Extraction, and Characterization of Carotenoids from 11 Allelopathic Plant Species as Potential Halal Food Colorants and Active Pharmaceutical Ingredients

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Abstract: Carotenoids are a class of natural product compound that are currently being used as colouring agents and widely used in food industry. Carotenoids are bioactive pigments obtained mainly from plants through dietary intake. They possess good features in terms of dietary supplement, food colourant, and polymer stabiliser. The presence of 4 main carotenoids, which are β-carotene, zeaxanthin, lutein, and violaxanthin, were determined in 4 classes of allelopathic plant groups namely trees, ferns, grasses and herbaceous plants. This research aims to explore the carotenoid's content and composition in 11 allelopathic species by HPLC analysis. *A. auriculiformis* (tree) was found to have the highest total carotenoid concentration (146.36 µg/g DW) that was substantially higher than all other species tested whereas the lowest total carotenoid concentration was found in *S. palustris* (fern) (3.76 µg/g DW). Lutein and β-carotene were detected highest in *A. auriculiformis* (tree), with 1024 ± 25.5 µg/g DW and 37.55 ± 3.16 µg/g DW, respectively. Violaxanthin and zeaxanthin were found substantially highest in *M. cajuputi* (tree) (5.02 ± 0.5 µg/g DW) and *S. palustris* (fern) (5.88 ± 0.19µg/g DW), respectively.

Keywords: Allelopathic species, carotenoids, active pharmaceutical ingredients, grass, fern, herb, tree.

INTRODUCTION

Carotenoids are a class of natural product compounds that are currently being used as colouring agents and widely used in food industry. Carotenoids occur widely in nature and, in general, these coloured compounds are obtained from fruits and vegetables. Currently, the carotenoids used in industrial processes are synthesized chemically, and some carotenoids are extracted from plants, algae, and animals [1,2]. Plants produce biochemical that are of importance in the healthcare, food, flavour and cosmetics industries. Whilst plant cell culture system or technology represents a potential renewable source of valuable medicinal, flavours, essences and colourants that cannot be produced by microbial cells or chemical syntheses. Currently, these and many other natural products are produced solely from massive quantities of whole plant parts [3,4]. Recent advances in molecular biology, enzymology, physiology and fermentation technology of plant cell cultures suggest that these systems will become a viable source of important natural products. Plant pigments are labile: they can be easily altered, and even destroyed. On the basis of their chemical structures, pigments can be classed into four families, i.e. tetrapyrroles (e.g. chlorophyll), carotenoids (e.g. beta-carotene), polyphenolic compounds (e.g. anthocyanins), and alkaloids (e.g. betalains). Colourants in plants arise from two main classes of pigments, carotenoids and anthocyanins. Anthocyanins are responsible for the water soluble vacuoles of pink, red, purple and blue pigments that are present in the coloured plant pigments whereas carotenoids are responsible for the orange and yellow lipid soluble pigments in plastids [5,6]. Colorants are often added in foods to enhance their visual aesthetics and to promote sales. Although the allowable amount of synthetic colorants is reduced for consumer healthiness reasons in recent years, many kinds of synthetic food dyes are still widely used all over the world due to their low price, high effectiveness and excellent stability [7,8]. As a consequence of these additional pigment needs, the demand in isolated natural colorants has increased as compared with synthetic dyes. However, this need cannot always be satisfied due to the limitation in the

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supply of raw materials because the production of pigments, using conventional plant cultivation methods, is influenced by climatic conditions, plant cultivars and varieties [9]. Consequently, part of plant pigment research is oriented in finding new sources of pigments. This quest is not only directed in finding natural alternatives for synthetic dyes, but also with the aim to discover new taxa and new procedures for the pigment production, for instance, from the different groups of allelopathic plant species such as, grasses, ferns, herbaceous plants and trees. Highlighting the fact that there is a high demand and consumer preferences for natural compounds, therefore, natural carotenoids are focused in this study. 4 types of plant groups from 11 allelopathic species were chooses, which are grasses, ferns, herbaceous plants and trees. Currently, there is no research that correlates between plant class and their carotenoid content. Therefore, the main subject of this study is determination of carotenoid concentrations based on their class, and comparison between plant classes.

MATERIALS AND METHODS

Sample Preparation

All 11 plant species were freeze-dried for 72 hours, after which the samples were grounded into a fine powder and stored at -20 °C until further analysis.

Extraction of Carotenoids

The extraction procedure essentially follows the methods described by Othman [10], with some modifications. 0.1 g of each powdered sample was rehydrated with distilled water and extracted with a mixture of acetone and methanol (7:3) at room temperature until colourless. 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

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 the 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 HPLC analysis, as described in detail by Othman [10].

Determination of Total Carotenoid Content

Total carotenoid concentration was determined by spectrophotometry, according to the method described by Othman [10]. The dried carotenoid was re-suspended in 500 µL of ethyl acetate for the determination of total carotenoid content. For spectrophotometric analysis, 50 µL of the re-dissolved sample was then diluted with 950 µL chloroform. Three different wavelengths λ; 480 nm, 648 nm, and 666 nm were used in measuring the carotenoid-containing solutions using Varian Cary 50 UV-Vis spectrophotometer. The total carotenoid content in chloroform was obtained by using the Wellburn equation [11] as described below:

$$Ca = 10.91A666 – 1.2A648 \quad (1)$$

$$Cb = 16.36A648 – 4.57A666 \quad (2)$$

$$Cx+c = (1000A480 – 1.42Ca – 46.09Cb) / 202 \ (\mu g / ml) \quad (3)$$

Ca = concentration of carotenoid at 666 nm, Cb = concentration of carotenoid at 648 nm, and Cx+c = total carotenoid concentration at 480 nm.

HPLC Analysis of Carotenoids

The HPLC analysis of carotenoids were performed on an Agilent model 1200 series that comprises 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 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 was 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 volume of each injection was 10 µL. 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 was expressed in terms of milligram per 1.0 g dry weight of freeze-dried matter (µg/g DW).

RESULTS AND DISCUSSION

Eleven allelopathic species from different plant groups of grasses (Lepironia articulata, Eleocharis ochrostachys, Dapsilanthus disjunctus), ferns (Stenochlaena palustris, Dicranopteris linearis Achrostichum aureum), herbaceous plant (Hanguana malayana) and trees (Acacia auriculiformis, Azadirachta indica, Melaleuca cajuputi) were selected. These 11 species exhibited highly significant differences in total and individual carotenoid content ($p < 0.0001$). A. auriculiformis was found to have the highest total carotenoid concentration (146.36 µg/g DW) that is substantially higher than all other species tested (Table **1**). In contrast, the lowest total carotenoid concentration was found in S. palustris (3.76 µg/g DW). Carotenoid analysis performed by HPLC system detected at least four major carotenoid peaks: violaxanthin, lutein, zeaxanthin and β-carotene. As shown in Table **1**, lutein and β-carotene are highest in A. auriculiformis, with 1024 ± 25.5 µg/g DW and 37.55 ± 3.16 µg/g DW, respectively. Violaxanthin and zeaxanthin are substantially highest in M. cajuputi (5.02 ± 0.5 µg/g DW) and S. palustris (5.88 ± 0.19µg/g DW).

All eleven species could be grouped into 3 classes depending on the accumulation of specific carotenoid pigments (Table **1**, Figures **1**, **2** and **3**). *D. linearis* and *A. auriculiformis* were found to have only two individual carotenoid pigments with a relatively high concentration of lutein. *E. ochrostachys* and *S. palustris* were detected to have three carotenoid pigments whereas the rest were found with all four types of carotenoids. These results established that carotenoid pigments composition and content vary with species. It was noticed that among all individual carotenoids, lutein is the major carotenoid in all 11 plant allelopathic species. According to [12], lutein and catechin are the major allelochemicals in plants. Hence, this proves that in terms of carotenoids, allelopathy plant species contain high lutein concentration because lutein itself is an allelochemical. Genotype and environment interactions have been reported to account for variation in free amino acids, protein, carotenoids and sugar composition [13-18]. Seasonal differences, growing conditions, locations, genotypes and postharvest storage conditions are among the factors that can be significantly affect the quality and nutritional value of plants [19-22]. The bioavailability of carotenoids is a complex issue and depends on many factors [23]. In this study of environment and genotype interactions, the data revealed that variations in total carotenoid content and the concentration of individual carotenoid pigments is due to strong relationship between genotype and plant group. This assumption is

Table 1: Distributions of Total and Individual Carotenoid Content (µg/g DW) in 11 Allelopathic Species

Botanical Name	Total Carotenoid (µg/g DW)	Violaxanthin (µg/g DW)	Lutein (µg/g DW)	Zeaxanthin (µg/g DW)	β-Carotene (µg/g DW)
Hanguana malayana	77.99	3.90 ± 0.01	491.1 ± 26.6	ND	11.77 ± 8.05
Dapsilanthus disjunctus	14.54	ND	17.27 ± 7.46	1.84 ± 0.11	1.12 ± 0.04
Eleocharis ochrostachys	25.50	3.44 ± 0.01	136.8 ± 10.6	3.82 ± 0.05	4.08 ± 0.71
Lepironia articulata	29.96	2.68 ± 1.09	94.46 ± 8.05	ND	4.64 ± 0.53
Stenochlaena palustris	3.762	1.70 ± 0.01	39.65 ± 2.66	5.88 ± 0.19	0.24 ± 0.23
Achrostichum aureum	15.48	ND	53.49 ± 6.64	2.15 ± 0.27	1.52 ± 1.39
Dicranopteris linearis	131.3	ND	716.8 ± 4.63	ND	18.58 ± 2.39
Rhizophora apiculata	18.51	ND	28.50 ± 3.35	2.13 ± 0.19	1.34 ± 0.14
Azadirachta indica	18.33	ND	102.6 ± 75.0	4.94 ± 0.36	4.01 ± 1.28
Melaleuca cajuputi	31.61	5.02 ± 0.5	189.3 ± 79.7	ND	7.273 ± 0.36
Acacia auriculiformis	146.36	ND	1024 ± 25.5	ND	37.55 ± 3.16

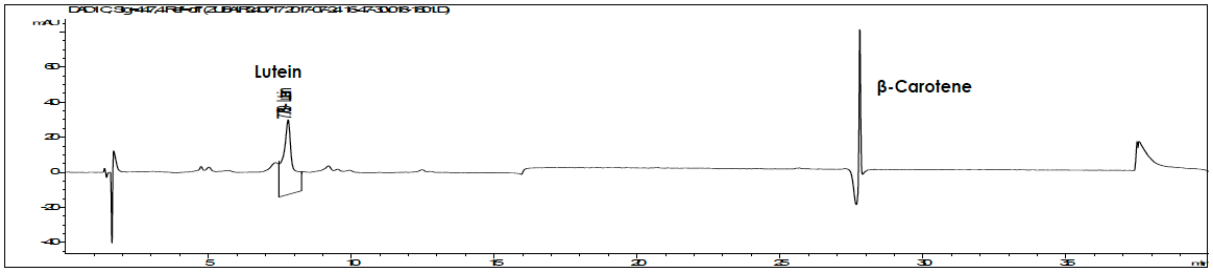

Figure 1: HPLC chromatogram of lutein and β-carotene in *Achrostichum aureum* of two carotenoid pigments group.

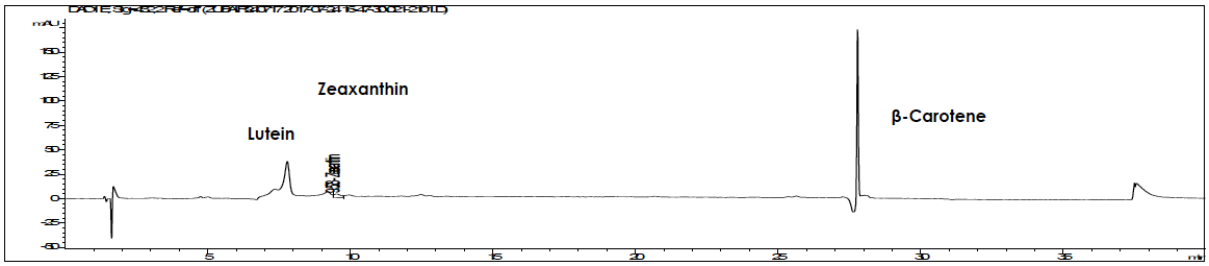

Figure 2: HPLC chromatogram of lutein, zeaxanthin and β-carotene in *Azadirachta indica* of three carotenoid pigments group.

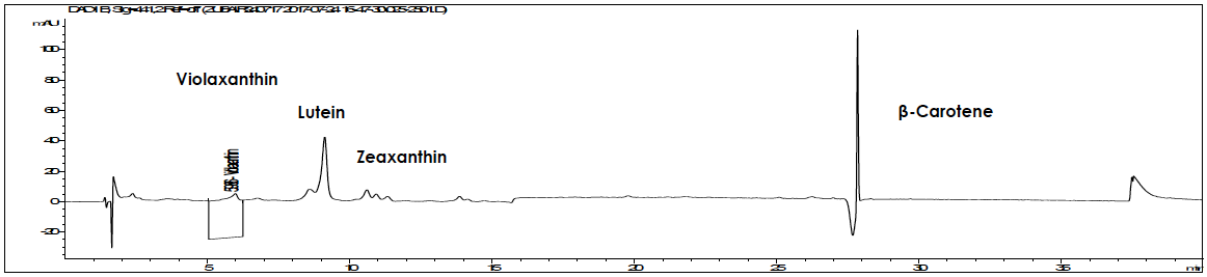

Figure 3: HPLC chromatogram of violaxanthin, lutein, zeaxanthin and β-carotene in *Eleocharis ochrostachys* of four carotenoid pigments group.

supported by [24], in their observations of 26 crops over a 43-year period growing seasons; where yield adaptability over time was controlled largely by weather and small variations from year to year in agronomical practices. In other words, major factors influencing yield are location, year and their interactions. A strong relationship and interaction between the intensity of the yellow colour in tuber flesh, total carotenoid content and growing locations has also been reported. [25] demonstrated that environmental factors can exert some influences on the expression of yellow tuber flesh intensity. The correlation between genotypes and environment can be indicative of the particular plant species that are best adapted to certain location. The bioavailability of carotenoids is a complex issue and depends on many factors such as location, year, cultivar and their interactions [26]. In this study on the influences of plant groups and genotype interactions on carotenoid accumulation, the data revealed that variations in both the total carotenoid content and the individual carotenoid compounds exhibit strong relationships between genotype, plant groups and environment (Figures **1-3**). Genotype x environment interactions on biochemical composition has been previously reported for phenolics accumulation in plants. There are two possible mechanisms that regulate the differences in carotenoid biosynthesis between allelopathic species:

i. The availability or the abundance of carotenogenic gene transcripts.

ii. The abundance or the presence of structures of sequestering or producing carotenoids.

Therefore, the differences in carotenoid profile from 11 allelopathic species from different growth habits can be explained by the regulation of genes especially zeaxanthin epoxidase (ZEP) and violaxanthin deepoxidase (VDE), presence of structure sequestering carotenoids and environmental stress. As stated by [27], cultivars which have a limited capacity to tolerate excessive light, exhibit an increased

susceptibility to photooxidative damage. In contrast, cultivars in which carotenoid content is much higher can specifically tolerate excessive light along with many environmental stress conditions by regulating ZEP and VDE. Selecting the appropriate cultivars for the appropriate environmental conditions and appropriate agronomic practices is not only important for yield production, but also for nutritional value and quality of targeted plant.

CONCLUSION

Carotenoid analysis of 11 allelopathic species detected four major carotenoids of violaxanthin, lutein, zeaxanthin and β-carotene. *A. auriculiformis* was found to have the highest total carotenoid (146.36 µg/g DW) whereas *S. palustris* had the lowest (3.76 µg/g DW). 2 species (*D. linearis* and *A. auriculiformis*) were found to have only two individual carotenoid pigments with a relatively high concentration of lutein. Other 2 species (*E. ochrostachys* and *S. palustris*) were detected to have three carotenoid pigments whereas the rest were found with all four types of carotenoids. This results established that carotenoid pigments composition and content vary with species and there is no relationship between carotenoid content and plant group.

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