Characterisation of Carotenoid and Total Retinol Equivalent Content in *Ulam* and Medicinal Species as Alternative Food Intervention to Combat Vitamin A Deficiency

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Abstract: Vitamin A deficiency (VAD) is one of the continuous leading causes of children and pregnant women death. To overcome this malnutrition which currently affected one-third of the world population, there is always renewed interest in exploring numerous dietary sources rich in carotenoids which some of them serve as pre-cursors to vitamin A (pro-vitamin A). It is important that affordable staple foods be as nutritious as possible because poverty limits food access for much of the developing world's population. Therefore, this study was aimed to explore various dietary sources for carotenoids in 28 *ulam* and medicinal species which are commonly consumed by the local folks. Carotenoid extraction using organic solvents was performed and analysis employed in this study through High Performance Liquid Chromatography revealed seven types of carotenoids in the food matrices; neoxanthin, violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene. Interestingly, these carotenoids profiles were found in varying concentration and composition in different species as well as in different period or season. Total carotenoids content quantified in all of the samples lies between 1.315 ± 0.007 to 190.301 ± 3.427 µg/g DW where *cekur manis* has the highest content. The total vitamin A activity (in terms of retinol equivalent, RE) of every species is also included in this study. The results suggested that at least 20 of the *ulam* and medicinal species may be used as alternative food intervention to eliminate VAD as a public health concern.

Keywords: Carotenoid, Retinol Equivalent, Vitamin A Deficiency, pro-vitamin A, *Ulam.*

INTRODUCTION

Human body normally obtain vitamin A either from animal-based substance known as preformed vitamin A (esters of retinols) or plant-based substance known as provitamin A carotenoid (β-carotene). Both are ready to use and derived from the breakdown of β-carotene which can be synthesized naturally by plants and microorganisms. Vitamin A possesses a broad-based preventive medicine agent in human diet either in terms of its health or nutritional impact. It quenches free radicals and prevents cellular oxidative damage, supporting the human immune system and normal development, and has anti-cancer activity [1-6]. The Recommended Dietary Allowance (RDA) for vitamin A is 1000 retinol equivalents, equal to 6 mg β-carotene, per day [7]. Insufficient vitamin A in the diet causes symptoms ranging from night blindness, xerophthalmia, keratomalacia to total blindness [8] and in some cases premature death [9]. It is also strongly correlated with an increased susceptibility to diarrhoea, respiratory diseases and childhood diseases such as measles [10].

The major carotenoids important to humans are α-carotene, β- carotene, lycopene, lutein, zeaxanthin and β-cryptoxanthin [11, 12]. Up to now, the well-established function of carotenoids in the human diet is the provitamin A activity associated with β-carotene, α-carotene and β-cryptoxanthin [13]. Therefore, improving nutritional quality of food crops and its ingredients for human consumption is one of the urgent health issues and high priority areas of research worldwide [14, 15]. On top of that, many people in most developing countries survive largely on plant-based diets or monotonous consumption such as cereals, legumes, starchy roots and tubers that are poor sources of vitamin A which can lead to malnutrition. It is important that affordable staple foods be as nutritious as possible because poverty limits food access for much of the developing world's population. So far, plant systems bear one of the greatest factories of vitamin production for the future. On this basis, it is ideal to manipulate provitamin A carotenoid synthesis in plants and as such this has become the target for human health rather than retinol synthesis [16].

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Until recently, horticultural and agricultural programs, in combination with biotechnology, have been used primarily for combating vitamin A deficiency [17]. Modern genetic alterations are being used to modify cereals such as rice and potato to contain high β-carotene and iron [18]. All these approaches have the potential to have a major impact on the micronutrient intakes of population groups who derive at least 50 % of their dietary energy from a single cereal staple, such as maize, rice or cassava [19]. There is now increasing interest in developing high yielding genotypes of indigenous wild plants resistant to drought and heat, which are rich sources of iron and zinc as well as provitamin A carotenoid [19-22]. *Ulam* or traditional vegetables of the Malays in Malaysia comprise more than 120 species representing various families, from groundcovers, shrubs up to large trees is one of those genotypes. The leaves, shoots, flowers, fruits up to roots or rhizomes of the vegetables are eaten fresh. They are consumed because of their taste, which adds variety and flavour to the diet, as well as for their health benefits. *Ulam* such as pegaga (*Centella asiatica*), daun selom (*Oenanthe javanica*), kacang botor (*Psophocarpus tetragonolobus*), ulam raja (*Cosmos caudatus*), daun kesum (*Polygonum minus*), daun kaduk (*Piper sarmentosum*), pucuk gajus (*Anacardium occidentale*) and tenggek burung (*Melicope ptelefolia*), are among the favourite *ulam* among Malays as well as rich sources of provitamin A carotenoid [23]. Unfortunately, little information is available about the nutritional values of *ulam* in Malaysia. According to [24] *ulam* refers to any vegetables that been eaten raw as salad or cooked in the Malaysian multiracial cultures. *Ulam* can also be cooked in dishes, come from various parts of the vegetable plants and are well known for improving one's health and also famous as they were claimed to exert anti-aging properties. Even though they are popular among local consumers, yet the availability of scientific findings on evaluation of their medicinal properties or specifically carotenoids activities are still lacking. Therefore, the aim of this research is to explore the composition and concentration of carotenoids in selected *ulam* species which are commonly consumed by the local folks as potential alternative sources to combat vitamin A deficiency especially in remote areas in Malaysia, as well as recommended amount to be taken per day.

MATERIALS AND METHODS

Sample Preparation

All edible parts of *ulam* samples (Table **1**) were freeze-dried for 72 hr, after which the samples were ground into fine powder and kept at -20°C until further analysis.

Table 1: List of Malaysian Traditional Vegetables (*ulam*) and Medicinal Species

Botanical name	Local name
Traditional Vegetables	
Allium cepa	Daun bawang
Allium tuberosum	Kucai
Anacardium occidentale	Gajus
Andrographis paniculata	Hempedu bumi
Apium graveolens	Saderi
Brassica chinensis	Sawi
Centella asiatica	Pegaga
Cosmos caudatus	Ulam raja
Daucus carota	Lobak merah
Durio zibethinus	Durian
Euodia redleyi	Tenggek burung
Ipomoea batatas	Ubi keledek
Lactuca sativa	Salad
Morinda citrifolia	Mengkudu
Murraya koenigii	Kari
Ocimum americanum	Kemangi
Ocimum basilicum	Selasih
Oenanthe javanica	Selom
Oroxylum indicum	Beko
Piper sarmentosum	Kaduk
Pluchea indica	Beluntas
Polygonum minus	Kesum
Sauropus androgynus	Cekur manis
Zea may	Jagung
Medicinal species	
Ficus deltoidea	Mas cotek
Pereskia sacharosa	Jarum tujuh bilah
Piper betle	Sirih
Ruta angustifolia	Garuda

Extraction of Carotenoids

The extraction procedure essentially follows the methods described by Othman [25], with some modifications. Each powdered sample weighed 0.1 g was rehydrated with distilled water and extracted with a mixture of acetone and methanol (7:3) at room temperature until colorless. The crude extracted was then centrifuged for 5 min at 10 000 g and stored at

4ºC in the dark prior to analysis. To extract carotenoids, an equal volume of hexane and distilled water was added to the combined supernatants. The solution was then allowed to separate and the upper layer containing the carotenoids was collected. The combined upper phase was then dried to completion under a gentle stream of oxygen-free nitrogen.

Determination of Total Carotenoid Content

Total carotenoid concentration was determined by spectrophotometry as described by Fatimah [26]. The dried carotenoid was resuspended in 300 µl of ethyl acetate and for determination of total carotenoid, 50 µl of the redissolved sample was then diluted with 950 µl chloroform for spectrophotometric analysis. Carotenoid containing solutions were measured at three different wavelengths, λ: 480 nm, 648 nm and 666 nm using Varian Cary 50 UV-Vis spectrophotometer. The Wellburn Equation [27] in chloroform was applied to obtain the total carotenoid content as described below:

$C_a = 10.91A_{666} – 1.2A_{648}$

$C_b = 16.36A_{648} – 4.57A_{666}$

$C_{x+c} = (1000A_{480} – 1.42C_a – 46.09C_b)/202$ (µg/ml)

Saponification

Samples were saponified with a mixture of acetonitrile and water (9:1) and methanolic potassium hydroxide solution (10% w/v). Base carotenoids were then extracted by addition of 2 ml hexane with 0.1% butylated hydroxytoluene (BHT), followed by addition of 10% NaCl until phase separation was achieved. The extracts were washed with distilled water, dried under a gentle stream of oxygen-free nitrogen and re-suspended in ethyl acetate for spectrophotometry and High Performance Liquid Chromatography (HPLC) analysis as described detail in Othman [25].

HPLC Analysis

The HPLC analysis of saponified carotenoids were performed on an Agilent model 1200 series comprised of a quarternary pump with autosampler injector, micro-degassers, column compartment equipped with thermostat and a diode array detector. The column used was a ZORBAX Eclipse XDB-C_{18} end capped 5 µm, 4.6x150 mm reverse phase column (Agilent Technologies, USA). The eluents used were (A) acetonitrile:water (9:1 v/v) and (B) ethyl acetate. The column separation was allowed via a series of gradient such as follows: 0-40% solvent B (0-20 min), 40-60% solvent B (20-25 min), 60-100% solvent B (25-25.1 min), 100% solvent B (25.1-35 min) and 100-0% solvent B (35-35.1 min) at a flow rate of 1.0 mL min-1. The column would be allowed to re-equilibrate in 100% A for 10 min prior to the next injection. The temperature of the column was maintained at 20°C. The injection volume is 10 µL each. Detection of individual carotenoids was made at the wavelengths of maximum absorption of the carotenoids in the mobile phase: neoxanthin (438 nm), violaxanthin (441 nm), lutein (447 nm), zeaxanthin (452 nm), β-carotene (454 nm), β-cryptoxanthin (450 nm) and α-carotene (456 nm). Compounds were identified by co-chromatography with standards and by elucidation of their spectral characteristics using a photo-diode array detector. Detection for carotenoid peaks was in the range of 350 to 550 nm. Individual carotenoid concentrations were calculated by comparing their relative proportions, as reflected by integrated HPLC peak areas, to total carotenoid content determined by spectrophotometry. The total and individual carotenoid concentration would be expressed in terms of milligram per 1.0 g dry weight of freeze-dried matter (µg/g DW).

RESULTS

Twenty-four traditional vegetables that have been consumed daily in Malaysian diet and four medicinal plant species that popular among Malay traditional midwifery practices representing diverse genetic backgrounds and growth habits were selected for this study (Table **1**). As a result, in the 28 traditional vegetables and medicinal plant species, the carotenoid content and composition range can be divided into six groups as detailed in Table **2**. There was positive relationship between total carotenoid content and types of carotenoid pigment. Cekur manis (*S. androgynus*) was found to have the highest total carotenoid content (190.30±3.43 µg/g DW), substantially higher than all other plant species tested. In contrast, the lowest total carotenoid concentration was found in lobak merah (*D. carota*) (1.31±0.01µg/g DW).

Carotenoid analysis performed by HPLC system detected at least six major carotenoid peaks: neoxanthin, violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carote and β-carotene. As shown in Table **2**, neoxanthin and violaxanthin were found highest in cekur manis (*S. androgynus*); lutein was highest in pegaga (*C. asiatica*), whereas zeaxanthin was detected highest in kaduk (*P. sarmentosum*). β-cryptoxanthin was found in trace element of all species analyzed. α- and β-carotene were detected in their highest level in durian (*D. zibethinus*) and selom

Table 2: Relative Distributions of Individual Carotenoid from One to Six Types of Carotenoid with 28 *ulam* and Medicinal Species

Species	Total Carotenoid (µg/g DW)	Neoxanthin (µg/g DW)	Violaxanthin (µg/g DW)	Lutein (µg/g DW)	Zeaxanthin (µg/g DW)	β-Cryptoxanthin (µg/g DW)	α-Carotene (µg/g DW)	β-Carotene (µg/g DW)
Species with 6 carotenoid pigments								
S. androgynus	190.30±3.43	142.40±3.57	28.06±0.65	15.57±0.32	nd	0.07±0.00	1.36±0.42	2.84±0.37
A. tuberosum	24.61±1.00	13.95±0.75	2.98±0.24	5.00±0.38	nd	0.06±0.00	0.75±0.08	1.86±0.27
Species with 5 carotenoid pigments								
C. asiatica	130.61±15.03	96.10±11.4	13.45±2.68	16.53±0.97	nd	nd	2.14±0.12	2.39±0.06
O. indicum	100.78±2.45	81.79±2.70	4.36±0.12	13.12±0.31	nd	nd	0.38±0.03	1.12±0.03
O. basilicum	95.28±3.25	65.16±3.22	17.97±0.50	9.66±0.96	nd	nd	0.53±0.11	1.95±0.24
F. deltoidea	13.77±1.04	6.87±0.76	2.38±0.03	3.26±0.04	nd	nd	0.38±0.18	0.87±0.13
Species with 4 carotenoid pigments								
P. sarmentosum	161.36±12.72	24.06±4.63	nd	12.58±1.28	123.45±12.3	nd	nd	1.27±0.29
O. javanica	144.48±4.93	115.55±4.09	11.06±0.70	14.80±0.44	nd	nd	nd	3.09±0.06
O. americanum	108.79±6.35	74.62±3.30	24.12±2.63	9.27±0.35	nd	nd	nd	0.78±0.16
Z. mays	61.53±5.55	nd	nd	1.54±0.03	58.87±5.38	0.05±0.00	1.06±0.14	nd
A. paniculata	46.83±2.43	26.39±1.84	9.71±0.38	8.97±0.24	nd	nd	nd	1.77±0.02
B. chinensis	27.00±2.73	18.75±1.84	nd	6.51±0.63	nd	0.04±0.00	nd	1.68±0.26
L. sativa	15.14±0.65	10.15±0.69	1.87±0.09	2.58±0.07	nd	nd	nd	0.54±0.02
Species with 3 carotenoid pigments								
P. sacharosa	79.83±1.80	nd	nd	5.64±0.05	72.32±1.89	nd	nd	1.86±0.15
M. citrifolia	57.11±1.94	nd	nd	5.20±0.00	52.08±0.25	nd	nd	1.31±0.01
M. koenigii	51.48±2.60	nd	nd	4.04±0.14	45.74±2.32	nd	nd	1.69±0.28
P. betle	18.30±0.08	nd	nd	15.49±0.10	nd	0.07±0.00	nd	2.74±0.10
A. graveolens	14.35±0.14	nd	nd	11.53±0.09	nd	0.06±0.00	nd	2.76±0.09
C. caudatus	12.59±0.27	nd	nd	9.60±0.32	nd	nd	1.56±0.16	1.43±0.05
P. indica	10.66±0.39	nd	nd	5.76±0.17	nd	nd	1.92±0.16	2.98±0.31
P. minus	7.40±0.38	nd	nd	4.16±0.11	nd	nd	0.71±0.08	2.53±0.25
R. augustifolia	6.45±0.56	nd	nd	6.00±0.11	nd	0.05±0.00	nd	1.10±0.01
Species with 2 carotenoid pigments								
A. occidentale	14.20±0.29	nd	nd	12.46±0.55	nd	nd	nd	1.74±0.28
E. redleyi	11.65±0.14	nd	nd	10.39±0.15	nd	nd	nd	1.30±0.03
A. cepa	6.90±0.04	nd	nd	4.83±0.02	nd	nd	nd	2.07±0.02
I. batatas	1.34±0.06	nd	nd	nd	nd	nd	0.38±0.09	0.97±0.06
D. carota	1.31±0.01	nd	nd	0.72±0.00	nd	nd	nd	0.60±0.01
Species with 1 carotenoid pigment								
D. zibethinus	5.04±0.25	nd	nd	nd	nd	nd	5.04±0.25	nd

nd – non-detectable.

(*O. javanica*) respectively. All 28 species could be grouped into one of several classes depending on the accumulation of specific carotenoid pigments (Table **2**). Two species were found to have six individual carotenoid pigments and four species were found to have five individual carotenoid pigments with a relatively high concentration of neoxanthin and lower concentrations of β-cryptoxanthin, α-carotene and β-carotene. However seven species were detected to have four of the six carotenoid pigments with a relatively high concentration of either neoxanthin or zeaxanthin.. A group of three and two carotenoid pigments only accumulated either zeaxanthin or lutein or lutein and β-carotene respectively, whereas another last group only contained one carotenoid pigment which is α-carotene.

In comparison to the total retinol equivalent as shown in Table **3** which meet the Recommended Dietary Allowance (RDA) for vitamin A of 1000 retinol equivalents per day [7], all species were found to accumulate between 90.37 to 656.59 retinol equivalents per g DW of samples. Beluntas (*P. indica*) had a higher RE content and at least 20 of the *ulam* and medicinal species may be used as alternative food intervention or to be further evaluated as potential candidates to eliminate VAD.

In general, the highest carotenoid concentrations, either in total or individual carotenoid pigments were detected in six carotenoid pigments group. It can be concluded that the total carotenoid is strongly associated with the concentration of individual

Table 3: Total Retinol Equivalent (RE) Content of 28 Malaysian Traditional Vegetables (*ulam*) and Medicinal Species

Botanical name	Local name	RE
Lactuca sativa	Salad	90.37
Zea mays	Jagung	93.07
Daucus carota	Lobak merah	99.64
Ocimum americanum	Kemangi	130.57
Ficus deltoidea	Mas cotek	145.77
Ruta angustifolia	Garuda	183.39
Ipomoea batatas	Ubi keledek	192.22
Euodia redleyi	Tenggek burung	209.97
Piper sarmentosum	Kaduk	211.43
Morinda citrifolia	Mengkudu	218.02
Oroxylum indicum	Beko	219.34
Murraya koenigii	Kari	281.69
Brassica chinensis	Sawi	284.36
Anacardium occidentale	Gajus	289.69
Andrographis paniculata	Hempedu bumi	294.36
Pereskia sacharosa	Jarum tujuh bilah	310.6
Allium cepa	Daun bawang	344.48
Cosmos caudatus	Ulam raja	368.52
Ocimum basilicum	Selasih	369.27
Allium tuberosum	Kucai	377.42
Durio zibethinus	Durian	427.05
Piper betle	Sirih	462.21
Apium graveolens	Saderi	464.54
Polygonum minus	Kesum	481.34
Oenanthe javanica	Selom	514.17
Centella asiatica	Pegaga	576.5
Sauropus androgynus	Cekur manis	592.45
Pluchea indica	Beluntas	656.59

carotenoid pigments especially neoxanthin and zeaxanthin. However, the relative distributions of individual carotenoids within each grouping did not necessary correlate to the levels of total carotenoids.

DISCUSSION

Previous studies on several *ulam* species as detailed in [26, 28-32] established that carotenoids profiles were predominantly neoxanthin, violaxanthin, lutein, β-carotene and zeaxanthin whereas the carotenoid profiles in *ulam* in this study were dominated by neoxanthin, violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carote and β-carotene. This result suggested that different plant species will react differently towards the stability of individual carotenoids accumulated in plants. Furthermore, the environmental conditions and response can also influence the presence of specific carotenoid compounds and their concentration in *ulam* species. There are two possibilities to explain these carotenoid profiles instability:

i. The conversion of other carotenoids such as violaxanthin, neoxanthin to zeaxanthin from the β-carotene and α–carotene branch point is due to irradiance stress condition from high-light exposure. As a result, zeaxanthin concentration will increase. This reaction will restrict the supply of precursors for abscisic acid (ABA) biosynthesis and the plant responds by increasing carotenogenic metabolic flux to compensate for this restriction [25, 33, 34]. In agreement with this Polle [35] also stated that zeaxanthin can successfully replace lutein and violaxanthin under irradiance stress condition.

ii. The presence and absence of zeaxanthin is in response to changes in pH. Acidity will trigger the de-epoxidation reaction by the conversion of violaxanthin and other precursors of ABA to zeaxanthin, whereas alkaline conditions will induce lutein or the supply of precursors for ABA biosynthesis which will lead to the conversion of zeaxanthin to violaxanthin, neoxanthin or other precursors for ABA biosynthesis through epoxidation reaction [25, 36]. Overall this clearly demonstrated that the environmental conditions can strongly influence the total and individual pigment content of carotenoids in plants, which can be significantly affecting the quality and nutritional value of *ulam* and medicinal plant species. Therefore, in addition to genotypic factors, environmental factors also play an important role in determining the accumulation of individual carotenoids in plants, especially in *ulam* and medicinal plant species. Clearly, further studies utilizing *ulam* and medicinal plant species grown under different environmental conditions is required to confirm this hypothesis.

CONCLUSION

In conclusion, of all the 28 *ulam* species tested, at least 20 of the *ulam* and medicinal species can be used as alternative food intervention to eliminate VAD as a public health concern. Also, we found that relative distributions of neoxanthin, violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene are genotype dependent. To sum up, 20 to 30 g fresh weight of *ulam* or traditional vegetables can be used to combat vitamin A deficiency due to these species meet the Recommended Dietary Allowance (RDA) for vitamin A of 1000 retinol equivalents per day.

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