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Characterization of Anthocyanins from Red Tamarind, *Tamarindus indica* Var. *rhodocarpa* using Spectral Analysis

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Abstract

Objective: To characterize anthocyanin from *Tamarindus indica* var *rhodocarpa* using spectral analysis for use in food colourants. **Results**: Nutritional parameters such as carbohydrate and protein contents were high in Red Tamarind unripen fruit extract with the values of 8.6g and 12.61g respectively. Low fat level with nil Cholesterol (BDL) is present in the extract. Other minerals and vitamins such as iron, calcium, vitamins A &C are rich in the extract. TLC separation of unripen fruit extract of red tamarind matched with the authentic anthocyanins pigments namely, cyaniding-3-glucoside, delphidin and pelargonidin. HPLC analysis also confirmed the presence of major anthocyanin pigments, cyaniding-3-glucoside, delphidin and pelargonidin. **Conclusion**: Anthocyanins are the important polyphenolic compounds present in Red Tamarind. The characterization of anthocyanins using TLC and HPLC analysis revealed the presence of three major anthocyanins pigments v: z., cyanidin-3-glucoside, pelargonidin, delphinidin. The red colour of the unripen fruit extracts of red tamarind is due to presence of cyaniding-3-glucoside with higher quantity of 94.21%. The pH of the extract is 2. Analysis of nutritional parameters revealed that the extract contains high level of protein and carbohydrate; rich in minerals and vitamins with low level of fat which support the daily human intake values, hence it may be considered to use as food colourant. Therefore, it is concluded that the presence of anthocyanin pigments cyaniding-3-glucoside in unripen fruit of red tamarind is responsible for red colour which is depending on the pH.

Keywords
Red tamarind**,** Anthocyanins, HPLC, Natural pigment, Food colourants.

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1. INTRODUCTION

Colours have always playing an important role in our day to day life as they give us the impression on fruits, foods, fabric and even medicines and it strongly influences every moment of our lives. As a result, eco-friendly non-toxic naturally occurring bio-colourants have gained re-emergence with vast applicability and are an integral part of human life since time immemorial. Plants are the reservoir of unique natural pigments that give them their colours. India, as a seventh biggest country sharing two percent of land area on the earth is endowed with some 500 varieties of plants that can yield natural colours [1] but only a few sources were

exploited commercially such as indigo, turmeric, annatto etc. Anthocyanins are the most spectacular plant pigments which have high demand to use as a food colour because of range of colours orange to red, purple and blue in flowers, fruits and vegetables which have the potential to be incorporated as food colourants, innocuous and beneficial health [2-5]. Consumer awareness about the demerits of synthetic colourants, the health benefits of natural colourants and due to the worldwide tendency towards the consumption of natural products lead to significant increase in interest towards natural colourants and the research in the field. The natural pigments have their own limitations like availability, colour yield, stability, and toxicity.

Anthocyanins are the largest group of water soluble polyphenolic flavonoids pigments, responsible for diverse pigmentation localized in various parts of the plants like fruits, vegetables and flowers that give these plants their brilliant colour. Incorporating anthocyanins is highly valuable for appearance and health benefits also anti-cancer, anti-inflammatory and vasoprotective effects preventing coronary heart diseases and improving visual activity [5-6]. Currently, anthocyanins reported in blue and purple corn are being used for making naturally coloured blue tortillas [4]. Radish and potato extracts have colour characteristics very similar to those of Allura red (a red dye used in food application) [5]. Hence exploration of anthocyanins from newer sources is warranted.

The red fruited variety of tamarind taxonomically known as *Tamarindus indica* var. rhodocarpa (red tamarind) is one such species found in India. It is a rare variant with limited distribution in southern states of India. The unripen stage of the fruits provides anthocyanins. The anthocyanins content of the unripen fruits of red tamarind is high (180 to 360 mg/g of unripe fruit), while comparing with other anthocyanin rich fruits like grapes (80-90 mg/g), cherry (70-75 mg/g) and jamun (120-130 mg/g) [7]. The anthocyanins from red tamarind unripen fruits are rich in antioxidant properties advocate a wide scope for utilizing it as a potential bio-colourants for use in food and cosmetic applications. Hence the present study aimed to characterize the anthocyanins from unripen fruits of red tamarind using spectral analysis.

2. MATERIALS AND METHODS

2.1. Sample collection

Extensive surveys have been conducted in length and breadth of Tamilnadu state and identified 18 trees in various districts of Tamilnadu. Geographical attributes (GPS marking) of each tree was recorded. Of the 18 trees identified, unripe fruit samples were collected from red tamarind tree in Pollachi, Coimbatore, Tamilnadu, India, situated between 10˚39’12.0” N latitude and 077˚02’03.6” E longitude. The collected fruits were labelled, transported to Chemistry and Bioprospecting laboratory, Institute of Forest Genetics and Tree Breeding, Coimbatore and stored at -20˚C for further study.

2.2. Extraction of anthocyanins

The unripen fruits of red tamarind were macerated thoroughly and extracted with acidified methanol (Methanol: HCL, 99:1, v/v) sequentially and incubated overnight in a shaker at low temperature (4ºc) to avoid the hydrolysis and degradation of acyl groups in anthocyanin structure. After 12 hrs of incubation, the mixture was centrifuged at 5000rpm for 10mins at 4ºc, and the clear supernatant was collected. The supernatant was concentrated under reduced pressure on a rotary evaporator (Heidolph WB 2000) below <40°C. Finally, the extract was purified by passed through Varian Bond Elut C_{18} solid phase extraction column and impurities were removed. The purified sample was refrigerated at 4˚C until further analysis.

2.3. Quantification of anthocyanins

Monomeric anthocyanins are main contributors to colour. However, the colours transform into other as a result of the development of polymeric pigments during ageing and maturation. Hence determination of monomeric anthocyanins content in the extract is a useful tool/ criteria in quality control of natural colourants. Monomeric anthocyanins undergo a reversible structural transformation as a function of pH (coloured oxonium from at pH 1.0 and colourless hemiketal from at pH 4.5) was determined using UV-Visible Absorption Spectrophotometry (Hitachi U-2000) at 530nm and 700nm by pH-differential method [8]. Two dilutions were performed for the sample with 0.025 M potassium chloride at pH 1.0 and with 0.4 M sodium acetate at pH 4.5. Diluted Samples were allowed to equilibrate for 15 min and recorded the absorbance at 530 and 700nm using a spectrophotometer calibrated with their corresponding buffer solutions as the blank. The difference in absorbance between the two pH values at two wavelengths was used to calculate anthocyanins content as cyanidin-3-glucoside with molecular weight of 449.2 g/mol. The anthocyanins

content was expressed as mg anthocyanins/gram of fruit.

2.4. Thin layer chromatography separation of anthocyanins

TLC was carried out to isolate the principle components present in the red tamarind unripen fruit extract. Preparative TLC plates (20 cm x 20 cm, Merck) were used for the separation of anthocyanins present in the extracts. Concentrated samples were prepared with methanol and spotted on the plate with capillary tube and allow drying and a distance of 1 cm was maintained in between each spotted samples. Loaded TLC plate was placed inside the TLC chamber pre-saturated with a solvent mixture of n-Butanol: Acetic Acid: Water (4:1:1, v/v/v, Upper phase). The samples were allowed to elute at a distance of 8cm and after the completion of the separation, the plate was viewed under UV light at both 254 nm and 366 nm. Solute front (florescent spots) and solvent was marked in order to determine the R_f (Retention factor) value of individual pigment band.

R_f =Distance travel by solute (cm) / Distance travel by solvent (cm)

The separated fractions were scarped from the TLC plate and purified with methanol, centrifuged and the resultant supernatant was separated and scanned under UV-Vis spectrophotometer to determine their specific wavelength (λ max and λ vis-max) for elution using high performance liquid chromatography.

2.5. High performance liquid chromatography (HPLC)

Hitachi model L-6200 Intelligent pump with Hitachi L-4000 UV-Detector and Pursuit C_{18} column with Data Ace workstation was used to determine the anthocyanins. Isocratic elution was performed with 0.01% formic acid, 22.5% HPLC grade methanol, 50% HPLC grade acetonitrile (v:v:v) as mobile phase. The mobile phase and the samples were filtered and sonicated before HPLC analysis. The flow rate was maintained at 1ml/min at a wavelength of 290nm and the injection volume of the sample was 20µl. The mobile phase and the samples were filtered and sonicated for use in HPLC system. The separated anthocyanins were detected and measured at 520nm. The identity and quantification of the anthocyanins were based on the congruence of retention times with the standards.

3. RESULTS AND DISCUSSION

The natural colour segment is one of the fastest growing markets of the food and cosmetics industries. More than 650 different anthocyanins have been identified [9-10]. In the present study, anthocyanins extracted from unripe fruits of red tamarind were quantified as 245mg/g using pH differential method. However, the pH differential method is a simple, laboratory method for determining the amount of anthocyannins following by AOAC's strict validation process [9]. The plant grown region and growth season have significant impacts on the composition of anthocyannins both quantitatively and qualitatively [11-12] reported that 181.2 mg/ 100 g of total anthocyanins content was quantified in the grape variety Vidal Black and 716.4 mg/ 100 in Catawba. [13] Reported that black raspberries own higher anthocyanins contents of 400 mg/100 g than blackberries 150 mg/100 g followed by the yellow raspberries 0–3.4 mg/100 g.

3.1. Nutritional analysis of Red tamarind

The nutritional values of extracts from unripen fruits of red tamarind is given in table 1.

The energy provided by the red tamarind extract was 94.65Kcal/kg while comparing to the grape extract (274/64 Kg/kcal). The carbohydrate and protein contents were high in red tamarind with maximum range of 8.6g-12.61g. [14] Reported the fruit extracts of grapes and strawberry have almost similar carbohydrate content range between 8.81mg/ml and 75mg/ml.

Table 1. Nutritional values of red tamarind extract

Parameters	Values	Minimum Daily Values
Energy	94.65Kcal/kg	-
Carbohydrate	8.6g	2.87%
Protein	12.61g	25.22%
Fat	1.09g	1.68%
Cholesterol	BDL	0%
Calcium	18mg/100g	1.80%
Iron	0.16mg/100g	0.89%
Sodium	37.11mg/100g	1.55%
Vitamin A	412.18U/100g	8.25%
Vitamin C	37.07mg/100g	53.45%

[14] reported that protein content of grape 0.16mg/ml extract has the lowest while muskmelon 1.6mg/ml extract have the highest which is comparable to the protein content of blackberry 2.0mg/ml. Red tamarind extract has a similar vitamin A content and vitamin C content between 412.18U/100g-37.07mg/100g, while comparing to the fruit extracts of blackberry 30-150. The extract of red tamarind follows almost all parameters that are required for daily intake values such as protein, carbohydrate, all fat along with minerals and vitamins such as calcium, sodium, iron and vitamin A and C.

TLC separation of the unripen fruit extracts of red tamarind gave us two different spots, by comparing with their corresponding standards bands of Cyanidin-3-glucoside and Pelargonidin (Figure1). Hence presence of cyaniding and pelargonidin was confirmed. [15] Reported that the anthocyanins namely cyanidin 3-O-(6"-O-α-rhamnopyranosyl-β-glucopyranoside) and cyanidin 3-O-(6"-O-α-rhamnopyranosyl-β-galactopyranoside) extracted from Mulberry fruit by TLC method. The two eluted bands were purified and subjected to wavelength scan in double beam scanning UV-Visible spectrophotometer. The separated anthocyannins band eluted through TLC were subjected to wave length scanning in UV visible spectrophotometer between 200 and 800 nm gave λmax at 220-240 and 200-220 respectively which confirmed the presence of anthocyannins in red tamarind. As a group of the flavonoid, anthocyannins have two cluster groups benzoyl and sinamoil groups due to which the anthocyanin compounds have two characteristic absorptions at a wavelength region 260-280nm (UV range) and 490-550 nm (visible). [16] Reported that the anthocyanins pigments from *Ficus padana* Bumf have two absorption peaks at 278nm in UV range and another was at 526nm in visible range.

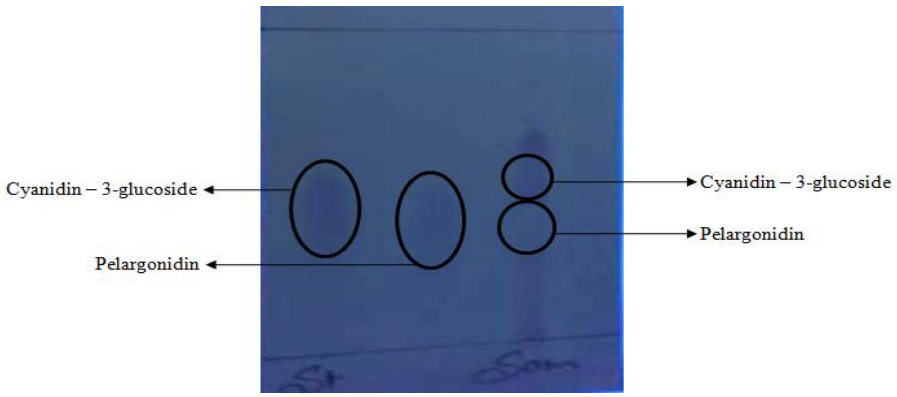

Figure 1. TLC separation of Red tamarind unripe fruit extract

HPLC is a tool to identify and quantify individual anthocyanins in a sample. Anthocyannins were identified by their respective retention time and characteristic chromatogram [17] reported 12 anthocyannins from Ayamurasaki cultivar of sweet potatoes and twenty-six anthocyanins pigments from purple sweet potato cell cultures by HPLC analysis. Cyanidin, pelargonidin and delphinidin were the major anthocyanins pigments identified in red tamarind through HPLC analysis in Table 1 & figure 1 & 2. Cyanidin-3-glucoside was also reported as the major anthocyanins in *Jabuticaba (Myrciaria cauliflora*), a tropical fruit [18]. Where as in Bilberry, five major anthocyanidins aglycones: delphinidin, cyanidin, petunidin, peonidin, and malvidin were reported through HPLC analysis [19]. Extracted phycocyanin from the blue algae, Spirulina (*Arthrospira platensis*). Cyanidin 3-glucoside, pelargonidin and delphinidin were quantified in unripe fruit extract of red tamarind and confirmed 94.219 %, 2.995 % and 2.249 % respectively in Figure.2. Similarly, in grapes malvidin-3-glucoside, delphinidin-3-glucoside, petunidin-3-glucoside, cyaniding-3- glycoside, and peonidin-3-were separated [20].

Delphinidin-3-rutinoside was the major component reported in the reddish color berries, and cyanidin-3-rutinoside in the black berry [21]. Pelargonidin appears as red-coloured pigment in nature and it differs from most of the anthocyanidins [22-23]. Pelargonidin gives red hue to few fruits and berries [9]. The authentic anthocyanins reported in red tamarind accounts for its red natural colourant.

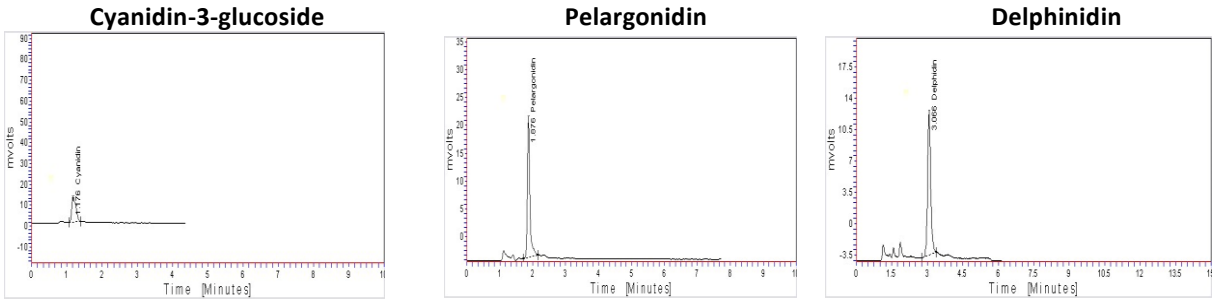

Figure 2. HPLC chromatogram of standards

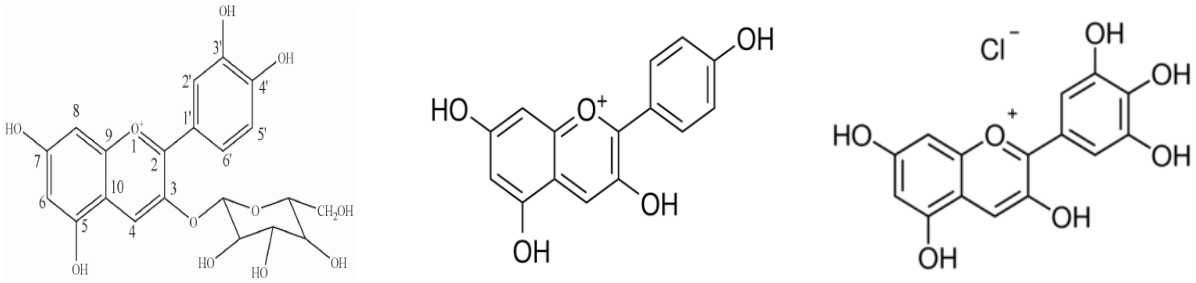

Figure 3. Structure of Standards

Table 2. HPLC quantification of anthocyanins from red tamarind unripe fruit extract

Name of the sample	Retention time (Mins)	Area (%)	Identified bioactive compound
Anthocyanins standard	1.192	100	Pelargonidin
Anthocyanins standard	1.176	100	Cyanidin -3-glucoside
Anthocyanins standard	3.066	100	Delphinidin
Red tamarind	1.176	94.219	Cyanidin -3-glucoside
Red tamarind	1.88	2.995	Pelargonidin
Red tamarind	3.033	2.249	Delphinidin

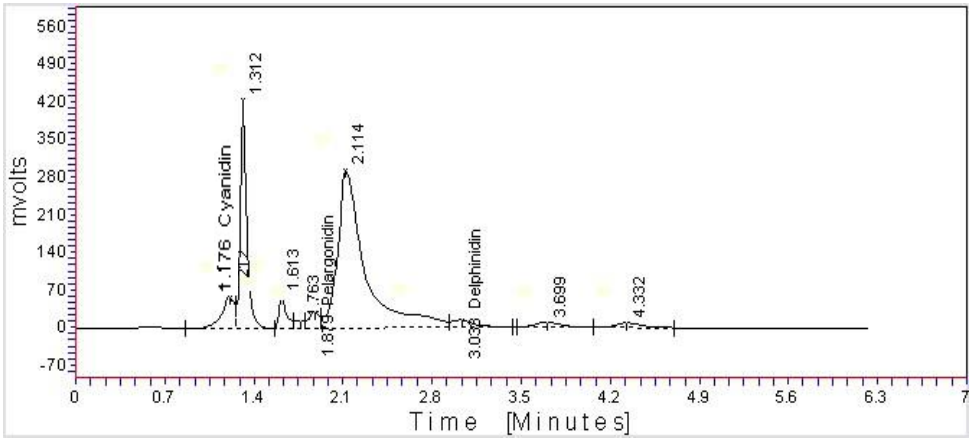

Figure 4. HPLC chromatogram of anthocyanins from red tamarind unripe fruit extract

CONCLUSION

Anthocyanins are the important polyphenolic compounds present in Red Tamarind The characterization of anthocyanins using TLC and HPLC analysis revealed the presence of three major anthocyanins pigments v: z., cyanidin3-glucoside, pelargonidin, delphinidin. The red colour of the unripen fruit extracts of red tamarind is due to presence of cyaniding-3-glucoside with higher quantity of 94.21%. The pH of the extract was 2

(Acidic). Therefore, it is concluded that the presence of anthocyanins pigments cyaniding-3-glucoside in unripen fruit of red tamarind is responsible for red colour which is depending on the pH. The stability study of the colour under various pH, temperature and lights regimes would help to consider the pigments of unripen fruits of red tamarind for use in various food colouring applications.

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