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SERIES NO. 1

IDENTIFICATION AND HPLC QUANTIFICATION OF CAROTENOIDS OF THE FRUIT PULP OF *CHRYSOPHYLLUM ROXBURGHII*

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Abstract: Carotenoids of the fruit pulp of *Chrysophyllum roxburghii* (Sinhala: *lavalu*) amounted to about 180 mgkg^{-1} by fresh weight. The carotenoids were isolated by open column chromatography (MgO:Celite 1:1) using mixtures of petroleum ether 40-60 °C and acetone and identified by UV/visible spectra, chemical tests, and High Performance Liquid Chromatography (HPLC) using authentic standards and a photodiode array detector (PAD). The major carotenoid was *trans*-violaxanthin (113 mgkg^{-1}). Also present was *cis*-violaxanthin, neoxanthin, β-cryptoxanthin monoepoxide, lutein, β-cryptoxanthin, ζ-carotene and β-carotene. The retinol equivalent of the pulp was only 68 RE/100 g. The study shows that *Chrysophyllum roxburghii* is not a good source of pro-vitamin A. Further as violaxanthin is reported to be not absorbed by humans, it is of no use as a dietary antioxidant. However, as *trans*-violaxanthin can be obtained in quantity in the pure crystalline state, directly from the column and has the benefit of eluting at 20% acetone away from most carotenoids, the compound will be useful as a standard for HPLC analysis of carotenoids from other fruits and leaves.

Key words: carotenoids, *Chrysophyllum roxburghii, Lavalu,* violaxanthin.

INTRODUCTION

Chrysophyllum roxburghii (Indian star apple) and *Chrysophyllum monopyrenum* (wild star apple) both known as *Lavalu* (Sinhala) are the two species of *Chrysophyllum* found in the Indian sub continent.[1] *C. lanceolatum* is a synonym for *C. roxburghii.*

No studies have been reported in published, refereed journals on the carotenoids of *C. roxburghii* fruit. Howerver, a thesis[2] states that the fruit has at least 18 carotenoids and a total carotenoid content of 112 mgkg^{-1} (as β-carotene). The carotenoids reported are phytoene, phytofluene, α-carotene, β-carotene, ζ-carotene, pro-γ-carotene, 5,6 epoxydihydro β-carotene, 5, 6, 5', 6' epoxy diepoxy tetrahydro β-carotene, β-cryptoxanthin, microxanthin, zeaxanthin, and 3-hydroxy sintaxanthin. Some of these are not listed in a recent compendium on carotenoids naturally occurring in plants.[3] Further, the experimental techniques and identification procedures used in the above study are not in line with modern analysis procedures. There is, therefore, a possibility that some of these are artifacts of isolation.

The present study deals with the characterization of the carotenoids of Sri Lankan *lavalu* (*Chrysophyllum roxburghii* G. Don) as part of our overall research program to study the carotenoid composition of Sri Lankan yellow fruits and leafy vegetables. It is especially important in Sri Lanka and other developing countries to identify as many yellow fruits to determine whether they can be used as a pro-vitamin A source or antioxidant. The objective of this study was to determine the carotenoid profile of *lavalu* using chromatography, chemical tests, UV spectrometry and High Performance Liquid Chromatography (HPLC) with authentic standards. HPLC with UV-visible photodiode array (PDA) detector was also used for the identification and quantification.

METHODS AND MATERIALS

Isolation of standards by Open Column Chromatography (OCC): Parsley (*Petroselinum crispum* L.) was used as the source of standards (neoxanthin, violaxanthin, lutein and β-carotene) because it is easy to extract and has high carotenoid content. To obtain the standards, the carotenoids were extracted with cold acetone, partitioned to petroleum ether, concentrated in the rotary evaporator and separated in an open column of MgO (Maerk Germany): Celite (1:1 activated for 2 h at 110 °C).[3,4]

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Preparation of column: A chromatographic glass tube was mounted on a suction flask. A small glass wool plug was placed at the bottom of the chromatographic tube. The separating medium (MgO: Celite 1:1) was added loosely up to a height of 20 cm and continuous moderate vacuum was applied from a water aspirator. A flat instrument was used to press down the absorbent and flatten the surface. The column was topped with a 1 cm layer of anhydrous sodium sulphate to ensure that no residual water gets into the adsorbent. The column was pre-equilibrated with petroleum ether (boiling range 40-60 °C).

Developing the column: The carotenoid solution was carefully poured into the column and the sample layer was allowed to reach almost to the surface of the sodium sulphate layer. The column was eluted with 100% petroleum ether, gradient of 1%-8% diethyl ether in petroleum ether and 1-50% acetone in petroleum ether.

An aliquot was taken from each isolate to verify purity by HPLC. All aliquots were dried under N_2 and immediately before injection, dissolved in 1 ml HPLC grade acetone, filtered through a 0.22 μm polyethylene teflon (PTEF) syringe filter (Millipore) directly to the sample vials and 10 μl injected into the HPLC. Once the desired purity was confirmed using HPLC, the concentration of the pure standards were determined spectrophometrically, using the following $A^{\%}_{cm}$ values: β-carotene, 2592 in petroleum ether; lutein, 2550 in ethanol; violaxanthin, 2550 in ethanol: neoxanthin, 2443 in ethanol. Aliquots of the standard mixtures were quantitatively transferred into screw-capped culture tube, dried under nitrogen and stored in freezer until use.

Calculation of carotenoid concentration:

$$X\ (\mu g) = \frac{A \times Y\ (ml) \times 10^6 \times \text{purity}}{A^{1\%}_{cm} \times 100}$$

X is weight (μg) of the carotenoid standard, Y is the volume of the solution that gives an absorbance of A at specified wavelength, $A^{1\%}_{cm}$ is the absorption coefficient of the carotenoid in the solvent used.

A_x = peak area of the carotenoid
C_s = concentration of the standard
A_s = peak area of the standard

Preparation of standard solution and construction of the standard curves for qualitative analysis of carotenoids from lavalu (violaxanthin, lutein, neoxanthin and β-carotene):

HPLC chromatogrms of the carotenoid standards are shown in Figure 1.

Construction of standard curve: Aliquots of the carotenoid isolates (in petroleum ether) were taken in volumes which gave the relative proportion found in the sample, mixed, concentrated, made up to 50 ml. For the standard curves, duplicate aliquots of 1, 2, 3, 4, and 5 ml were transferred to culture tubes, dried under N_2, and just before injection, dissolved in 1 ml of acetone and filtered through a 0.22 μm PTFE syringe filter (Millipore); 10 μl was injected into the HPLC equipment.

Identification and quantification of the carotenoids of lavalu (Chrysophyllum Roxburghii G. Don):

Qualitative analysis: Ripe fruits of *lavalu* were selected from Piliyandala and Kadawata areas in Sri Lanka. The fruits were hard, oval shaped with a small point at the apex. The diameter of the fruits varied from 5-13 cm with 4-8 seeds arranged steliately inside the flesh. Fruits were quartered and seeds and peel removed from two opposite sections of each fruits and the pulp homogenized in a blender. Fruit carotenoids are complex, qualitatively different and esterified. Analysis was carried out according to Rodriguez–Amaya (1999).[3] The procedure involved the following. Homogenized fruit pulp (125 g) containing carotenoids was extracted with cold acetone, partitioned to petroleum ether, saponified with an equal volume of 10% KOH in methanol at room temperature overnight, washed, concentrated, dried with Na_2SO_4 and separated on a MgO:celite as described above. Identification of carotenoids were carried out by the methods of Rodriguez-Amaya (1999).[3b] This study involved the combined use of the retention times, co-

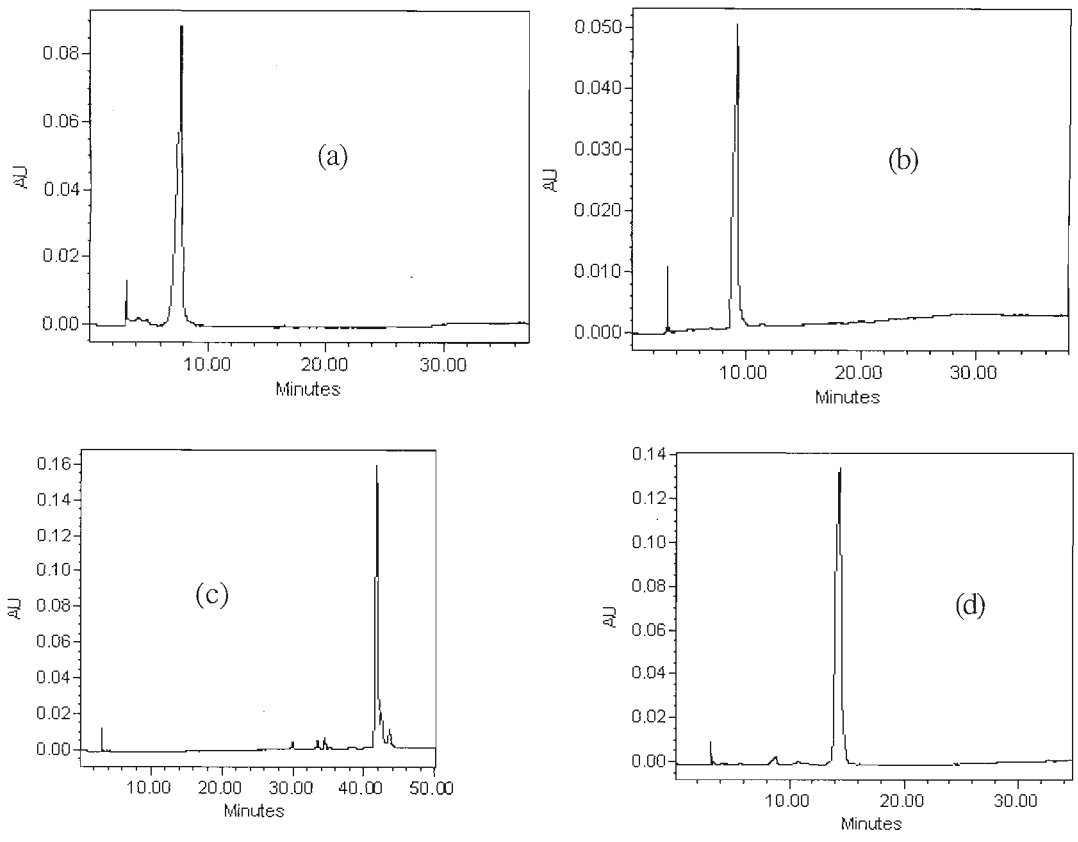

Figure 1: HPLC chromatograms of the carotenoid standards from Parsley (a) neoxanthin (b) violaxanthin (c) β-carotene (d) lutein

chromatography with authentic samples of neoxanthin, violaxanthin, lutein and β-carotene, the visible absorption spectra, and for xanthophylls, chemical tests such as acetylation with acetic anhydride of secondary hydroxy groups (as in lutien, violaxanthin, neoxanthin), methylation with acidic methanol of allylic secondary hydroxyl groups (as in lutein), and epoxide-furanoid rearrangement of 5,6, epoxy groups (as in violaxanthin and neoxanthin)

Quantitative analysis: To determine the mean carotenoid composition four batches of samples were analysed in duplicate. For each sample, fruit were quartered, seeds and peel were removed from two opposite sections of each fruit and the pulp was homogenized in a blender. Approximately 5 g (weighed accurately) were used for extraction. Extraction and saponification were carried out as described under qualitative analysis. Then 5.0 ml of sample was evaporated to dryness immediately before injection to the HPLC, dissolved in a solution of acetone after filtering using a syringe filter.

HPLC conditions: The HPLC analysis was performed on Waters separation module (model 2690) equipped with quaternary pump, four channel in-line vacuum degasser, and auto sampler injector, controlled by Millenium 2010 workstation using a monomeric C_{18} column (Waters Spherisorb S_3 ODS2), 3 μm, 4.6 x 150 mm. The mobile phase consisted of acetonitirile, methanol, and ethyl acetate containing 0.05% of TEA (triethylamine) used at a flow rate of 0.5 ml/ min. A gradient was applied from 95:5:0 to 60:20:20 in 20 min, maintaining this proportion until the end of the run. A UV-visible photodiode array detector (Waters model 996) with a millennium 32 software was used.

Chemical tests: Chemical tests were conducted to verify the type and position of substituents in xanthophylls and iodine-catalyzed isomerization reaction was carried out to verify the geometric configuration as follows[3]:

Acetylation of primary and secondary hydroxyl groups: The carotenoid (about 0.1 mg) was

dissolved in 2 ml pyridine and 0.2 ml acetic anhydride was added. The reaction mixture was left in the dark at room temperature for 21 h. The carotenoid was then transferred to petroleum ether, concentrated, and applied on a silica thin layer plate. The developing solvent was 5% methanol in toluene. The number of hydroxy groups were determined by the number of acetyl derivative TLC spots as the derivatisation agent was limiting.

Methylation of allylic hydroxyl groups: The carotenoid was dissolved in 5 ml methanol and a few drops of 0.2 N hydrochloric acid was added. The reaction was allowed to proceed at room temperature in the dark for 3 h. The carotenoids were then transferred to petroleum ether and subjected to TLC as described above.

Epoxide-furanoid rearrangement: The carotenoids were dissolved in ethanol and UV/visible spectrum (300-600 nm) was recorded. A few drops of 0.1 N hydrochloric acid was added. The spectrum was again recorded after 3 min. For each epoxide group a twenty nanometer hypsochromic shift was manifested.

Iodine-catalized cis-trans isomerization: A few crystals of iodine were dissolved in petroleum ether. The carotenoid spectrum was recorded in petroleum ether and a drop of the iodine solution was added. The spectrum was taken after 3 min exposure to light. The λ_{max} of *trans* will shift 3-5 nm to a lower wavelength and of *cis* to a 3-5 nm higher wavelength.

RESULTS AND DISCUSSION

Figure 2 shows a HPLC chromatogram of the *lavalu* carotenoids and Table 1 represents identification. The main carotenoid in *lavalu* was identified as violaxanthin. Quantification of

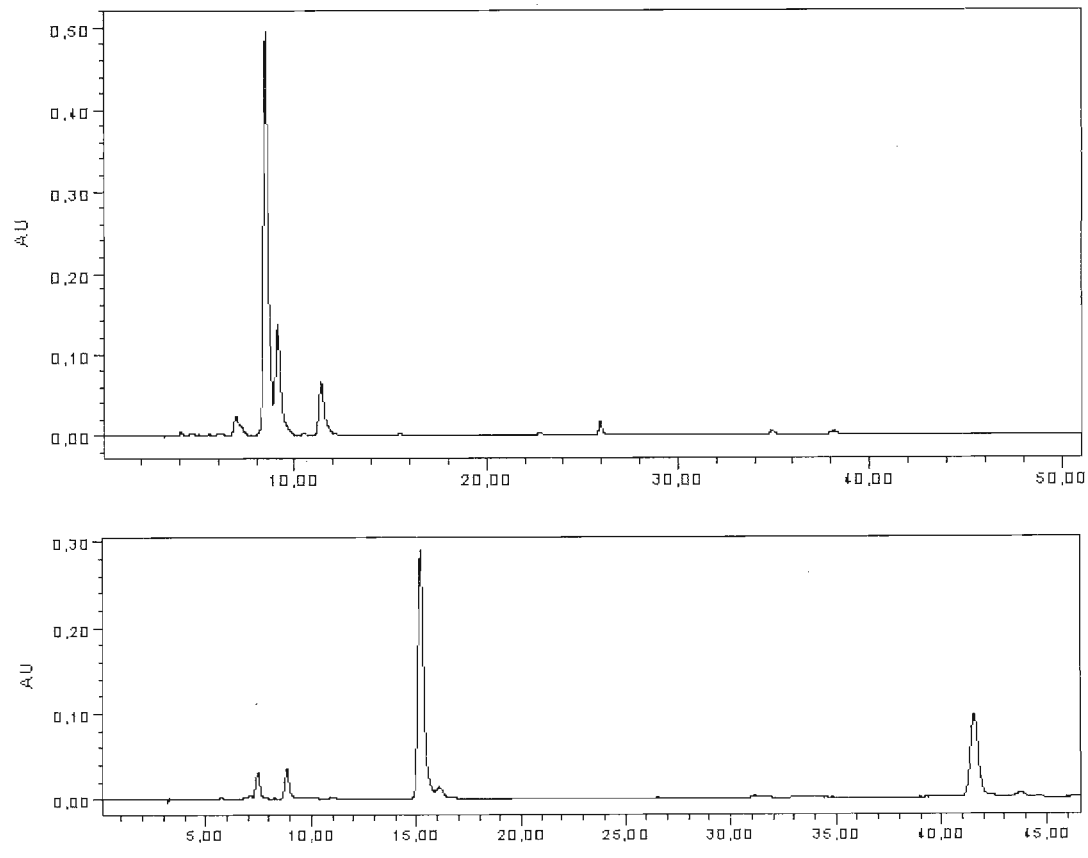

Figure 2: HPLC chromatogram of the (a) isolated standards (b) carotenoids of an extract of *lavalu* (*Chrysophyllum lanceolatum*). Peak identifiction
1. neoxanthin 2. violaxanthin 3. *cis*- violaxanthin 4. β-cryptoxanthin monoepoxide 5. lutein 6. β-cryptoxanthin 7. ζ-carotene 8. *trans*-β-carotene

Table 1: Properties of lavalu carotenoids

Peak	% of total carotenoids	possible carotenoid	λ_{max} in petroleum ether	λ_{max} in mobile phase	Chemical tests
1	3.0%	neoxanthin	413, 436, 465	414,438.6,466.6	Positive to 5,6 epoxide test(1 group), Positive to acetylation test (3 OH groups) Positive to *trans* form
2	62.9%	*trans*-violaxanthin	416,439,469	417.5,441.6,470.8	Positive to 5,6 epoxide test(2 groups) Positive to acetylation test (2 OH group) Negative to methylation,Positive to *trans* form
3	19.7%	*cis*-violaxanthin	328,408,437,465.8	327.8,408,437.4,465.8	Positive to 5,6 epoxide test (2 groups) Positive to acetylation test (2 OH groups) Negative to methylation,Positive to *cis* form
4	11.2%	β-cryptoxanthin monoepoxide	423.3, 447.6,475.8	423,447.8,476	Positive to 5,6 epoxide test(1 group), Positive to acetylation test (1 OH group) Negative to methylation, Positive to *trans* form
5	0.3%	lutein	422,445,473	420, 447, 475	Positive to acetylation test (2 OH groups) Positive to methylation, Positive to *trans* form
6	1.6%	β-cryptoxanthin	428.1,454.4,481.0	(424), 454.4,481.2	Positive to acetylation test (1 OH group) Negative to methylation, Positive to *trans* form
7	0.6%	ζ-carotene	379.4,400.6,424.8	395, 400.7,425.9	Positive to *trans* form
8	0.7%	β-carotene	(424),448,476	(425), 454.9,480.3	Positive to *trans* form

Peaks 1,2,5 and 8 identified using isolated standards. The rest of the peaks UV/visible spectra in mobile phase (using PDA detector).

neoxanthin, violaxanthin, lutein and β-carotene was carried out using HPLC. Table 2 shows the contents of the carotenoids of *lavalu*.

The technique used in a previous study[2] is cause for concern. It was felt that by not washing the carotenoids at the earliest, immediately after saponification could have caused artifacts. Further identification in that study was supported by only 3 standards (for 18 isolates). The non-identification of the major compound violaxanthin in that study[2] was surprising, as it should have eluted in the acetone: petroleum ether 20: 80 fraction.

Table 2: Carotenoid concentrations in *lavalu* (*Chysophyllum roxburghii*)

Carotenoids	Concentration ($mgkg^{-1}$ wet weight) Mean ± SD
violaxanthine	31.3 ± 5.5
neoxanthin	113.3 ± 17.2
lutein	0.4 ± 0.1
β-carotene	1.4 ± 0.3

n=4

The present study shows that violaxanthin (a non pro-vitamin A carotenoid) dominates. This includes *trans*-violaxanthin (62% w/w) and *cis*-violaxanthin (20% w/w) of total carotenoids. Percentages given for individual carotenoids were from HPLC readings (Millennium 32 software) directly. *Cis*-violaxanthin was identified using *cis* peak at 328 nm of UV/visible spectrum and iodine catalised isomerisation reaction. All *trans* - violaxanthin was obtained directly from the column as pure crystals. Since it is very polar and easily isolated pure, it could be used as standards in carotenoid analysis.

The percentage of β-carotene in C. *roxburghii* 0.8% and other pro-vitamin A carotenoids were 11%. This represents very low retinol equivalent (68 RE/100 g). As such it cannot be advocated as pro-vitamin A supplement as done in Phillipines (Rodeiguez-Amaya, personal communication). Further, the structural features of the carotenoids make bioconversion studies with animals or humans futile.

There is also a doubt that violaxanthin is not absorbed as such by humans[4], and this may be the reason for there being no information regarding its health implications. Therefore antioxidant action of these fruit carotenoids is also questionable.

Perhaps the best use of the *lavalu* carotenoids is as a food colourant for oil based foods.

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