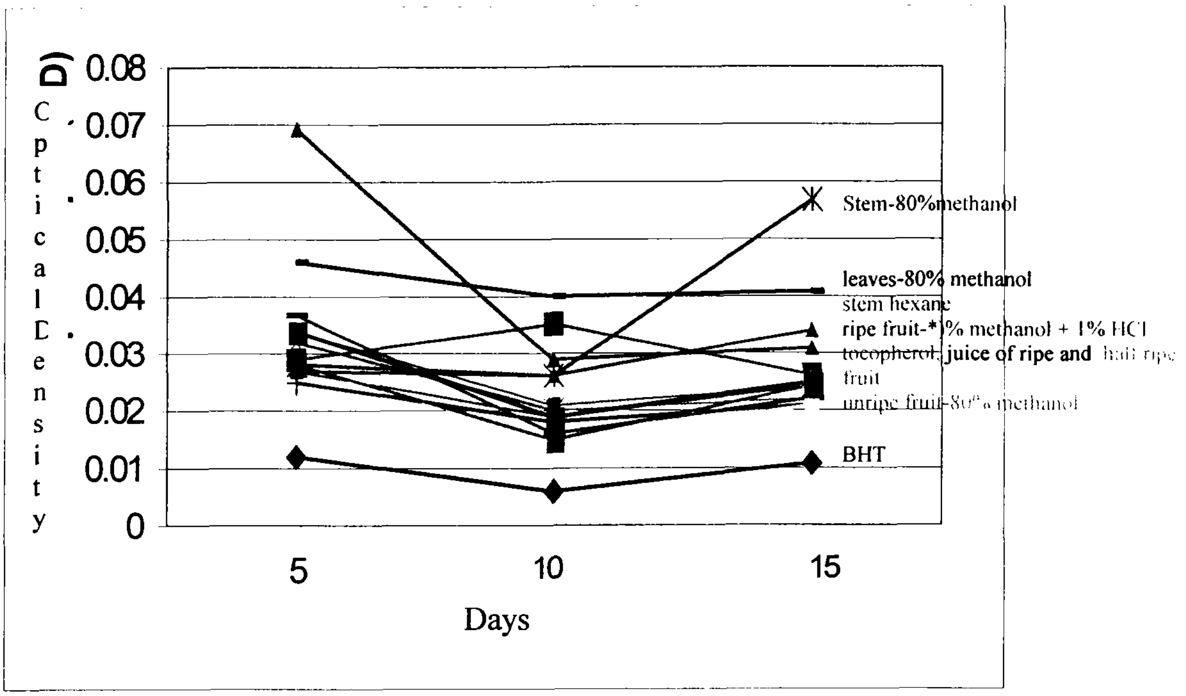


Figure 2. Antioxidant activity of *Carissa carandas* extracts using TBA method



RESULTS AND DISCUSSION

The result revealed that all of the *Carissa carandas* extracts showed strong levels of inhibitory activity towards lipid oxidation. The FTC method measures the amount of peroxide produced during the initial stage of lipid oxidation.

Figure 1 indicated that the antioxidant activity was still stable until 10th days of incubation. The lowest optical density value was indicated by the chloroform extract of the unripe fruit, followed by the 80% methanolic extract of the stem and the half ripe fruit juice. The later stage of lipid oxidation, where peroxide composes to form carbonyl compound are measured using TBA method. The optical density value of the 80% methanolic extract of unripe fruit and the half ripe fruit juice are the lowest after 10 days of incubation (Figure 2). Flavonoid compounds from the half-ripe fruit juice were isolated and characterized as the extract performed the best activity in both methods. The identification of major flavonoid content was made based on the co-chromatography comparison with authentic markers, rf values, colours, ultra violet and mass spectrum analyses. The major compound in the extract was tentative identified as Apigenin 6-*C*-rhamnosil-7-*O*-rhamnoside. After 4 hours hydrolysis treatment, Apigenin 6-*C*- rhamnoside and rhamnose were identified. The UV spectrum analysis of this compound revealed the absence of NaOAc shift indicating that the 7-hydroxyl was substituted by the rhamnose. The minor components found in the extract are Pelargonidin 3-*O*-glucoside, Chrysoeriol 7-*O*-glycoside and Quercetin 3-*O*-methy-7-*O*-glucoside.

Table 1 Identification of flavonoid glycosides in *Carissa carandas*

Flavonoids	Hydrolysis		Colour		Rf values			HPLC retention time
	aglycone	sugar	UV	UV + NH_3	BAW	15% HOAc	H_2O	
Pelargonidin 3-O-glucoside	Pelargonidin	glucose	Yellow	Bright yellow	44	42	0	15.5
Chrysoeriol 7-O-glucoside	Chrysoeriol	glucose	Yellow	Yellow	38	11	0	10.8
Quercetin 3-O-methyl 7-O-glucoside	Quercetin 3-O-methyl	glucose	Orange	Bright orange	33	7	0	15.6
Apigenin 6-C-rhamnosil 7-O-rhamnoside	Apigenin 6-C-rhamnoside	rhamnose	Deep purple	Bright greenish yellow	70	70	60	22.8

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