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Extraction of Anthocyanins from Seasonal Flowers and Biological Activities of its Phytochemicals

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Abstract

Anthocyanins are water soluble vacuolar pigments that, depending on their pH, may appear red, purple, or blue found in plants. In the current study anthocyanin colors were extracted from locally available flowers of the *Pyrostegia venusta, Butea monosperma, Cassia auriculata, Tecoma stans and Woodfordia fruticosa* using acidified methanol as a solvent. Obtained extracts were evaluated for their phytochemicals and assessed for its bioactivity such as antimicrobial (*E coli, Staphylococcus aeries and Bacillus subtilis*) and antioxidant properties. This flower extracts has shown considerably significant activities along with their coloring property. The results show the potential usage of these natural colorants in various coloring applications including food coloration, alternative to synthetic colors. The study also explores the utilization of easily available sources and value addition to waste products.

Key words: Anthocyanin, Natural colorant, Medicinal plants, Antioxidants, Antimicrobials

Introduction

Colour is one of most important properties of food and beverages and is basis for their recognition and acceptability. Normally, food colours are due to naturally occurring pigments, but synthetic colorants are often added to confer the desired colour to the final product. Although synthetic colour had been favoured over the past 100 years, their use has been reduced due their harmful health effects in the past four decades. Consumer attention have turned towards naturally derived colours as a viable alternative to synthetic ones leads several avenues in search of pigment from various natural sources. Several natural food colors such as anthocyanins, betalains, chlorophylls, phycocyanins etc. were extracted from fruits, vegetables and other natural sources.[1-3]

Anthocyanins are extracted from a wide variety of sources such as fruits (grapes, red

raspberry, cranberries),[4,5] Vegetables (red cabbage, red radish, sweet potato)[6,7] and petals of some flowers.[8,9] Anthocyanin are considered as potential replacements for synthetic counterparts due to their bright attractive colour shades and ease of solubility that allows their incorporation into aqueous food system along with their known health benefits.[10] Anthocyanins pigments extracted from flowers, fruits, and vegetables are traditionally used as dye and food colorant. Besides being used as natural colorants, some of the anthocyanin-rich flowers and fruits have been traditionally used as medicine to treat various diseases. Plant anthocyanins have been widely studied for their medicinal values and known for anti-diabetic, anti-cancer, anti-inflammatory, anti-microbial, and anti-obesity effects and in addition to prevention of cardiovascular diseases (CVDs).[11-16]

Anthocyanins are the phenolic subclasses in the form of glycoside known as the aglycone. Anthocyanins are grouped into 3-hydroxyanthocyanidins, 3-deoxyanthocyanidins, and O-methylated anthocyanins. The most common types of anthocyanins are cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin. Acylated anthocyanins are also detected in plants besides the typical anthocyanins.[17]

In the present study, the intense colored (Yellow, Pink and Red) flowers are used as an inexpensive sources of anthocyanins with potential to be used as natural, innocuous, and health beneficial colorants. The study involves isolation of anthocyanins from flowers of four selected plants *Pyrostegia venusta,Tacoma stans, Cassia arucilata, Woodfordia fruticosa andIpomoea cornea* and screening of biological activities. There are several scientific evidences with reference to these plants which could be potentially exploited for the extraction of various bioactives besides food colorants from these plants.

Material and Methods

Sample Collection

The flowers of the plants *Pyrostegia venusta, Tacoma stans, Cassia arucilata, Woodfordia fruticosa and Ipomoea* cornea were collected from the nearby locality in and around Manasagangothri campus in Mysore, India. The flowers were cleaned, dried under shade and stored for further use.

Anthocyanin Extraction

The pigment from the flowers was extracted in acidified 50% methanol using a food processor. The solid to liquid content was maintained at 1:2 ratios. The extracted pigment was filtered using cheese cloth to remove the fibrous particles and then it was centrifuged at 10,000 rpm for about 10 min to remove the tiny suspended particles. The obtained supernatant (anthocyanin extract) was stored at 4° C.

Anthocyanin Estimation

The pH differential method was used for quantitative determination of anthocyanins as reported by Guisti (2003).[18] Each of two 0.2 ml aliquots was diluted with 2.8 ml of pH 1.0 buffer (125 ml of 0.2 N KCl and 385 ml of 0.2 N HCl) or pH 4.5 buffer (400 ml of 1 N sodium acetate, 240 ml of 1 N HCl and 360 ml distilled water) solutions, respectively. The samples were incubated for 30 minutes in dark and the absorbance was measured at 510 nm using UV / VIS spectrophotometer. Total anthocyanin pigments were determined from absorbance in pH 1.0 buffer, while monomeric anthocyanins were determined from the differences between absorbance in pH 1.0 and 4.5 buffers.

The anthocyanin content in all the extract was calculated using the following equation

Anthocyanin pigment (mg/100ml)

$$= \frac{A \times Mw \times DF \times 100}{\varepsilon \times L}$$

where A= A_{510} (pH 1.0)–A_{510} (pH 4.5), Mw is the molecular weight of anthocyanin (433.2 g/mol), DF is the dilution factor, ε is the extinction coefficient (31,600 L/cm mol) and Lis the path length (1 cm).

Phytochemical Analysis

Qualitative estimations of phytochemicals were carried out according to standard procedures reported in the literature.[19]

Test for Carbohydrates

To 2ml of plant extract, 1ml of Molisch's reagent (α-naphthol dissolved in ethanol) and few drops of concentrated sulphuric acid were added. Presence of purple or reddish color indicates the presence of carbohydrates.

Test for Tannins

To 1ml of plant extract, 2ml of 5% ferric chloride (5% w/v solution of ferric chloride prepared in 90% alcohol) was added. Appearance of a dark green or deep blue colour indicated the presence of tannins.

Test for Flavonoids

To 2ml of the extract, a few drops of 10% ferric chloride solution were added. A green blue or violet colouration indicated the presence of Flavonoids.

Test for Alkaloids

To 2ml of plant extract, 2ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids.

Test for Quinones

To 1ml of extract, 1 ml of concentrated sulphuric acid was added. Formation of red color indicates presence of quinones.

Test for Phenols

To 1 ml of the extract, 2 ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of blue or green color indicates presence of phenols.

Test for Terpenoids (Salkowski Test)

To 0.5ml of extract, 2ml of chloroform was added, followed by a further addition of 3ml of conc. H2SO4 to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

Test for Cardiac Glycosides (Keller-Killiani Test)

To 0.5 ml of extract, 2 ml of glacial acetic acid and few drops of 5% ferric chloride were added. This was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of cardiac glycoside.

Test for Proteins

Theextract was taken with 2 ml of water and 0.5 ml of concentrated HNO_3 was added to it. Yellow colour is obtained if proteins are present.

Test for Triterpenoids

To 1.5ml of extract, 1ml of Libemann –Buchard Reagent (aectic anhydride concentrated sulphuric acid) was added. Formation of blue green color indicates presence of triterpenoids.

Test for Coumarins

To 1 ml of extract, 1ml of 10% NaOH was added. Formation of yellow color indicates presence of coumarins.

Test for Anthraquinones

To 1ml of plant extract few drops of 10% ammonia solution was added, appearance pink color precipitate indicates the presence of anthraquinones.

Test for Saponins

To 2 ml of plant extract, 2 ml of distilled water was added and shaken in a graduated cylinder for 15 minutes lengthwise. Formation of 1 cm layer of foam indicates the presence of saponins. The persisted frothing on warming was taken as an evidence for the presence of saponins.

Test for Sugars (Fehling's test for Free Reducing Sugar)

About 0.5g of each extract was dissolved in distilled water and filtered. The filtrate was heated with 5ml of equal volumes of Fehling's solution A and B. Formation of a red precipitate of cuprous oxide was an indication of the presence of reducing sugars.

Test of Steriods (Salkowski's test).

To 1 ml of the extract was added to 1 ml of chloroform in a test tube. Concentrated sulphuric acid was carefully added from the sides of the test tube, to form a layer. A reddish brown colour at the interphase indicated the presence of steroids.

Test for Glycosides

To 2 ml of plant extract, 3 ml of choloroform and 10% ammonia solution was added. Formation of pink color indicates presence of glycosides.

Phlobatannins

To 1ml of plant extract few drops of 2% HCL was added appearance of red color precipitate indicates the presence of phlobatannins.

Antibacterial Activity

Antibacterial activity of the extracts was carried out by agar well diffusion assay reported by Gonelimali et al.(2018)[20] with some modifications. Petri dishes were prepared by pouring 20 ml of sterilized nutrient agar media under aseptic condition and allowed to solidify. After solidification of the media,

100 µl of standardized test microbial inoculum of *S. aureus, B. subtilisand E. coli* were inoculated and spread uniformly using sterile spreader. 6 mm diameter agar is drawn from plate to form a well using sterile cork borer. Antibiotic Streptomycin was used as positive control. After keeping at 4 °C for 4 hours for the diffusion of anti-bacterial metabolites, thereafter plates were incubated at 37 °C for 24 h. The diameter of the inhibition zone around the well is measured in millimeter (mm) and the average of three repeated agar discs were taken to assess the strength of antibacterial activity.

Antioxidant Assay

DPPH Scavenging Assay

The free radical scavenging capacity of the extracts was determined by DPPH method reported by Williams et al.(1995)[21] with minor modifications. The DPPH radical solution (300µM) was prepared in ethanol kept in dark. 0.2 ml of Different concentrations of test samples 2.8 ml of DPPH was added. The tubes were incubated for 30 min at room temperature and the absorbance was recorded at 517 nm. Ascorbic acid (AA) was used as positive control. The free radical scavenging activity of samples was expressed in percentage and each sample was analyzed in triplicate. The free radical scavenging activity was calculated by using the following equation:

$$\text{Scavenging activity (\%)} = \frac{[Aa-(Ab-Ac)]}{Aa} x100$$

where Aa is the absorbance of the control solution of DPPH (without anthocyanin extract), Ab is the absorbance of the mixture containing anthocyanin extract as well as DPPH, and Ac is the absorbance of the blank solution without DPPH.

Results and Discussion

Anthocyanin estimation

Estimation of anthocyanin is carried out using pH

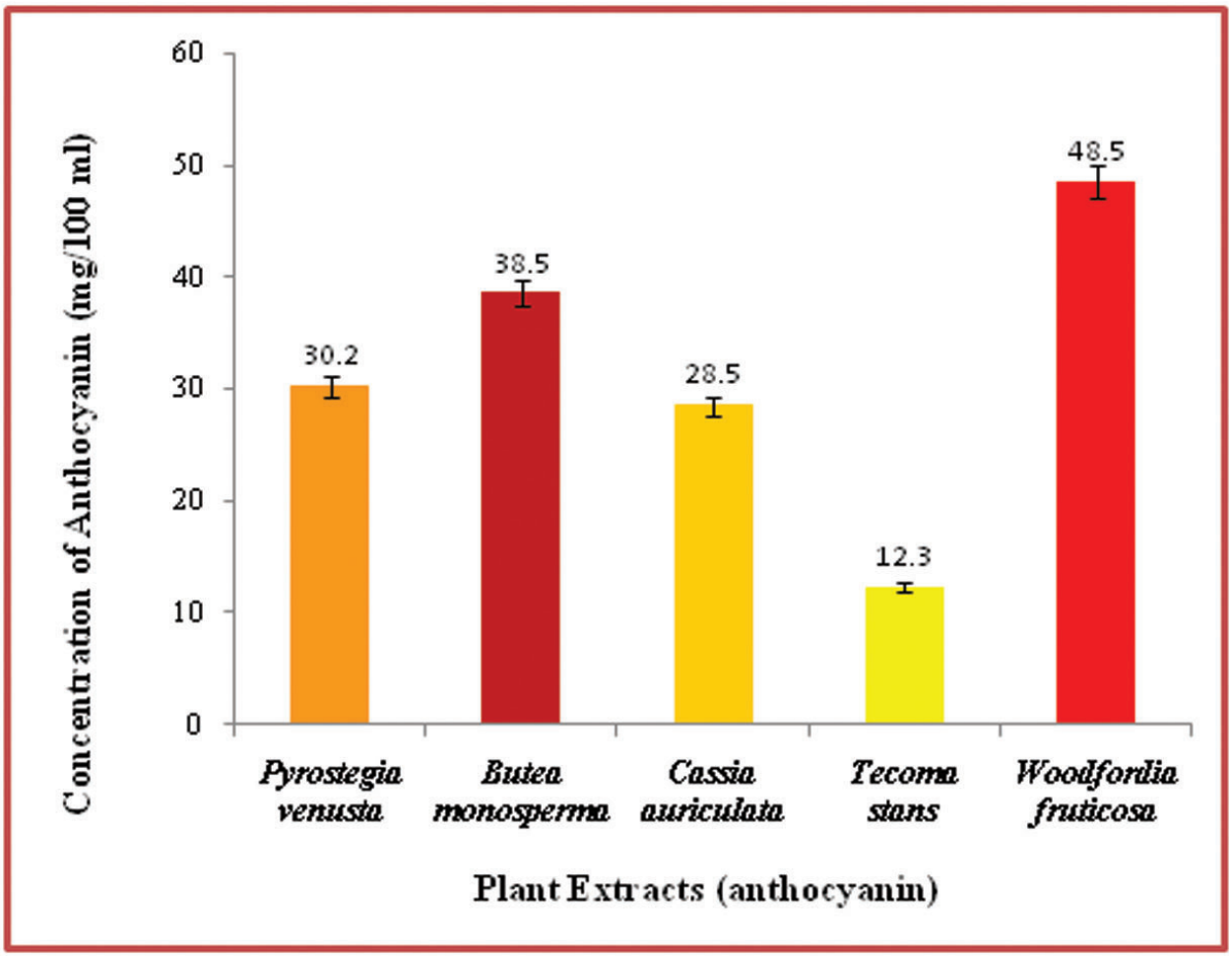

Fig. 1: Concentration of anthocyanins extracted from flowers

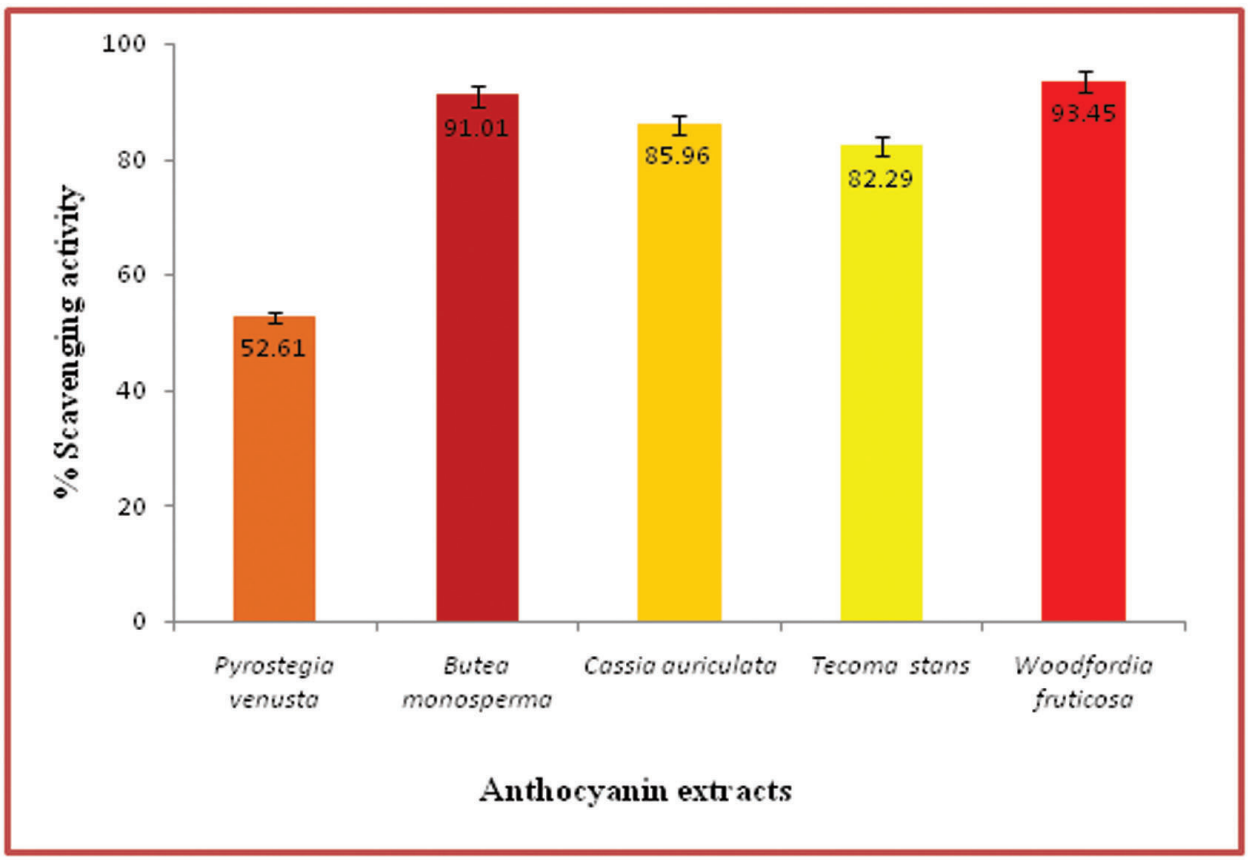

Fig. 2: Radical scavenging activity (%) of flower extracts (anthocyanin)

differential method as per the standard protocol and calculated the concentration employing the equation given. The obtained results were presented in Fig. 1. Highest concentration of anthocyanin 48.5 mg/100ml is observed in flower extract of *Woodfordia fruticosa* and lowest concentration of anthocyanin 12.3 mg/100ml is with flower extracts of *Tecoma tans*. Similarly, other extracts were found to be *Pyrostegia venusta* is 30.2 mg/100ml, *Butea monosperma* is 38.5mg/100ml and *Cassia auriculata* is 28.5mg/100ml.

Phytochemical Tests (qualitative analysis)

The phytochemical analysis of methanol extracts of flowers *Pyrostegia venusta, Butea monosperma, Cassia auriculata, Tecoma stans* and *Woodfordia fruticosa* were carried out employing standard protocols. The results are tabulated in Table 1. The presence of the Tannins, Saphonins, Flavonoids, Quinones, cardiac glycosids, phenols were observed in all extracts of flowers. The extracts of *Butea monosprma* and *cassia auriculata* has shown the presence of steroids. The presences of the phytochemicals are the indications of their bioactivities they have.

Antimicrobial Activity

Antibacterial activity was carried out using disc diffusion method. Three different concentrations of the compounds (10, 20, 30 µl) along with standard Streptomycin (1mg/ml, 10 µl sample) was used as a positive control. These plates were incubated for 24 hrs at 37 °C. Zone of inhibition was recorded in millimeters (mm). Observed results were tabulated in Table 2.

Inhibition of bacterial growth around the sample was observed in all the samples studied which shows the antimicrobial potential of the anthocyanin extracts. The best antimicrobial activity was observed at *Woodfordia fruticosaflower* extract which has shown highest inhibition against *Staphylococcus aureus*, zone of inhibition observed was around 2.6mm. The best antimicrobial activity of *Pyrostegia venustaflower* extract was against *Staphylococcus aureus* (2.2mm). The best activity of Buteamono sperma flower extract was against Bacillus subtilis (2.2mm). The best activity of *Cassia auriculataflower* extract was against *Staphylococcus aureus* (1.6mm). The best activity of Teccoma stans flower extract was against Bacillus subtilis (1.6mm). It was observed that zone of inhibition

Table 1: Phytochemical analysis of anthocyanin extract

Sl. no.	Name of the flower	*Pyrostegia venusta*	*Butea monosperma*	*Cassia auriculata*	*Tecoma stans*	*Woodfordia fruticosa*
1	Carbohydrate	-	-	-	-	-
2	Tannins	+	+	+	+	+
3	Saponins	+	+	+	+	+
4	Flavonoids	+	+	+	-	+
5	Alkaloids	-	-	-	-	-
6	Quinones	+	+	+	+	+
7	Cardiac glycosides	+	+	+	+	+
8	Trpinoids	-	-	-	-	-
9	Tritrpinoids	-	-	-	-	-
10	Phenols	+	+	+	+	+
11	Coumarin	+	+	+	+	+
12	Steroids	-	+	+	-	-
13	Phytosteroids	-	-	-	-	-
14	Protein	-	-	-	-	-
15	Sugar	-	-	-	+	-
16	Anthoquinones	-	-	+	-	-

Table 2: Antimicrobial activity (Zone of inhibition)

Flowers of different plants used	*Zone of inhibition (mm) of bacterial colonies			
	Concentration	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus subtilis*
Pyrostegia venusta	Control	2.0	2.0	2.0
	10µL	0.8	1.0	0.8
	20µL	1.0	1.8	1.4
	30µL	1.6	2.2	1.9
Butea monosperma	Control	2.0	2.1	2.0
	10µL	1.0	0.8	1.1
	20µL	1.4	1.5	1.6
	30µL	1.6	2.0	2.2
Cassia auriculata	Control	2.0	2.0	1.9
	10µL	0.6	0.7	0.8
	20µL	1.0	1.0	1.0
	30µL	1.4	1.6	1.5
Teccoma stans	Control	2.1	2.0	2.0
	10µL	0.5	0.5	0.6
	20µL	0.6	0.7	1.0
	30µL	0.8	1.0	1.6
Woodfordia fruticosa	Control	2.0	2.0	2.1
	10µL	1.0	1.2	1.2
	20µL	1.6	1.9	1.7
	30µL	2.0	2.6	2.4

.Data based on the average of triplicates *in vitro*

was increased with increased content. The antimicrobial activities were varied with respect to the extract and the organism studied. The activity may be due to the content and also depending on the phytoconstituents of each extracts.

Antioxidant Activity

DPPH Free Radical Scavenging Activity

The free radical scavenging activity of flowers of *Pyrostegia venusta, Butea monosperma, Cassia auriculata, Tecoma Stans and Woodfordia fruticosa* was carried out using 2,2-diphenyl-picrylhydarzyl whose free radical were to be scavenged by the sample extract using the ascorbic equivalence standard. All the samples tested were shown significantly good radical scavenging activity. However the % scavenging activity varies with the samples (Fig. 2).

The best radical scavenging activity was observed in *Woodfordia fruticosa extrct* compared to other flower extracts which exhibits 93.45% antioxidant activityfollowed by *Butea monosperma* (91.0%) and lowest was with *Pyrostegia venusta* extract (52.61%). The results indicate the variation of % antioxidant activity may be due to the variation in anthocyanin concentrations and also presence of the variousphy to chemicalsin different extracts.

Because of the growing awareness on importance of naturally derived products over synthetic ones, several attempts have been made on processing of natural colors from various natural sources. The current study is also demonstrates the possibility of isolation of natural pigments from easily available flowers from selected plants which are also known for some medicinal properties. Freely available flower sources such as *Pyrostegia venusta, Butea monosperma, Cassia auriculata, Tecoma stans and Woodfordia fruticosawere* rich in anthocyanin and could be used as good sources of producing different shades of colours. Further, the study also explores the usage of these extracts not only to color, but also its other health benefits such as potent antioxidant and antimicrobial properties. Natural antioxidants have demonstrated beneficial effects in maintenance of health, management of age related diseases, ameliorating due to the harmful effects of toxic agents both chemical and physical. There are several evidences available to understanding of effects of natural antioxidants.

Conclusion

Increasing demand for natural food colorant in comparison to synthetic one is mainly due to the health promoting properties besides its coloring nature. The market for natural food colours is estimated to increase by approximately 10% annually leading for the exploration of newer sources for these products from natural origin. In this view this anthocyanin pigments were extracted from naturally available sources which are rich in pigments and the studies revealed the significant levels of biological activities of these extracts.

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