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***SHORT COMMUNICATION***

# STUDIES ON THE CAROTENOIDS AND *IN VITRO* ANTIOXIDANT CAPACITY OF PALMYRAH FRUIT PULP FROM MANNAR

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**Abstract**: The pulp of palmyrah (*Borassus flabellifer*.L) fruits collected from Mannar in the North-West of Sri Lanka was used for the study. The 4 types of palmyrah fruit collected in Mannar, represented >95% of all fruit types of palmyrah. Carotenoids of palmyrah fruit pulp were separated by open column chromatography. Identification and quantification of the carotenoids were carried out using a scanning spectrophotometer with the aid of a standard uv-visible spectral bank for carotenoids. Type I, II-A and III fruit pulps showed spectra dominated by phytoene and neurosporene and were largely non-provitamin A. Type II-B fruit pulp showed spectra high in phytofluene and had a retinol equivalent of 9.8.100$g^{-1}$ fresh weight from β-carotene and β-zeacarotene. The pulp contained both hydrophilic and lipophilic antioxidants with total antioxidant varying from 2.4-21.8 trolox equivalent.$g^{-1}$ determined by the 2-2$^1$ azino-bis-benzothiozoline-6-sulphonic acid free radical cation ($ABTS^{+\cdot}$). The antioxidant levels measured as trolox equivalents of the separated carotenoids gave values generally consistent with the extent of conjugation of double bonds.

**Keywords**: ABTS, antioxidants, carotenoids, Palmyrah fruit pulp, trolox equivalent.

The earliest report[1] (in 1986) stated that the yellow colour of Palmyrah Fruit Pulp (PFP) was due to carotenoids but provided no evidence. A later study[2] which measured the total carotenoid (unseparated) spectra of pulp from over 50 palmyrah fruits from all parts of the island reported that, although their colour varied from pale yellow to dark red, the total (mixed) carotenoid spectra were similar. However the total (unseparated) carotenoid content varied from 2-253 mg 100$g^{-1}$ dry weight. A report from Bangaladesh[3] quoted a total value of 7.6 mg carotenoids 100$g^{-1}$. In 2001[4], the carotenoids from a bulk sample of PFP from Kalpitiya were separated for the first time and found to contain α-carotene, ζ –carotene, lycopene and β-zeacarotene. Another report[5] gave nearly identical results from a bulk sample from Hambantota giving a retinol equivalent of 159. 100 $^{-1}$ dry weight.

The main stands of palmyrah occur in the Jaffna peninsula, Mannar and the Wanni. In Jaffna the stands showed only type I and type II fruits. The Wanni was inaccessible to the workers and they were also constrained to study PFP from Mannar. This is the first study on carotenoids from pulp that had been seperated from fruits classified according to morphological type.[2]

The only report on the antioxidant effect of PFP is an *in vivo* study of a sample of unknown origin.[6]

The objectives of this study were to: -

(i) Quantify the separated carotenoids of palmyrah fruit pulp from fruit types I, II-A, II-B and III[2] of Mannar (type IV[2] is found only in Kalpitiya) in order to determine their retinol equivalent.

(ii) Determine the *in vitro* antioxidant capacity of the total and lipophilic fractions of PFP and of their separated carotenoids.

Ripe palmyrah fruits (n=90) collected on the first day of fall was separated into the types I, II and III as described previously. Type II was divisible into 2 types. The type II-B fruit was distinct from II-A as it had a flatter distal end, with a large yellow marking. The rest of the fruit was black. It was noted that type II-A did not have a flattened distal end and had 3 symmetric yellow spots about 1.5–2 cm in diameter. This difference had not been described previously.

The fruits of types I, II-A, II-B and III were bagged separately using polythene and fertilizer bags and transported to Colombo within 20-24 h. These were stored at -20°C and not opened until

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the day of extraction of pulp. To extract the pulp the tough pericarp was removed and the pulp was scraped out with a spoon under dark conditions. No water was added. The pulp of fruits (n=6) of each type was filtered through muslin to separate the fibrous material, and pooled, homogenized and stored at –20°C under $N_2$. This pulp was used for antioxidant and carotenoid assays within 3 d of extraction.

The PFP sample (50 g) was ground into a slurry with celite (5-7 g) and cold acetone (50 mL x4) using a motar and pestle. The acetone extract was filtered through filter funnel (3 mm gauge) under suction. The filtrate was extracted into petroleum ether (PE) (60°C–80°C) (25 x4 mL) containing diethyl ether (DEE) (2 mL) and washed with a flow of distilled water until the aqueous layer was colourless. The petroleum ether layer was further washed with distilled water until it was devoid of acetone. The petroleum ether layer then rotor evaporated to 10 mL, and further evaporated (to 1 mL with $N_2$ flushing. The sample was stored at -20°C, under $N_2$.

A column, 14 cm x 1.5 cm diameter was packed with 1:1 mixture of celite 545 and MgO (AR) as matrix and covered with a layer of anhydrous sodium sulphate (1 cm). After equilibrating with petroleum ether (60°C–80°C) the carotenoid sample (1.0 mL) was eluted under the solvent gradient with increasing polarity from petroleum ether (PE) / diethyl ether (DEE) (1%, 2%, 4%, 6% DEE/PE) to petroleum ether / acetone (AC) (1%, 2%, 4%, 8%, 10%, 15%, 20%, 30%, AC/PE). The fractions (10 mL) were scanned with a double beam UV visible spectrophotometer (Shimadzu model 1601) for identification of characteristic of absorption patterns of carotenoids. Carotenoids were identified using (i) Peak wavelengths, (ii) Ratio of wavelengths (iii) Eluent solvent mixture as described above and (iv) Standard spectra.[7] Knowing the volume containing each carotenoid and the $A^{100}_{1cm}$ of each carotenoid, quantification was achieved.[7] Retinol Equivalent (RE) was calculated using 6μg and 12 μg carotenoid containing 2 and 1 β-ionone moiety respectively as being equal to 1RE.

A mixture of $ABTS^{+\cdot}$ [2, 2- azino-bis - (ethylbenzothiazoline–6sulphonic acid), di ammonium salt (2.27 mL) and $K_2S_2O_8$ (11.5 mL)] reacted for 16 h and generated the cationic free radical[8]. Then $ABTS^{+\cdot}$ solution was diluted with phosphate buffer saline solution to obtain an initial absorbance of 1.5 at 730 nm. Standards (100 mL) were reacted with the $ABTS^{+\cdot}$ solution (2900 mL) for 30 min. Absorbance was measured over 30 min at 5 min intervals. Fresh $ABTS^{+\cdot}$ solution was used in each analysis. Trolox 6 hydroxy–2,5,7,8,-tetramethyl chromane - 2-carboxylic acid (vit E analogue) (2.5 mM) was used as the standard. A standard curve of reduction in absorbance versus mg trolox gave $r^2 = 0.993$.

PFP (1.0 g) was extracted into methanol (5 mL x 3) and separated by centrifuging in a microcentrifuge. The collected extract was divided into two equal volumes. The methanol in one portion was evaporated to dryness using $N_2$ flow. The resulting residue was dissolved in methanol (1.0 mL) and aliquots were tested for the reduction in absorbance as before.

The remaining portion of methanol extract was extracted with petroleum ether (60°C-80°C) (5 mL x 2). The extract was evaporated to dryness by $N_2$ flushing and the residue dissolved in methanol (1-20 mL) as required. The antioxidant capacity of mixed carotenoids was determined as the procedure described above. Individual carotenoid samples obtained from open column chromatography (and identified) were evaporated to dryness as before, dissolved in methanol (5.0–50.0 mL) and $ABTS^{+\cdot}$ reducing capacity was measured.

Carotenoid content is shown in table 1. Type I, II-A and III showed a similar profiles and had insignificant retinol equivalent. The other noteworthy characteristic of the above type was the high phytoene (first carotenoid on biosynthetic pathway) and low phytofluene (second carotenoid on biosynthetic pathway). Type II-B on the other hand had high phytofluene, no phytoene but contained pro-vitamin carotenoids (retinol equivalent = $9.8.100g^{-1}$ fresh weight)

**Table 1: Carotenoid profiles of PFP types**

| | Content (μg. 100 $g^{-1}$ FW PFP) | | | | |
|---|---|---|---|---|---|
| Carotenoids | I | II A | III | II B | Antioxidant activity Trolox eq. $g^{-1}$ PFP |
| Phytoene | 286 | 461 | 353 | ND | 27 |
| Phytofluene | 30.6 | 48.8 | 9.0 | 474 | 13 |
| β -carotene | 4.2 | 5.0 | ND | 43.8 | 840 |
| ζ-carotene | 64.6 | 176 | 19.5 | 165 | 480 |
| Neurosperene | 79.8 | 96 | 183 | 690 | 235 |
| β zeacarotene | ND | ND | ND | 30 | 250 |
| Unidentified | ND | ND | ND | 35 | 200 |
| Retinol equivalent (100$g^{-1}$ FW) | 0.7 | 0.8 | 0.0 | 9.8 | |

ND= Not Detected.

Results show that the carotenoid profiles (in distribution and amounts) of PFP from Mannar differ from those reported for bulk samples from Kalpitiya[4] and Hambantota[5]. What is interesting is that from the pulp of the 4 main fruit types from Mannar, two carotenoid patterns emerge. It appears that the conversion from phytoene to phytofluene is apparently slow in types I, II-A & III giving a build-up of phytoene. In type II-B the biosynthetic pathway apparently proceeds faster in the phytoene to phytofluene step which probably has an influence on the entire profile resulting in higher pro-vitamin A carotenoids in type II-B. This may also be influenced by the high concentration of neurosporene which is the branch point of the carotenoid biosynthesis pathway (Table 1). Retinol equivalent is significant only in type II-B.

The total antioxidant capacities of the types I, II-A, II-B and III were 5.4, 2.4, 15.4 and 21.8 trolox equivalent $g^{-1}$PFP (FW) respectively and their lipophilic antioxidant capacities were 3.2,0.6,8.1 and 14.0 trolox equivalent $g^{-1}$ PFP respectively the latter contributing 25-75% of the total antioxidant capacity.

It was clear that hydrophilic antioxidants are present. Absence of browning in PFP on standing for days could rule out the presence of polyphenols. However, PFP is known to have 15-20 mg 100$g^{-1}$ vitamin $C^{content}$. The study of hydrophilic antioxidants were not pursued further as vitamin $C^{content}$ could account for water soluble antioxidant capacity. The total lipophilic antioxidant capacity was highest in type II-B as would be predicted from the structure of the respective carotenoids present.

The trolox equivalent should be proportional to the number of conjugated double bonds. Although this is generally so, this is not so for phytoene (too high) and phytofluene (Table1). Phytoene (colourless) is the first to emerge from the column and it is conceivable that it can be contaminated by a non-uv-visible absorbing very lipophilic antioxidant contaminant. The inversion of values for nuerosporene and ζ-carotene are

harder to explain. This was despite care being taken to ensure that carotenoids in methanol were maintained at low concentrations so that they are soluble in the $ABTS^{+\cdot}$ reaction mixture.

## CONCLUSION

There are two distinct patterns of carotenoid profiles for PFP from Mannar. Only type II-B had significant pro vitamin A potential.

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