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Chemical nature, stability and bioefficacies of anthocyanins from fruit peel of *syzygium cumini* Skeels

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Abstract

In the present study, anthocyanin pigments from *Syzygium cumini* fruit peels were characterized and evaluated for their antioxidant efficacy, and stability as extract and in formulation. Total anthocyanin content was 216 mg/100 ml of extract which is equivalent to 230 mg/100 g fruit on a dry weight basis. Three anthocyanins were identified as glucoglucosides of delphinidin (1), petunidin (2) and malvidin (3) by HPLC–ESI–MS. The antioxidant capacity of the extract was tested using models, such as DPPH·-scavenging, reducing power assay, lipid peroxidation in rat brain, liver, liver mitochondria, testes and human erythrocyte ghosts. The extract showed 78.2% DPPH·-scavenging at 2.5 ppm, while BHA exhibited only 41.6% activity at the same concentration, thus proving it to be a more efficient free radical-scavenger than the widely used BHA. One ppm of the extract was equivalent to 3.5 μM ascorbic acid, as estimated by reducing power assay. Inhibition of rat brain lipid peroxidation was 94.4% at 5.0 ppm concentration. It was almost equally active in all the biological models, except human erythrocyte ghost cells, where it showed only 48% inhibition at 5.0 ppm. The extract was quite stable at 0 °C with 11% loss in 4 weeks, while the pigment loss in the antitussive formulation was only 13% at 30 °C at the end of 8 weeks. The high antioxidant activity and relatively high stability of the pigments make *S. cumini* a potential source of natural colourant as well as antioxidants.
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Keywords: Anthocyanins; Indian black plum; Java plum; Delphinidin; Electrospray ionization; Malvidin; Mass spectrometry; Petunidin

1. Introduction

Indian black plum or Java plum is a tropical edible fruit obtained from the trees of *Syzygium cumini* Skeels (Syn. *Eugenia jambolana* Lam.; *Eugenia cumini* Druce; Fam; Myrtaceae). The fruits are oblong berries, deep purple or bluish in colour with pinkish pulp, having various medicinal properties and used in Ayurveda as a stomachic, astringent, antiscorbutic, diuretic, antidiabetic, and in chronic diarrhea and enlargement of spleen (Achrekar, Kakliji, Pote, & Kelkar, 1991; Morton, 1987; Nadkarni, 1954). The fruit concentrate of *S. cumini* has a very long history of use for various medicinal purposes and currently has a large market for the treatment of chronic diarrhea and other enteric disorders, including its use as an antimicrobial (Migliato, 2005). The leaves are found to reduce radiation-induced DNA damage in cultured human peripheral blood lymphocytes (Jagetia & Baliga, 2002). Though different parts of this species are used in herbal formulations, very few reports are available on the systematic characterization of chemical components of the fruit.

There have been different reports of the anthocyanin composition of the fruits. According to one of the earliest reports, the deep purple colour of the fruit is due to anthocyanins, namely delphinidin-3-gentiobioside and malvidin-3-laminaribioside, along with petunidin-3-gentiobioside (Venkateswarlu, 1952). Sharma and Sheshadri (1955)

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reported the presence of cyanidin diglycoside and glycosides of petunidin and malvidin. Another recent report on *S. cumini* Lamarck revealed the presence of malvidin-3-glucoside and petunidin-3-glucoside in the Brazilian variety (Lago, Gomes, & da Silva, 2004). All these studies involved preliminary techniques, such as chemical tests, paper chromatography and UV–visible spectrophotometry. HPLC, coupled to ESI–MS, has proved to be a powerful tool for the characterization of anthocyanins, involving minimal sample preparation (Giusti, Rodriguez-Saona, Griffin, & Wrolstad, 1999), and, with the current availability of this technique, proper confirmation of the chemical nature of each anthocyanin is possible. Therefore, the present study aims at the identification of the anthocyanins of *S. cumini* by HPLC, coupled with ESI–MS analysis for the first time.

In addition to their colourful characteristics, anthocyanins are known to possess excellent antioxidant properties (Kong, Chia, Goh, Chia, & Brouillard, 2003). Anthocyanins from different sources have been reported to inhibit lipid peroxidation, and platelet aggregation (Ghiselli, Nardini, Baldi, & Scaccini, 1998), and possess anti-tumor (Kamei, Hashimoto, Koide, Kojima, & Hasegawa, 1998), antimutagenic (Yoshimoto et al., 1999), and hepatoprotective (Obi, Usenu, & Osayande, 1998) properties. Therefore, the present study was also focussed on evaluating the antioxidative properties of the extract through *in vitro* chemical and cellular models. The low stability of anthocyanins at high temperature and light conditions (Furtado, Figuekiredo, Chaves das Neves, & Pina, 1993) is a limiting factor in their application as colorants. Therefore, the present study assesses the stability of the pigment as an aqueous extract and in a formulation under different light and temperature conditions.

2. Materials and methods

2.1. Chemicals

Ascorbic acid, α,α-diphenyl-β-picryl hydrazyl (DPPH·), butylated hydroxyl anisole (BHA) and trifluoroacetic acid (TFA) were procured from Sigma-Aldrich (Steinheim, Germany). Acetonitrile and water, used in HPLC analysis, were of HPLC grade (Merck, Darmstadt, Germany). XAD-7 (Amberlite polymeric adsorbent of 20–50 mesh) was purchased from Fluka (Germany). Methanol, butanol, acetic acid and trichloro acetic acid (TCA) were of analytical grade and were purchased from Qualigens, India. All other reagents and chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.2. Plant material

The peels of fully ripe berries obtained from the local market were manually separated and immediately transferred to solvent for pigment extraction.

2.3. Extraction of anthocyanins

Anthocyanins were extracted with 0.1% HCl in methanol (Francis, 1986) by way of soaking the fruit peel in a 10-fold volume of the solvent for 3 h on an orbital shaker set at 100 rpm (25 °C ± 1). After filtration, the residue was repeatedly extracted until the filtrate obtained was nearly colourless. The extracts were combined and concentrated in a Buchi Rotavapor (Flawil, Switzerland) under vacuum at 30 °C (±1 °C), and partitioned against ethyl acetate before application onto an Amberlite XAD-7 column (Andersen, Viksund, & Pedersen, 1995). The column eluate (henceforth referred to as "extract") was concentrated to 100 ml and the total anthocyanin content was determined by the pH differential method (Lee, Durst, & Wrolstad, 2005).

2.4. Quantification of total phenolics in anthocyanin extract

Since, this extract may contain other phenolics, in addition to anthocyanins, the total phenolics were determined, using the method described by Velioglu, Mazza, Gao, and Oomah (1998). An aliquot (25 μl) of the suitably diluted extract was mixed with 225 μl methanol and 1.0 ml of aqueous Folin-Ciocalteau reagent (0.2 M) and allowed to stand at room temperature (27 °C ± 0.5 °C) for 5 min. One ml of 6% w/v sodium carbonate solution was added to the mixture, followed by incubation for 90 min at room temperature and the absorbance was measured at 725 nm, using a UV–visible spectrophotometer (Shimadzu 160 A, Japan). A calibration curve of gallic acid was prepared (ranging from 0.001 to 0.01 mg ml^{-1}). Results were determined from a regression equation of the calibration curve ($y = 0.0945\times$; $R^2 = 0.9971$) and expressed as mg gallic acid equivalents (GAE) per 100 ml of the extract.

2.5. Paper chromatography

Partially purified anthocyanins were separated on Whatman No. 3 chromatographic paper. Known volumes of the extract were applied directly to several sheets of paper and descending chromatography was carried out in butanol: acetic acid: water (4:1:5). Three distinct, mauve-coloured bands were eluted with methanol: acetic acid: water (90:05:05); the eluates collected and concentrated under reduced pressure in a Buchi Rotavapor at 30 ± 1 °C.

2.6. Analysis of sugars

An aliquot of the partially purified anthocyanin extract was dissolved in 2 ml of 2 N HCl and heated to 100 °C in a screw-capped tube for 90 min. Subsequently, the extract was cooled and extracted with amyl alcohol. The aqueous layer, containing hydrolysed sugars, was concentrated under vacuum and chromatographed on Whatman paper (Francis, 1986) and also analysed by HPLC–MS.

2.7. HPLC–MS Analysis

The partially purified samples as well as anthocyanins separated on paper, were diluted suitably with methanol, filtered through a 2 μm membrane filter (Millipore, USA) and analysed by HPLC, using a Waters Alliance 2695 HPLC equipped with an auto sampler and coupled with a Waters 2696 photodiode array detector and a Q-TOF Ultima™ mass spectrometer, utilizing the electro spray ionization (ESI–MS) interface (Waters Corporation, Manchester, UK). The chromatographic separation was performed on a Wakosil II C_{18} reverse phase stainless steel column, 250 × 4.6 mm i.d., 5 μm (SGE, Australia) with a guard column of the same material. The mobile phase (0.6 ml min^{-1}) consisted of (A) water/acetonitrile (95:05, v/v) and (B) water/acetonitrile (50:50, v/v) adjusted to pH 2.5 (with TFA). The gradient was: 0 min, 15%B; 0–20 min, 15–30%B; 20–25 min, 30–35%B; 25–35 min, 35–40%B; 35–42 min, 40%B; 42–43 min, 40–100%B; 43–48 min, 100B; and 48–49 min, 100–15%B, followed by 5 min for equilibrium at 15%B. Chromatograms were acquired at 520 nm. Samples (20 μl) were analysed in duplicate. Positive ion spectra of the column eluate were recorded in the range of *m/z* 20–2000 at a scan rate of 2 s/cycle under the following conditions: collision energy 10.0; capillary voltage 35 V; cone voltage 100 kV; source temperature 80 °C; desolvation temperature 150 °C; cone gas flow 0.4 l/min; desolvation gas flow (8.3 l/min). Argon was used as the collision gas. Data acquisition and processing was performed using MassLynx™ 4.0 SP4 software (Micromass).

HPLC–MS of the extract and its ethyl acetate fraction were carried out in negative ion mode to analyse other phenolics present in them. Chromatographic separation was performed on a Phenomenex Gemini 5 μm C_{18}110 A reverse phase column, 250 × 4.6 mm i.d. Mobile phase consisted of (A) 3% aqueous acetic acid and (B) methanol with a gradient of 100% A for 1 min, 56%B for 24 min and 100% A for the next 2 min at a flow rate of 1.0 ml/min. Chromatograms were acquired at 280 nm.

2.8. Free radical-scavenging activity

The antioxidant activity of the anthocyanin extract was measured on the basis of its ability to scavenge the stable DPPH·. Different concentrations (0.5–2.5 ppm) of the extracts in methanol (2.0 ml) were treated with 0.5 ml of a 0.5 mM solution of DPPH· in methanol. Absorbance at 517 nm was determined after 20 min and the percentage scavenging activity was calculated against a reagent blank (Murthy, Singh, & Jayaprakasha, 2002).

2.9. Reducing power assay

The reduction of ferric to ferrous ion by the extracts is an indication of the potential antioxidant property. The reducing power of the extracts was determined by the method of Gulcin (2005). Different concentrations of the extract (0.5–5.0 ppm to the final concentration) in methanol (1.0 ml) were diluted with 2.5 ml of phosphate buffer (0.2 M; pH 6.6) and mixed with 2.5 ml of 1% aqueous potassium ferricyanide. After incubation at 50 °C for 20 min, 2.5 ml of 10% trichloroacetic acid were added to the mixture. An aliquot of the reaction mixture were diluted with an equal amount of distilled water and absorbance was measured at 700 nm after treatment with 0.5 ml of 0.1% aqueous $FeCl_3$. Increased absorbance of the reaction mixture indicated an increase in reduction capability.

2.10. Anti-lipid peroxidation assays

Lipid peroxidation was assessed (Halliwell & Gutteridge, 1989) in rat brain, liver, liver mitochondria, testes and human erythrocyte ghost cells. For the preparation of substrate, brain, liver and testes were obtained from normal Wistar strain rats and washed in ice-cold saline. The liver was perfused with ice-cold saline before homogenization. A 10% w/v homogenate of the tissues was prepared separately in ice-cold 1.15% KCl using a Teflon Potter–Elvehjem glass homogeniser. The homogenate was centrifuged at 1000× g for 10 min and the supernatant was used for study. Rat liver mitochondria were prepared according to the method of Hogeboom (1955). Mitochondrial fraction was finally suspended in 1.15% KCl, so as to contain approximately 1 mg of protein per ml of suspension.

2.11. Preparation of human erythrocyte ghost

Small amounts of human erythrocytes were prepared from 10 ml of freshly drawn blood by sedimentation, at unit gravity, through 4 volumes of 0.75% (w/v) dextran T-500 at room temperature in Tris-buffered saline (TBS), pH 7.5 (10 mM Tris-HCl and 150 mM NaCl). The cells were then washed three times in 10 volumes of TBS supplemented with 10 mM glucose, before being resuspended to a 20% (v/v) suspension, with respect to the packed-cell volume (Kuhlman, 2000).

2.12. Measurement of lipid peroxidation by thiobarbituric acid (TBA) Assay

Protein estimation in the above three preparations was done by the method of Lowry, Rosenberg, Farr, and Randall (1951) and an aliquot containing 500μg protein equivalent was used for lipid peroxidation assay, as described by Halliwell and Gutteridge (1989). Briefly, aliquots of the tissue homogenates were treated with different concentrations of the extracts in methanol (0.5 ml) followed by 1.0 ml each of 10 μM $FeSO_4$ and 0.1 M ascorbic acid and incubated at 37 °C for 60 min. One ml each of 28% TCA and 1% TBA were added to the reaction mixture and heated for 15 min at 95 °C. After cooling on ice and centrifuging the samples, the absorbance of the supernatant was read at 532 nm. The reaction mixture without the extract served as a control.

2.13. Formulation of salbutamol syrup and determination of colour stability

Since, anthocyanins are hydrophilic compounds, they can be easily incorporated into syrups, which is one of the most accepted form of pharmaceutical formulations. The high antioxidant property of the pigments may confer additional prophylactic advantage, along with the therapeutic property of the active ingredient in the syrup. Salbutamol syrup (antitussive) was prepared according to Howard (1996) and its stability was studied for 8 weeks under light (2000 Lux) and dark conditions at different temperatures (20, 30 and 45 °C). Similarly, the stability of the anthocyanin extract in 1% aqueous solution (pH 3.0) was monitored for 4 weeks at 0, 10, 20, 30 and 45 °C under dark conditions. For the stability studies, the test samples (10 ml) were dispensed into screw-capped tubes and stored. Each tube was used for one spectral measurement only, so as to minimize the contact with oxygen. The stability was assessed by measuring the remaining pigment using spectrophotometry at an absorption maximum of 520 nm (Singha, Baugher, Twonsed, & D'souza, 1991).

3. Results and discussion

3.1. Composition

The results of spectrophotometric analyses of the extract revealed a total phenolic content (inclusive of anthocyanins) of 560 mg GAE/100 ml and an anthocyanin content of 216 mg/100 ml, the latter being equivalent to 230 mg/100 g fruit on a dry weight basis. Thus, the total anthocyanins in *S. cumini* are equivalent to blue berries and black currants that contain 230 and 229 mg/100 g, on a dry weight basis, respectively and are 1.6-times higher than that of black berries (141 mg/100 g) (Moyer, Hummer, Finn, Frei, & Wrolstad, 2002). The blue and black berries have recently been regarded as premier nutraceutical commodities of great commercial value. Since, *S. cumini* trees are perennial, with enormous yields of berries, the information found through the present study appears to partly complement its application in various nutraceutical products.

The analysis of the extract by paper chromatography showed three distinct mauve-coloured bands. The acid hydrolysis of the *S. cumini* extract, followed by paper chromatography and ESI–MS, revealed the unique presence of glucose, thus indicating that glucose may be the only sugar involved in the formation of these anthocyanin glycosides. No acids were found on alkaline hydrolysis of the anthocyanins, thus suggesting the non-acylated nature of the anthocyanins.

HPLC, coupled to MS, has been a powerful tool for the characterization of anthocyanins from various sources. A good HPLC separation was achieved by direct injection of the partially purified extract (Fig. 1). Three major compounds were identified as glucoglucosides of delphinidin (1), petunidin (2) and malvidin (3), based on previous work (Venkateswarlu, 1952) and also supported by their respective MS. The major anthocyanins, corresponding to peak 1, 2 and 3, represented about 23%, 35% and 38%, respectively of the total peak area revealed at 520 nm. ESI–MS of each peak resulted in clear and characteristic fragmentation patterns (Table 1). A typical ESI positive MS shows two ions: the protonated molecular ion $[M + H]^+$ and a fragment ion $[M + H - X]^+$ arising from loss of the sugar moiety. However, since the anthocyanins have a natural residual positive charge, one observes a true molecular ion $[M]^+$ and a fragment ion $[M - X]^+$ which is of the underivatised aglycone. The value of X, based on the difference between the molecular ion and fragment, gives a clue to the nature of the sugar molecule (Abdel-Aal, Young, & Rabalski, 2006). The compound 1 produced ions at *m*/*z*

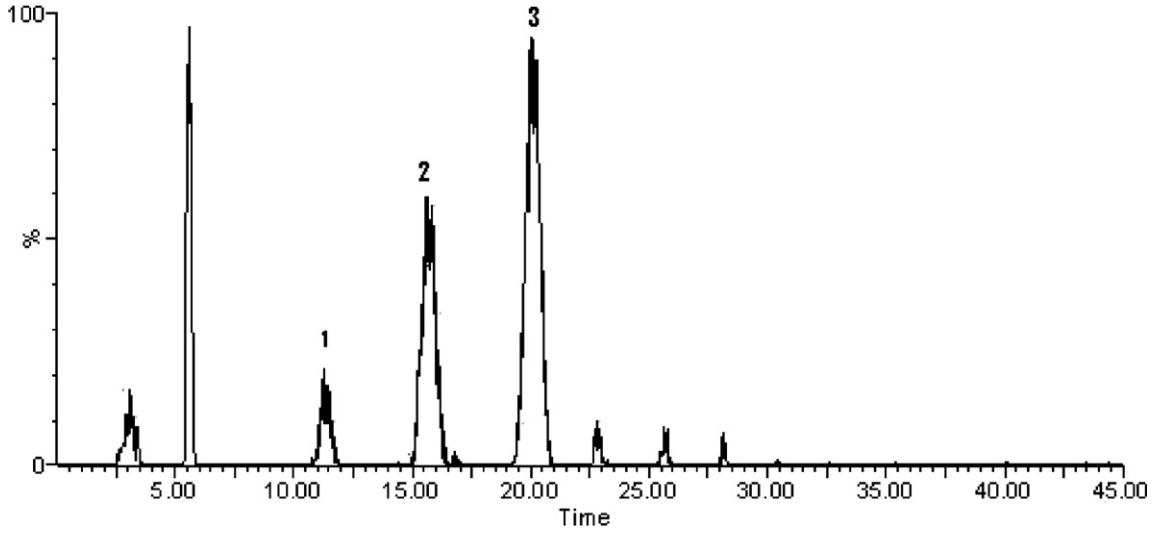

Fig. 1. HPLC separation of anthocyanins of *S. cumini*. (1) delphinidin-diglucoside; (2) petunidin-diglucoside and (3) malvidin-diglucoside. Detection was done at 520 nm. Other conditions are explained under the Section 'Materials and methods'.

Table 1
Assignment of M^+ ions for the anthocyanins and their fragments

Compound	Retention time (min)	ESI(+)–MS/MS *m/z*		
		M^+	$[M^+–Glu]^+$	$[M^+–Glu–Glu]^+$
Delphinidin-diglucoside	11.2	627 (100)	465 (40)	303 (100)
Petunidin-diglucoside	15.6	641 (90)	479 (50)	317 (100)
Malvidin-diglucoside	19.8	655 (90)	493 (50)	331 (100)

Values in parentheses represent the relative abundance of the ions.

627, *m/z* 465 and *m/z* 303. Similarly, compound 2 showed ions at *m/z* 641, *m/z* 479 and *m/z* 317 and compound 3 produced ions at *m/z* 627, *m/z* 493 and *m/z* 331 (Table 1 and Fig. 2a–c). This suggests that the aglycones are delphinidin (*m/z* 303), petunidin (*m/z* 317) and malvidin (*m/z* 331) for compounds 1, 2 and 3, respectively and the differences of *m/z* 162 and *m/z* 321 from the aglycone in the two fragments (in all three compounds) suggest the presence of 2 hexoses. The mass spectrum of these compounds revealed fragments resulting from the sequential loss of two glucose [M^+−162 Da] molecules. However, since both the sugar units are glucose (confirmed by paper chromatography of the acid hydrolysate in comparison with the authentic standards), it is not possible to determine the sequence of their elimination (Oliveira, Esperanca, & Almoster Ferreira, 2001).

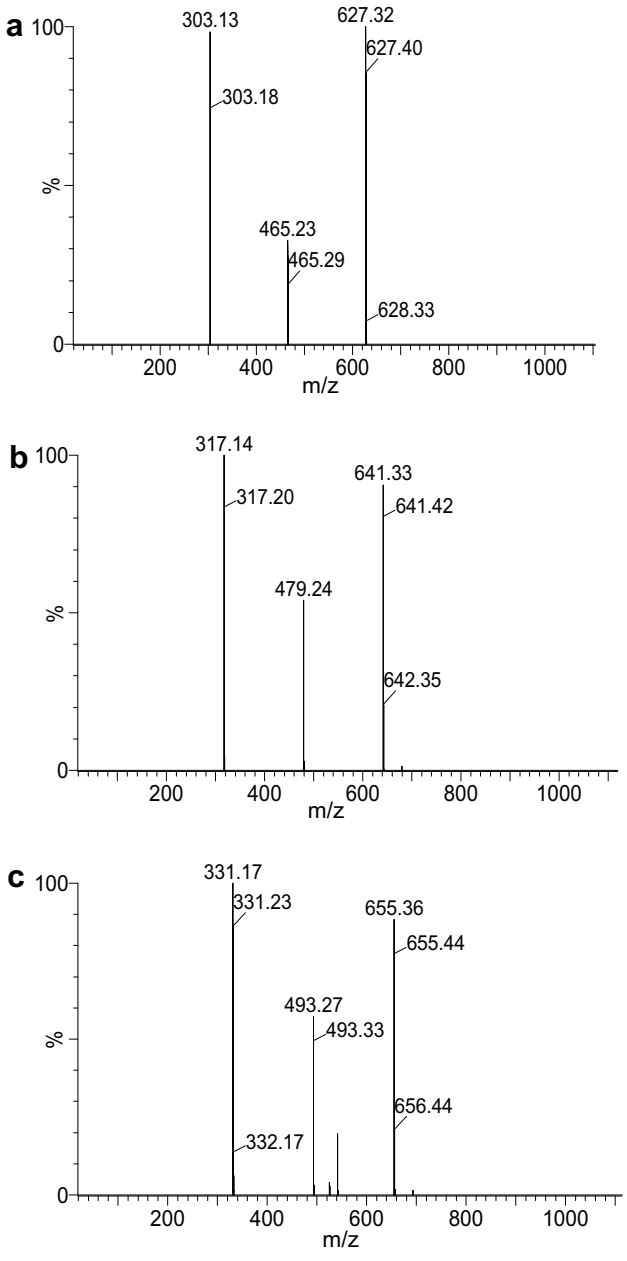

Fig. 2. ESI–MS spectra of anthocyanins of *S. cumini*. (a) delphinidin-diglucoside; (b) petunidin-diglucoside and (c) malvidin-diglucoside.

Upon paper chromatography of the anthocyanin extract, only three anthocyanin pigments of intense mauve colour were observed. When the methanolic solutions of individual spots were analysed by ESI–MS, only the same three anthocyanins were observed as were identified in the partially purified total extract.

The HPLC pattern of the phenolic components of the anthocyanin extract and the ethyl acetate fraction are shown in Fig. 3a and b. The ethyl acetate fraction of the anthocyanin extract exhibited more peaks with higher intensity than did the anthocyanin extract. This suggests that most of the other phenolics were extracted into ethyl acetate fraction during the partial purification of anthocyanin extract before its application onto the Amberlite XAD-7 column. In addition, from the X-axis values of both the chromatograms (Fig. 3a and b; analysed under identical conditions), it could be inferred that the phenolic content in the anthocyanin extract was less than 8% of that in the ethyl acetate fraction. Therefore we suggest that activity of the extract might be mainly due to the anthocyanins and the other phenolics play only a minor role in contributing to the antioxidant activity.

3.2. Antioxidant activity

DPPH$^{\cdot}$-scavenging assay is useful for rapid analysis of free radical-quenching efficacies of various plant extracts (Murthy et al., 2002). DPPH$^{\cdot}$, a stable free radical with an unpaired electron, shows a strong absorption band at 517 nm, its solution appearing deep violet in colour. As the electron becomes paired off, which happens in the presence of an antioxidant (electron/hydrogen donor), the absorption vanishes (Blois, 1958). Thus, the faster the reaction, the more potent is the free radical-scavenging ability

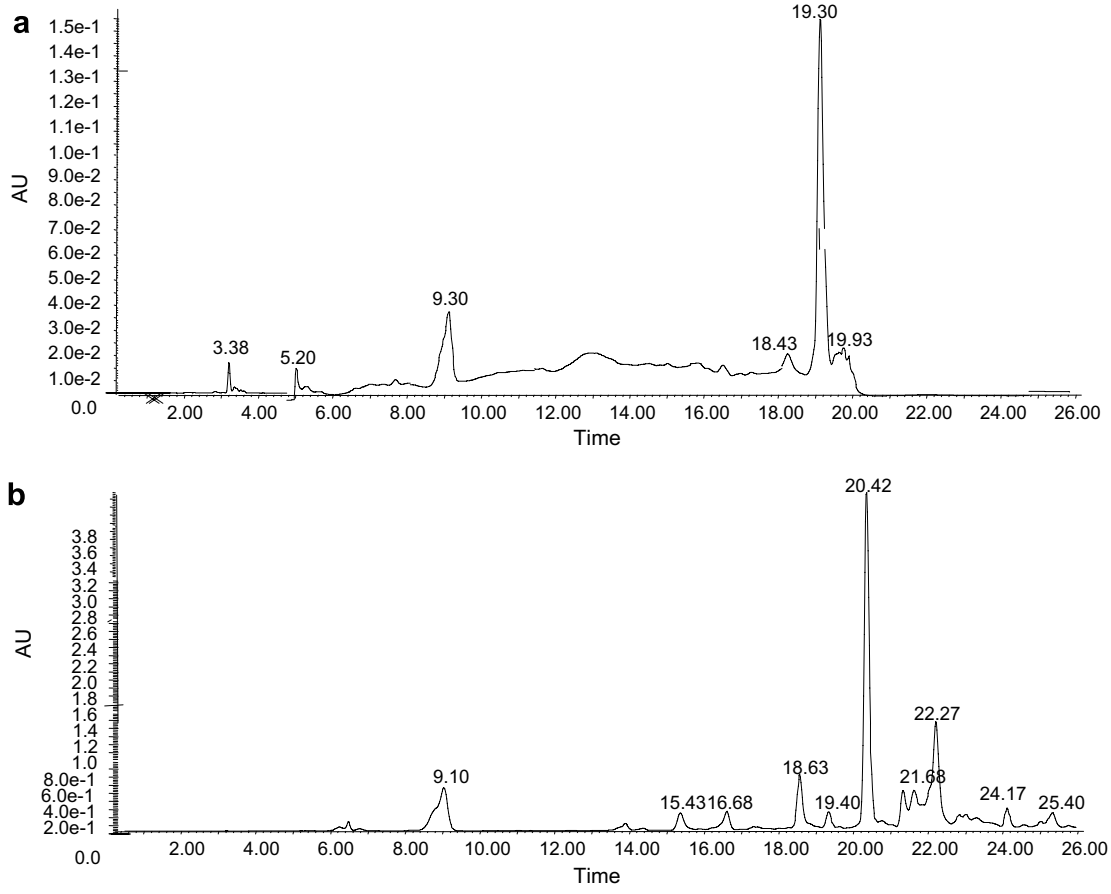

Fig. 3. LC/MS chromatograms of (a) anthocyanin extract and (b) ethyl acetate fraction of anthocyanin extract. Chromatograms were recorded at 280 nm.

Table 2
Antioxidant activity of anthocyanin-rich extract of *S. cumini* fruit peel[a]

Conc. (ppm)	% DPPH·-scavenging activity	Reducing power[b]	% Protection against lipid peroxidation				
			Brain	Liver	Testes	LM[c]	EG[d]
0.5	27.0 ± 1.71	2.16 ± 0.72	68.3 ± 0.22	26.1 ± 1.55	25.8 ± 1.65	25.5 ± 1.57	5.86 ± 0.02
1	46.8 ± 0.72	3.50 ± 0.21	71.2 ± 2.31	57.4 ± 1.08	43.2 ± 0.33	44.1 ± 0.85	32.4 ± 0.65
1.5	62.4 ± 1.02	5.86 ± 0.72	73.6 ± 1.25	62.6 ± 1.23	50.1 ± 0.98	67.1 ± 1.23	35.2 ± 1.68
2.0	71.4 ± 0.85	8.05 ± 1.05	74.7 ± 1.54	67.2 ± 0.98	56.7 ± 1.65	70.5 ± 1.66	38.8 ± 2.03
2.5	78.2 ± 1.03	9.72 ± 0.72	86.7 ± 1.80	70.0 ± 1.55	63.4 ± 2.02	72.8 ± 2.56	40.8 ± 1.65
5.0	–	–	94.5 ± 0.11	83.3 ± 1.33	72.3 ± 2.65	86.3 ± 2.05	48.6 ± 1.23

[a] Data are expressed as mean ± SD ($n = 3$).
[b] Reducing power is expressed as μM ascorbic acid equivalents.
[c] Liver mitochondria.
[d] Erythrocyte ghost cells.

(Amarowics, Pegg, Rahimi-Moghaddam, Barl, & Weil, 2004). In the present study, the pigment extract quenched the DPPH· in a dose-dependent manner. At 2.5 ppm, the activity of the extract was 78.2%, while BHA exhibited only 41.6% activity (data not shown) at the same concentration (Table 2), thus proving the extract to be a potent free radical-scavenger than the widely used BHA. A recent study has reported a 50% DPPH·-scavenging with 168 μg/ml of the hot aqueous infusion prepared from fruit peels of *S. cumini* dried for 7 days (Banerjee, Dasgupta, & De, 2005). However, our study establishes that the fresh fruit peel extract offers the same effect at concentrations as low as 1.39 μg/ml suggesting that the drying and heating process might lead to the loss of thermolabile bioactive substances e.g. anthocyanins and other polyphenols.

The reduction capacity of the extracts is directly proportional to the green/blue colour produced, due to the reduction of Fe^{3+}/ferricyanide complex to the ferrous (Fe^{2+}) form. The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Gulcin, 2005). The Fe^{3+}–Fe^{2+} transformation, in the presence of the extracts, increased with increase in concentration of the extracts.

One ppm of the extract was equivalent to 3.5 μM of ascorbic acid in bringing about Fe^{3+}–Fe^{2+} transformation (Table 2). This again proves the superiority of the anthocyanin-rich extract of *S. cumini* over the traditional antioxidants.

The introduction of phenolic compounds into the lipid-rich bio-membranes effectively inhibits the lipid oxidation caused by the chain-propagating lipid peroxyl radicals and ferrous generating enzymatic systems present on them (Pazos, Lois, Torres, & Medina, 2006). Anthocyanins from various sources, being polyphenolic in nature, are known to cause similar effects against various oxidative reactions (Kong et al., 2003; Cooke, Steward, Gescher, & Marczylo, 2005). In the present study, the *S. cumini* extract was tested for its ability to inhibit the iron-induced lipid peroxidation in rat brain, liver, liver mitochondria, testes and human erythrocyte ghost cells. The data (Table 2) indicated that the pigment extract was most effective (94%) against brain lipid peroxidation at 5.0 ppm compared with rat liver (83%), mitochondria (86%) and testes (72%), erythrocyte ghost cells (48%) being least responsive to anthocyanin treatment. This could possibly be due to the involvement of a different mechanism in erythrocyte membranes, which may act as barriers to large molecules. From the data, it can also be observed that the EC_{50} of the extract was less than 0.5 ppm for brain homogenate while it was more than 5.0 ppm for erythrocytes. The homogenates of liver and liver mitochondria were almost equally protected against peroxidation by the anthocyanin extract, indicating a possible similarity in the mechanism involved (Table 2). BHA showed approximately 80% activity against all the systems (data not shown), which is very close to that of the anthocyanins. The extent of protective effect exerted by the anthocyanins in different tissues is in the following order: rat brain > liver mitochondria > liver > testes > human erythrocyte ghost cells. In another study, a 400-fold higher quantity (222 μg/ml) of the hot water extract of dried peel of black plum was required to cause a similar effect, and further decrease of activity occurred when the dried fruit peel was stored for 6 months (Banerjee et al., 2005). The lower efficacy in the latter study is probably due to the loss of anthocyanins during heat processing. Through the present study, it has been clearly established that the fresh peel extract of *S. cumini* is a powerful antioxidant that can find various food applications.

Stability of a coloured compound is very important in retaining the appearance of the product in which it is incorporated and hence the customer acceptance, as well as the bio-efficacy. Naturally occurring colorants, such as anthocyanins from black carrot, grape, red cabbage, are now frequently used in foodstuffs and pharmaceuticals due to their

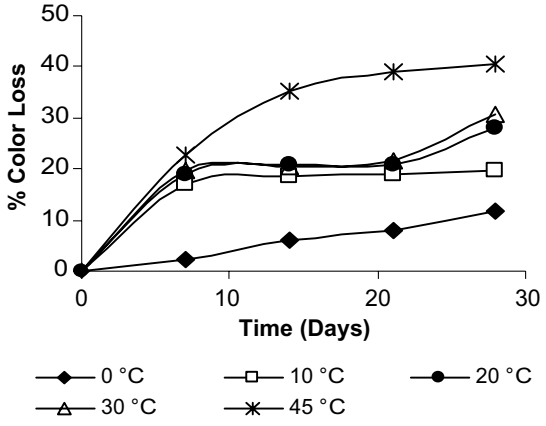

Fig. 4. Stability of anthocyanin-rich extract of *S. cumini* fruit peel. Aqueous solution (1%) of the extract (pH 3.0) were stored in screw-capped vials. Samples from two of the vials were taken afresh every week and absorbance measured at 520 nm.

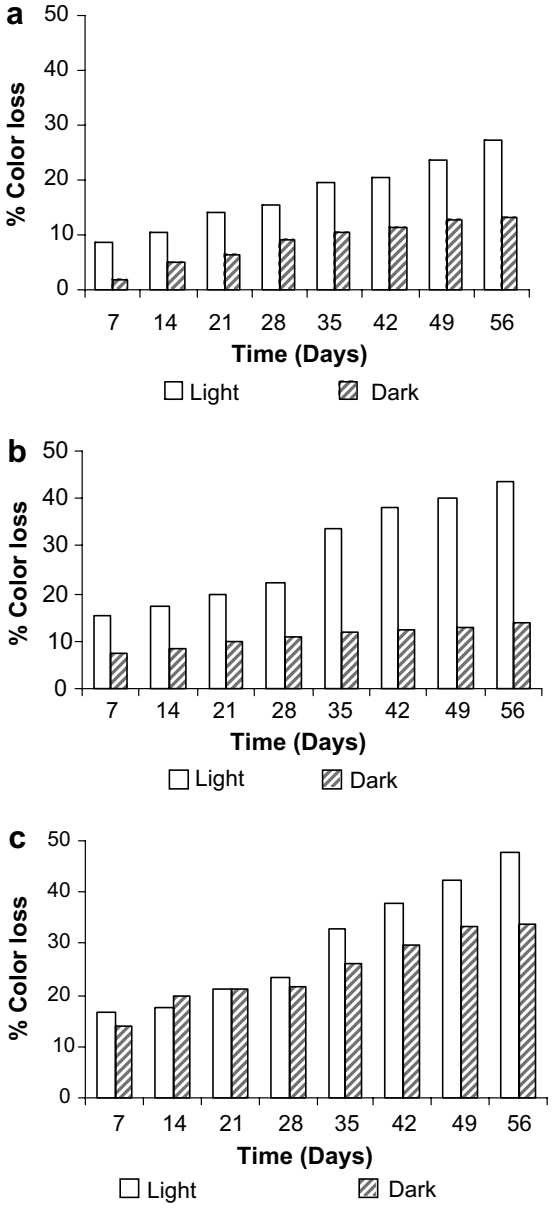

Fig. 5. Loss of colour in the salbutamol formulation when stored at (a) 20 °C; (b) 30 °C and (c) 45 °C under light (2000 Lux) and dark conditions. The formulation was stored in screw-capped vials. Samples from two of the vials were taken afresh every week and absorbance measured at 520 nm.

additional health benefits (Markakis, 1982; Teresa, Buelga, & Gonzalo, 2002). However, anthocyanins are relatively unstable, especially when non-acylated (Kirca, Ozkan, & Cemeroglu, 2007) and are prone to degradation in the presence of light, high temperature and varying pH (Cemeroglu, Velioglu, & Isik, 1994; Fossen, Cabrita, & Anderson, 1998). Therefore, the present study aimed at determining the colour stability in the concentrated extract, as well as in a pharmaceutical preparation containing salbutamol, a widely accepted antitussive. Thermal stability of the pigment extract stored in the dark was studied at 0, 10, 20, 30 and 45 °C for 4 weeks and the syrup containing anthocyanins extract was studied at 20, 30 and 45 °C in the dark and in the presence of a continuous illumination of 2000 Lux for 8 weeks. The colour loss in both the extract and the syrup increased with increase in temperature/time and more so in presence of light in the case of syrup. At the end of 4 weeks, the colour losses in the extract were 11.8 and 19.6% at 0 °C and 10 °C, respectively (Fig. 4). The syrup exhibited a 13% loss when stored at 20 °C and 30 °C in the dark (Fig. 5a and b) after 8 weeks. The presence of light, along with high temperature, accelerated the loss of colour in the syrup (Fig. 5c). However, there was no observable difference in the colour of the extract/formulation to the naked eye. Since, the syrups are generally stored in amber-coloured containers, the pigment from *S. cumini* appears to be useful for similar formulations that are stored on the shelf at room temperature. However, the extract is more stable when stored at 0 °C.

4. Conclusions

The present work reports the chemical nature of anthocyanins present in the berries of *S. cumini* by HPLC–MS for the first time. The high antioxidant activity of the extract at extremely low concentrations makes *S. cumini* a potential source of antioxidants, as well as a natural colourant. The *S. cumini* anthocyanins, like all the other anthocyanins, have the advantage of high solubility in aqueous mixtures, imparting an attractive colour that makes easy their incorporation into numerous aqueous food and non-food formulations, including pharmaceuticals. *S. cumini* extract is a rich source of anthocyanins whose content is equivalent to that of blue berries and black currants and higher than that of blackberries, all widely acclaimed anthocyanin-rich edible fruits (Singha et al., 1991). Like grape anthocyanins, that are sold commercially as oenocyanin, the peel powder of *S. cumini* may also be employed as a colorant for foods and pharmaceuticals.

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