ISSN (Online): 2091-2609
DOI Prefix: 10.3126/ijasbt

International Journal of Applied Sciences and Biotechnology (IJASBT)

Short Communication

Characterization of Pigments from the Berries of *Syzygium caryophyllatum:* Novel Source of Anthocyanins

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Abstract

Extract was prepared from the pulp and peels of the *Syzygium caryophyllatum* berries. At pH ≤2, the extract showed prominent red coloration and at pH values above 7, the extract attained bluish-green coloration. These typical characteristics indicate presence of anthocyanin pigments in the berries. The yield of the anthocyanins was around 1.9mg per gram wet weight and, around 43% of the anthocyanin pigments appear to be present in polymerized forms. At pH 1.0, the pigments absorbed maximally at 515nm and at pH 9.0, the pigments were found to absorb to a maximum extent in the range of 380-400nm. The colors at alkaline pH were unstable, while the red coloration under acidic conditions was relatively stable when exposed to fluorescent light and temperature of 50°C. Mass spectrum of the extract showed predominance of malvidin and petunidine derivatives in the extract. *Syzygium caryophyllatum* berries can serve as reservoirs of anthocyanin pigments.

Keywords: *Syzygium caryophyllatum*; anthocyanins; stability of pigments

Introduction

Natural and synthetic pigments are employed extensively in cloth, medicine, food, cosmetics and various other industries. Toxic potential of most synthetic pigments and the environmental concerns have propelled researchers to search for colorants from natural sources. Pigments derived from natural sources are not only useful in their contribution to enhance the aesthetic appeal, but are also safe and may also add to the nutraceutical value of the product. Chromophores with conjugated systems comprising of carotenoids, anthocyanins, betalains etc. and metal-coordinated porphyrins such as chlorophylls are the major pigments found in plants. Anthocyanins are often the major coloring components which impart colors and hues ranging from orange to blue to petals, fruits and leaves. Anthocyanins harvested from flowers, fruits, and vegetables have found use as dye and food colorant. Anthocyanins reportedly possess antidiabetic, anticancer, anti-inflammatory, antimicrobial, and anti-obesity effects. Anthocyanins are basically substituted glycosides of salts of

Cite this article as:

P. Shetty et al. (2019) Int. J. Appl. Sci. Biotechnol. Vol 7(2): 291-297. DOI: 10.3126/ijasbt.v7i2.24631

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Peer reviewed under authority of IJASBT

phenyl-2-benzopyrilium (anthocyanidins). Anthocyanidins are grouped into 3-hydroxyanthocyanidins, 3-deoxyanthocyanidins, and O-methylated anthocyanidins. Cyanidin, Delphinidin, Pelargonidin, Malvidin, Peonidin, Petunidine are the commonly found anthocyanidin constituents of anthocyanin pigments. The glycosylated and /or acylated anthocyanidins result in formation of anthocyanins (Delgado-Vargas *et al.*, 2000; Horbowicz *et al.*, 2008). Acyl residues such as coumaric acid, caffeic acid, malonic acid etc. are often linked to the available hydroxyl residues of the glycosyl moieties. Thus, great number of combinations of glycosylation and/or acylation can exist in nature, and their interaction with other molecules and/or media conditions, result in generation of wide array of colors of anthocyanin pigments. An estimate of >400 anthocyanins is known to occur in nature. Inspite of this large number, anthocyanins are generally known to exhibit typical absorption characteristics. Presence of eight conjugated double bonds carrying a positive charge results in Intense red or orange under acidic conditions. With increasing pH, they lose color and start gaining blue-green coloration as the pH becomes alkaline. The Intensity and shades of the color is influenced by the number of hydroxyl and methoxyl groups. Bluish color is affected by presence of hydroxyl groups, while presence of more methoxyl residues is reported to contribute intense red color. Glucose is often the most commonly fund sugar. Rhamnose, xylose, galactose, arabinose, as well as rutinose are the other sugars which occur in anthocyanins. At a pH 2, anthocyanin solutions show absorption absorption maxima in the ultraviolet region (260-280 nm) and two in the visible region at 415 and 490-540 nm (Horbowicz *et al.*, 2008; Khoo *et al.*, 2017; Wahyuningsih *et al.*, 2017)

Purple colored dark berries of *Syzygium caryophyllatum* have remained unexplored. These pigmented berries are edible and leave the mouth especially the tongue and the palms intensely colored on contact. The present investigation was undertaken to characterize the pigments present in these berries.

Materials and Methods

All the reagents used were of analytical grade.96 well plates were procured from Tarsons Products Pvt Ltd. Silica gel 60 F_{254} plates were procured from MERCK, Germany and SIGMA –ALDRICH, China.

Preparation of Extract

Syzygium caryophyllatum berries were collected from the trees in the local region. The fruits were washed, seeds were removed and extracts from the peel and pulp were prepared in D/W. The extract was stored in freezer.

Absorption Spectra of the Extracts

To 50µl of the appropriately diluted sample, 150 µl of 0.2M buffer (pH 1.0, KCL-HCl; pH 4.5, acetate; pH 9, Glycine-NaOH buffer) was added to the wells in 96well plates. Absorption spectrum of the extract was scanned from 380-750nm at the intervals of 5nm.

Effect of Light and Temperature on the Absorption Spectrum

Appropriately diluted extract 50 µl was added to 150 µl of 0.2M buffer ranging from pH 1 to pH 9 (pH 1.0-2.0, KCL-HCl; pH 3-5, acetate; pH 5.5-8.5, phosphate and pH 9, Glycine-NaOH buffer) in 96 well plates. The plates were placed below the fluorescent light at a distance of 30cm/ in dark/ at 50°C for 1h. The absorbance at 0min and at the end of 1h was noted.

Relative Quantification of Anthocyanins

Approximate quantification of the pigments was carried out by employing the pH differential method using the following formula (Giusti and Wrolstad, 2001):

$$A=(A_{515}-A_{700})_{pH\ 1.0} - (A_{515}-A_{700})_{pH\ 4.5}$$

A_{515} is the extinction at the wavelength maximum, and A_{700} is the absorbance due to various phenolics and other interfering compounds which are likely to be present in the extract. The total monomeric anthocyanin content was calculated considering the molar extincton coefficient of the malvidin monoglucoside (≈36000).

Estimation of Polymeric Color

To 25µl of the extract, 0.98ml of 0.1 M KCl-HCl buffer, pH1 containing 3.5mg of potassium meta-bisulfite was added. The absorbance values of the bisulfite treated extract at 420, 515 and 700nm were noted immediately after addition of bisulfite. Colour density of untreated control was calculated as follows, Color density = $[(A_{420nm} – A_{700nm}) + (A_{515nm} – A_{700\ nm})]$ x dilution factor

The percent of polymeric form of anthocyanins present in the bisulfate treated sample was calculated based on following formulas (Giusti and Wrolstad, 2001).

Polymeric color = $[(A_{420nm} – A_{700\ nm}) + (A_{515nm} – A_{700\ nm})]$ x dilution factor

Percent polymeric color = (polymeric color/color density) x100.

Determination of Anti-Oxidant Capacity, DPPH Scavenging Ability and Phenolics Content

To the sample 1ml, 0.5ml of Potassium ferricyanide (1% w/v) was added followed by incubation at 50^0C for 20min. TCA (10%) 0.5ml was added followed by centrifugation at 1000rpm for 6min. One ml of the supernatant was diluted to 2ml and 0.15ml of Ferric chloride (mg/ml) was added. Absorbance was read at 700nm. Reducing ability was calculated in terms of Vitamin C.

To 1 ml of 0.002 % DPPH (in absolute methanol), equal volume of the appropriately diluted extract was added. After incubating for 30min in dark, the absorbance was read at 517nm. Radical scavenging capacity was calibrated in terms of Vitamin C.

Phenolic content of the extract was estimated using Folin Cio-calteau reagent. Phenol was used as the standard.

Thin Layer Chromatography

To 100µl of the extract, 150 µl of 5N NaOH was added and left in dark for 10min, followed by addition of 120 µl 5N HCl. Periodate oxidation of the glycosyl residues was carried out by treating the extract with 5mM sodium periodate for 30min followed by neutralization of excess periodate with 10mM PEG 6000. The extract subjected to alkaline hydrolysis and periodate treatment were spotted (extract obtained from 20mg of the sample) onto silica gel plates and chromatogram was developed using the upper organic layer of butanol: acetic acid: Water (4:1:5) mixture as the solvent system. The spots were observed after 6h.

Mass Spectrometry

The extract prepared in 80% methanol containing 0.01N HCl was subjected to Mass spectrometric analysis (MALDI) using Bruker Autoflex Spec. Alpha cyano 4 hydroxycinnamic acid (in equal volumes of CH3CN and 0.1% TFA in water) was used as the matrix. Premix of 2µl each of matrix and extract was applied by dried droplet method. The analysis was performed in reflectron mode with smart beam –II solid state laser for a mass range of 200-1000.

Results and Discussion

Syzygium caryophyllatum tree grows extensively in the forests of south India. The berries though edible, have not found much use because the pulp layer is very thin. The berries and their colored pigments therefore, have not received due attention. The extract prepared in D/W was subjected to characterization of the pigments. Effect of pH on the color of the extract was studied. As observed in Fig. 1, at pH 1.2, the color of the extract was intense red. With increasing pH, the red/pink color of the solution faded and at pH 5.5, the solution showed traces of pinkish hue. However, as the pH approached neutral pH the solution became colored. At pH 7 the color was purplish (color of the berry) and at higher pH values, the solution attained bluish-green coloration. After 5h at ambient temperature (30±1°C), respective colors at pH values above 6.5 especially at pH 9 faded to a significant extent. The red color seen in the highly acidic range was stable even after 24h of incubation. The hue of bluish-green coloration observed at 0h under alkaline conditions, disappeared at the end of 24h. This is typical of anthocyanin pigments as anthocyanins are considered to occur in several equilibrium forms. In strongly acidic solutions (pH ≤ 2), the flavylium cations of the anthocyanins predominate, exhibiting red coloration. As the acidity decreases, the anthocyanins acquire neutral and/or ionized quinonoidal basic forms as a consequenceof deprotonation. As a result, the flavylium cations form convert to the colourless hemiketal (alternatively hemiacetal or carbinol pseudobase) and chalcone forms. At higher pH, the aromatic acylated anthocyanins are reported to exhibit bluish/greenish coloration. The characteristics of the pigments are typical of anthocyanin pigments. Thus, it appears anthocyanins are the major pigments present in the extract of *S caryphyllatum* berries.

The absorption spectrum in the visible region of the extract at pH values of 1, 4.5 and 9 is shown in Fig. 2. It can be seen that at pH 1.0, the absorption of the pigments is maximum at 515nm. At pH 9.0 however, the pigments absorb maximally in the range of 380-400nm.

Fig. 1: Effect of pH and time on the colour of the *Syzygium caryophyllatum* extract

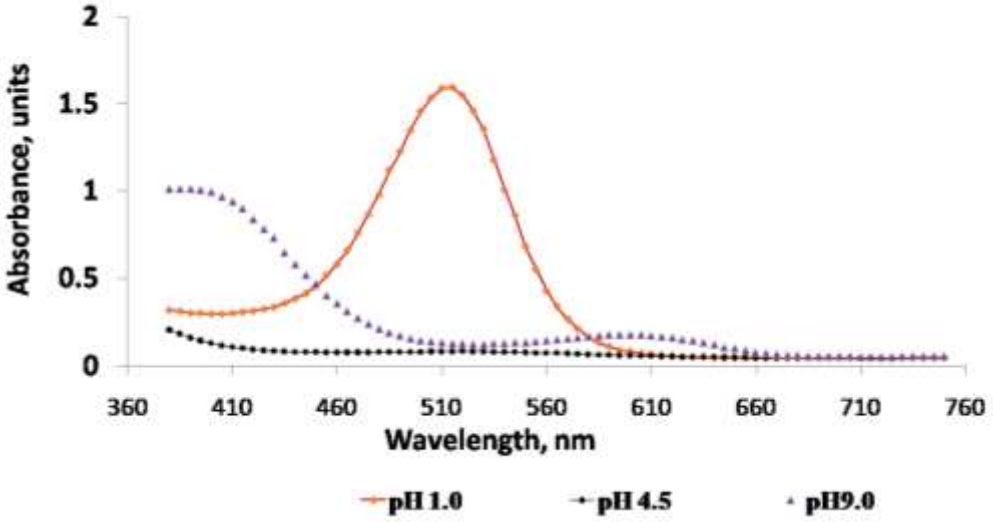

Fig. 2: Absorption spectra of the *Syzygium caryophyllatum* extract

Co-pigmentation or polymerization of anthocyanins with other phenolic compounds is known to occur in nature. These polymerized anthocyanins are known to confer stability to anthocyanins against environmental/chemical factors such as heat, light, and SO_2. Anthocyanin pigments are reported to form a colorless sulfonic acid adduct with bisulfite. However, polymerized colored anthocyanin-tannin complexes are resistant to bleaching by bisulfite, whereas the monomeric anthocyanins will lose their color rapidly. The absorbance at 420 nm of the bisulfite-treated sample serves as an index for browning. Around 43% of the anthocyanin appears to be in polymerized forms. The yield of anthocyaninin pigments by relative quantification (pH differential method) was found to be around 1.9 mg per gram wet weight of the fruit (peel+pulp). Phenolic content of the extract was found to be relatively high around 17.65mg/g wt weight. Anthocyanins are well documented for their medicinal properties. DPPH scavenging ability and reducing power approximating 2.5mg and 3.4mg of Vit C was observed respectively in gram wet weight.

Light and heat are reported to affect the stability of anthocyanins. In the present investigation, the extracts maintained at various pH values were exposed to fluorescent light while the control for each was kept at ambient temperature for 1h in dark. Effect of heat was studied by incubating the samples at 50°C for 1h. The absorption spectra for the sets are shown in Fig. 3. The color of the pigments incubated in dark remained relatively stable for an hour (Fig. 3A). At higher pH of 9, exposure to light appears to initiate degradation of the pigments as seen by the reduction in extinction (6-9%) at 405 and 440nm (Fig. 3B). However, this degradation was found to be more significant (17-18%) in samples incubated at high temperature (Fig. 3C). Red coloration of flavyllium cations remained unaltered to a significant extent in almost all the samples. It was interesting to note that in the initial one hour of incubation at pH 8, the A_{405nm} of the pigment was found to increase to an extent of around 20-25% in samples kept in dark and in presence of light. An intensification of around 10% was observed in samples maintained at 50°C. It must be noted that as shown in Fig. 1, at pH 7 and 9 significant alteration/ reduction in color was observed at pH 7 and 9 at the end of 5h of incubation. However, at pH 8, the reduction was not as significant. Overall, the results exhibited in Fig. 1 corroborates well with the absorption spectra shown in Fig. 3. Effect of exposure to UV light yielded results similar to that of sample exposed fluorescent light (results not shown). The use of these pigments in acidic foods can be considered, as the red color is relatively stable.

The pigments subjected to alkali treatment was loaded onto silica gel plate. Treatment with alkali is known to render the ester bonds labile, which couple the organic acid moietes to the sugar residues, thereby deacylating the acylated anthocyanins (Moreno *et al.*, 2005; Tatsuzawa *et al.*, 2012). Periodate treatment is known to affect the glycosyl residues by oxidative cleavage of the bond between adjacent carbon atoms carrying hydroxyl groups (Shetty and Jaffer, 2017). As seen in the chromatogram (Fig. 4), the major spot in the alkali treated sample migrated faster than that in the untreated and the periodate oxidized anthocyanins. The color of the spot in untreated sample remained purplish, while the color of the spots in the chemically treated samples were altered. The results therefore appear to support the reports which state that glycosylation and acylation of anthocyanidins confer stability to the color/hue of anthocyanin pigments.

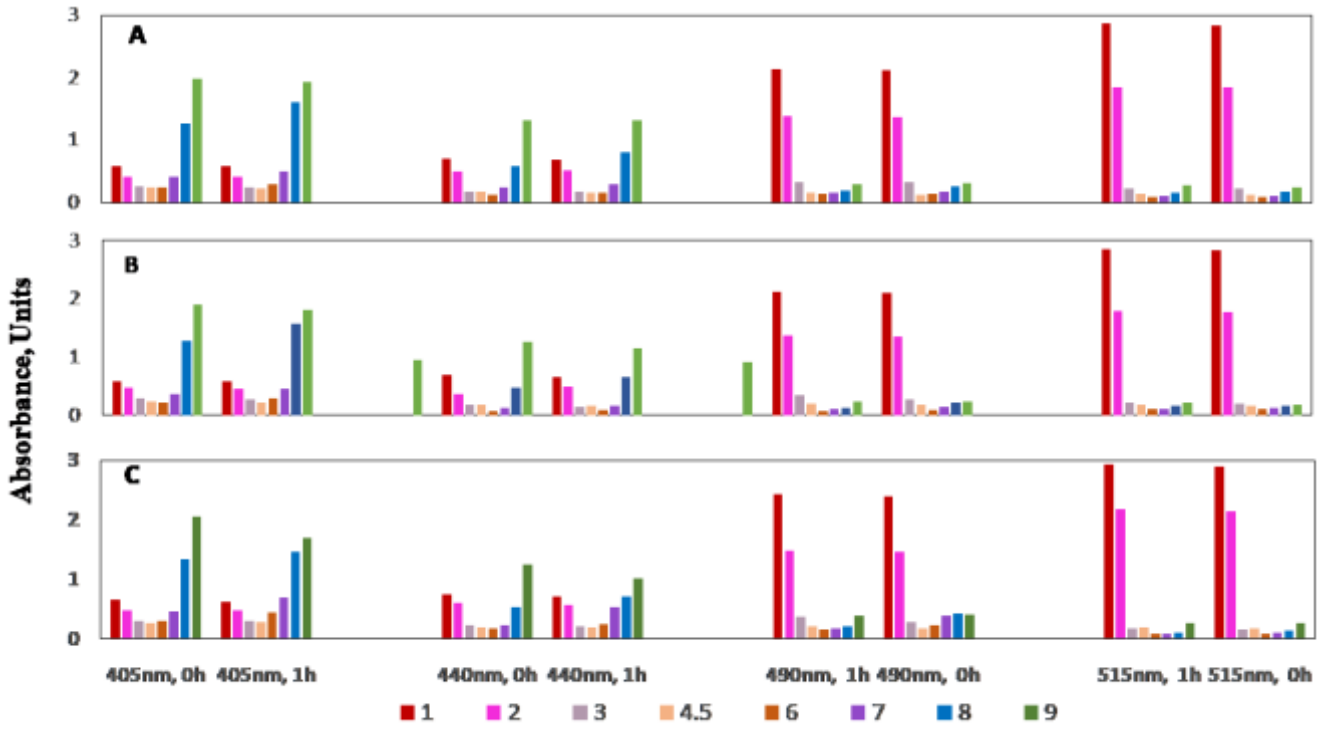

Fig. 3: Effect of Light and high temperature on the absorption spectrum of *Syzygium caryophyllatum* extract
A: Extract incubated in dark; B: Extract exposed to fluorescent light; C: Extract incubated at 50°C

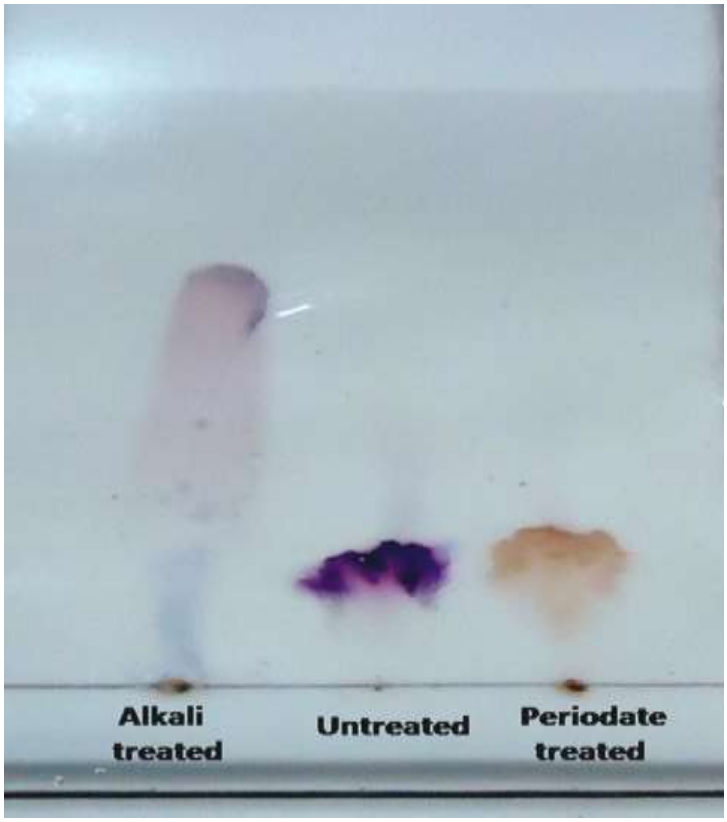

Fig. 4: Thin layer chromatogram of the extract subjected to chemical treatments

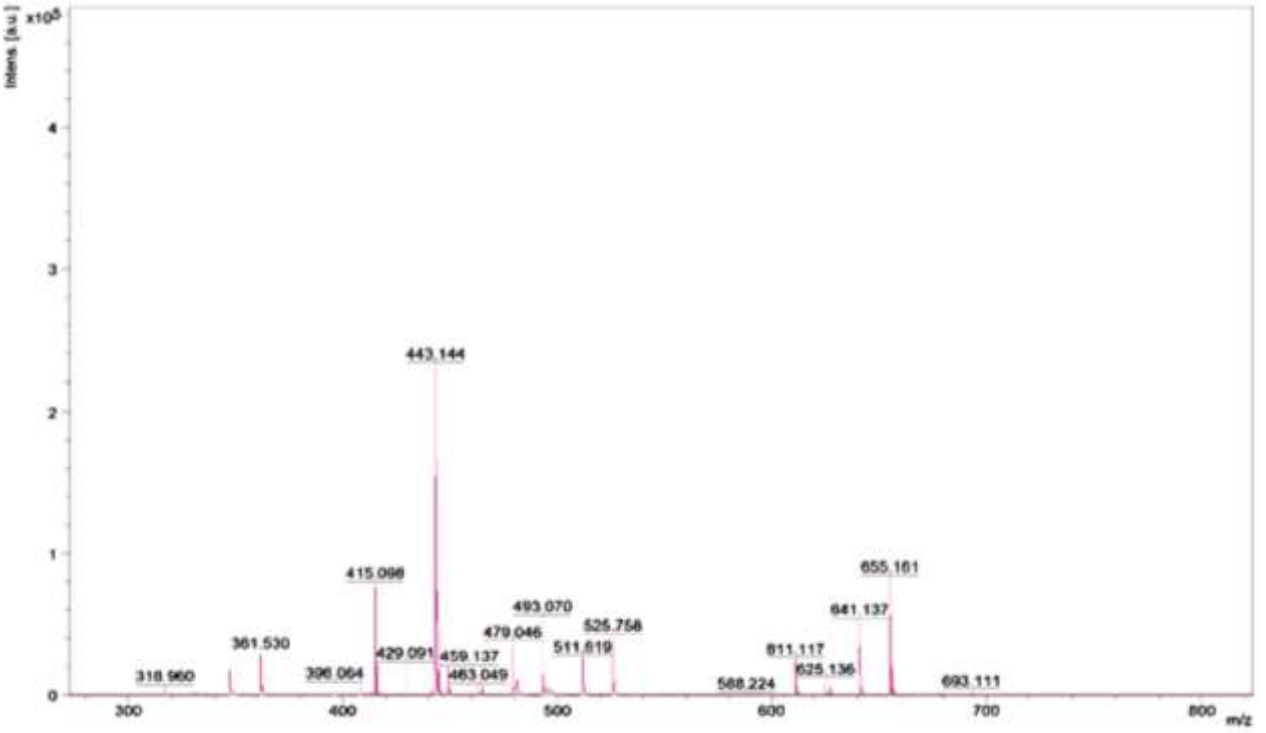

Fig. 5: Mass spectrum of the *Syzygium caryophyllatum* extract

Fig. 5 represents the mass spectra of the compounds present in the extract. A search (https://pubchem.ncbi.nlm.nih.gov/compound/ or http://phenol-explorer.eu/search) for the possible anthocyanin pigments corresponding to the molecular masses (m/z±0.4) exhibited in the mass spectra is presented in Table 1. Masses corresponding to the monoglycosides of malvidin, petunidine and peonidine were observed. Appearance of a mass of 611.12 can be due to the presence of any of the anthocyanin pigments presented in the Table 1. Apart from the monoglycosides, the diglycosides and acylated derivatives are the predominant forms documented to be present in many fruits.

The higher molecular masses of 655.16 and 641.137 and monoglycosides of mass 493.07 and 479.046 were the major anthocyanins identified in the extract. Cyanidin pigments are reportedly purplish red in colour, and malvidin and petunidins are purple violet (Młodzińska, 2009; Martin *et al.*, 2017). Cyanidin derivatives are reportedly the most common forms of anthocyanins present in fruits. However, in S caryophyllatum extract, malvidin and petunidin derivatives appear to be the major anthocyanins present. colored pigments and therefore, these pigments may be responsible for the dark purple colour of the S caryophyllatum berries.

Table 1: Analysis of the mass spectra of the extract

Molecular mass observed in the spectrum	**Anthocyanin pigment corresponding to m/z**
463.05	Peonidin monoglycoside
479.05	Petunidin monoglycoside
493.07	Malvidin monoglycoside
611.12	Cyanidin diglycoside, Cyanidin 3 gentiobioside
625.14	Peonidin diglycoside, Peonidin 3 gentiobioside, Petunidin 3 rutinoside
641.14	Petunidin diglycoside, Delphinidin feruloyl glucoside, Petunidin 3 gentiobioside
655.16	Malvidin diglycoside, Malvidin 3-(6-P-caffeylglucoside)

Conclusion

S. caryophyllatum berries were found to contain significant amount of anthocyanins. Malvidin and petunidin derivatives are the likely contributors to the colour of these berries. These trees grow extensively in and around the coastal regions of Karnataka. The berries though edible,

have not gained much popularity as the fleshy edible part surrounding the seed is a thin layer. The dark purple peel is relatively thick and hence, is rich in anthocyanin. These neglected berries can therefore find commercial use as sources of anthocyanins.

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