Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(5): 1616-1620
Received: 13-07-2018
Accepted: 15-08-2018

Manjula GS
Ph. D scholar, College of Horticulture, Bengaluru, Karnataka, India

Krishna HC
Assistant professor, College of Horticulture, Bengaluru, Karnataka, India

Karan M
Assistant Horticulture Officer, Governament of Karnataka, India

Chirag Reddy M
Agronomist- coffee, Louis Dreyfus Company, Bengaluru Karnataka, India

Sadananda GK
Assistant professor, College of Horticulture, Bengaluru, Karnataka, India

Correspondence
Manjula GS
Ph. D scholar, College of Horticulture, Bengaluru, Karnataka, India

Biochemical changes in extracted anthocyanin pigment from roselle (*Hibiscus sabdariffa* L.) calyces for edible colour during storage

Manjula GS, Krishna HC, Karan M, Chirag Reddy M and Sadananda GK

Abstract
An investigation was carried out to know the biochemical changes in the extracted pigment from roselle (*Hibiscus sabdariffa* L.) calyces for edible colour during storage. For knowing the storage stability of extracted pigment, it was kept for 3 months in both ambient and refrigerated conditions. In storage under ambient and refrigerated condition, a decreasing trend were observed in the anthocyanin content, total phenols, water activity in the extracted pigment obtained by different methods of extraction upon storage under both conditions. At 90 days after storage at ambient and refrigerated condition, the anthocyanin content of 802.62 and 1091.92 mg 100 ml^{-1} and total phenols of 4.29 and 5.56 mg ml^{-1}, were found be highest in treatment ethanol acidified with 1.5 N HCl. The water activity was found be highest 0.91 and 0.92 in the treatment of hot water extraction during 90 DAS under both conditions respectively. An increasing trend was observed in the total soluble solids of extracted pigment obtained by different methods of extraction upon storage and it was highest 29.30 and 28.93 °B was found in treatment ethanol acidified with 1.5 N HCl under both ambient and refrigerated conditions. Overall from the present investigation, the treatment of ethanol acidified with 1.5 N HCl was found to be the best with highest anthocyanin retention and total phenols compare to all other treatments which can be used for large scale extraction of biocolour from roselle calyces.

Keywords: Anthocyanin, total phenols, correlation, edible colour, roselle calyces

Introduction
Roselle (*Hibiscus sabdariffa* L.) is a multi-use plant, belongs to the family Malvaceae, widely distributed in tropical regions, especially in the Middle Eastern countries and generally considered as a medicinal plant. The calyces, also known as natal sorrel, (Anon., 1999; Mohamed *et al*., 2012; Plotto, 2004) [2, 12, 14] are potentially a good source of antioxidant agents such as anthocyanins and ascorbic acid. Roselle calyx is a rich source of dietary fiber, vitamins, minerals and bioactive compounds such as organic acids, phytosterols and polyphenols.

Anthocyanins derived from two greek words *anthos* and *kyanose* which means flower and blue, respectively, are the largest group of water-soluble pigments in the plant kingdom and belong to the family of compounds known as flavonoids which are part of an even larger group of compounds known as polyphenols. These are found in the vacuoles of different plant cells in the form of glycosides. There are about 400 known anthocyanic glycosides (Mazza and Miniati, 1993) [10]. The most common aglycones of anthocyanins are pelargonidin, delphinidin, cyanidin, peonidin, petunidin and malvidin. Anthocyanins, the colouring pigments are considered secondary metabolite and as well as a food additive (INS163).

Anthocyanins are responsible for the red, purple and blue hues in fruits, vegetables, flowers and grains. They also play important roles such as attractants for insect pollinators and helps inseed dispersal and are widely distributed in thehuman diet. The estimated daily intake has been found tobe 12.5 mg in the United States, because this natural pigments are used quite safely in food; create more attractive colours for food products among the natural food colours. Optimizing health and performance through the diet is believed to be one of the largest and most lucrative markets in the US, and throughout the world (Giusti and Wrolstad, 2003) [6]. Therefore, they can beincorporated as a functional food ingredient into our diet.

Therefore, finding the most efficient extraction and separation method, as well as the full characterization of obtained bioactive compounds from natural matrices are a major challenge for researchers in the food, pharmaceutical, and cosmetic industry. The extraction efficiency of bioactive components from plant materials is affected by different factors, such as the extraction techniques, solvents, time, temperature, solvent-to-plant material ratio and many others. However, a suitable extracting method and solvent are crucial for ensuring an efficient extraction of the targeted nutraceuticals from plant material (Goli *et al*., 2005) [7]. Theoretically, the optimal extraction method should be simple, safe, reproducible, inexpensive and suitable for industrial application.

Material and Methods

Considering the abundance of anthocyanin content in the roselle calyces and their application in the food industry as edible biocolour, the experiment was carried on extraction of anthocyanin from roselle calyces through different methods using solvents, enzymes, fermentation and hot water has been carried out in the Department of Postharvest Technology. Solvents used are Ethanol acidified with hydrochloric acid, citric acid and acid. Changes in anthocyanin, total phenols, water activity and total soluble solids are recorded at 30 days interval regularly in both ambient and refrigerated condition.

Anthocyanin was measured through recoding optical density of the filtrate at 535nm using spectrophotometer (Make: SYSTRONICS, Model: UV/VIS Spectrophotometer 117). Directly anthocyanin extract is used for the determination of total soluble solids by using hand refractometer (Make: Erma Optical Works Ltd., Tokyo, Japan, 0-32°B range). The values were corrected at 20°C and expressed as °Brix (Anon., 1984) [1]. For water activity, 2 ml extractant is put into sample cup and the probe was connected to the indicator (Model: Hygrolab, Make: ROTRONIC instuments) and direct reading was recorded. Total phenols were estimated according to procedure given by Singleton and Rossi (1965) [9]. Phenols react with the oxidizing agent phosphomolybdate in Folin-Ciocalteau reagent and form a blue colored complex, molybdenum blue which is measured at 700nm.

Results and Discussion

Anthocyanin content

Significant difference among the treatments for the anthocyanin content present in extracted pigment which are stored in ambient condition represented in table 1, the highest anthocyanin was found in the treatment ethanol acidified with 1.5N HCl (802.62 mg 100 ml^{-1}) after 90 DAS which was decreased from 1638.17 mg 100ml^{-1} at time zero (T_0) and the lowest was found in the T_6- Hot water extraction (202.86 mg 100 ml^{-1}) at 90 DAS which was reduced from 372.21 mg 100 ml^{-1} at T_0, this may be probably due to non-enzymatic browning reactions and also the formation of 5-hydroxi methyl furfural and as a result, clarity and quality of colour were lost (Ruangsri *et al.*, 2008). The treatment T_6- hot water extraction showed the highest (54.50%) and lowest (27.20%) retention was recorded in the treatment fermentation of calyces. This may be because at higher pH stability of the anthocyanin reduced, hence degradation is faster.

Data presented in the table 2 revealed that after 90 DAS, the maximum and minimum anthocyanin content in treatment ethanol acidified with 1.5 N HCl (T_1) were 1091.92 mg 100 ml^{-1} and 802.62 mg 100 ml^{-1} obtained at refrigerated and ambient condition respectively. Storage temperature, increase in pH, prolonged storage time which had a significant effect on the stability of anthocyanin (Bordignon *et al.*, 2006, Rad and Yavarmanesh, 2006). Same results were obtained by Sharifi and Hassani, (2012) in the study of extraction methods and stability of colour extracted from barberry pigments.

Total phenols

The changes in the total phenols varied significantly among the treatments during storage stability study of extracted anthocyanin pigment from roselle in both ambient and refrigerated conditions represented in the table 3 and 4.

The total phenols decreased from initial T_0 to 90 DAS in both ambient and refrigerated condition. The highest loss (77.32%) of total phenols was found in the treatment T_5 (Fermentation of calyces) and lowest (48.17%) in the treatment T_4 distilled water with 0.2 per cent pectinase during ambient storage. Highest loss of total phenols may be due to more pH in the extracts and loss of anthocyanins during storage.

Similarly in refrigerated condition, highest (68.72%) loss of total phenols was found in the treatment fermentation of calyces (T_5) and lowest (41.55%) wasin the treatment T_4 distilled water with 0.2 per cent pectinase. The variation in the total phenols in the same treatment which were stored in the ambient and refrigerated storage condition was mainly due to temperature, as at higher temperature degradation of phenols increased. This decrease in the total phenols of extracted pigment is mainly due to degradation in anthocyanin content in the pigment that are major flavonoids which are part of an even larger group of polyphenol compounds (Mazza, 2007) [11].

Table 1: Effect of extraction methods on anthocyanin content (mg 100 ml^{-1}) during ambient storage condition

Treatments		T_0	30 DAS	60 DAS	90 DAS	Retention (%)
T_1	Ethanol acidified with 1.5N HCl (85:15)	1638.17	1334.76	1292.43	802.62	48.99
T_2	Ethanol with 2% citric acid	1241.27	920.22	884.94	365.15	29.42
T_3	Ethanol with 2% acetic acid	1311.24	967.26	926.10	441.58	33.68
T_4	Distilled water with 0.2% pectinase	979.60	821.44	741.43	391.02	40.04
T_5	Fermentation of calyce	1093.68	597.41	512.74	297.53	27.20
T_6	Hot water extraction	372.21	281.06	235.78	202.86	54.50
S. Em±		2.15	1.18	2.60	1.72	-
CD at 5%		6.40	3.51	7.72	5.12	-

DAS: Days after storage T_0: Time zero

Table 2: Effect of extraction methods on anthocyanin content (mg 100 ml^{-1}) during refrigerated (4°C) storage condition

Treatments		T_0	30 DAS	60 DAS	90 DAS	Retention (%)
T_1	Ethanol acidified with 1.5N HCl (85:15)	1638.17	1471.18	1380.63	1091.92	66.65
T_2	Ethanol with 2% citric acid	1241.27	1028.42	1009.10	575.65	46.37
T_3	Ethanol with 2% acetic acid	1311.24	1118.38	1063.69	652.09	49.73
T_4	Distilled water with 0.2% pectinase	979.60	957.85	895.52	556.84	56.84
T_5	Fermentation of calyce	1093.68	1004.31	828.49	552.13	50.48
T_6	Hot water extraction	372.21	326.21	275.77	243.43	65.40
S. Em±		2.15	1.45	1.63	2.29	-
CD at 5%		6.40	4.31	4.87	6.82	-

DAS: Days after storage T_0: Time zero

Table 3: Effect of extraction methods on total phenols (mg GAE ml^{-1}) during ambient storage conditions

Treatments		T_0	30DAS	60DAS	90DAS	Loss (%)
T_1	Ethanol acidified with 1.5N HCl (85:15)	12.84	8.91	5.32	4.29	66.58
T_2	Ethanol with 2% citric acid	6.99	4.21	3.45	3.37	51.78
T_3	Ethanol with 2% acetic acid	8.11	5.25	3.87	3.79	53.26
T_4	Distilled water with 0.2% pectinase	6.04	4.77	3.25	3.13	48.17
T_5	Fermentation of calyce	9.88	4.31	2.71	2.24	77.32
T_6	Hot water extraction	2.29	1.68	1.28	1.09	52.40
S.Em±		0.34	0.16	0.01	0.01	-
CD at 5%		1.02	0.47	0.04	0.04	-

DAS: Days after storage T_0: Time zero

Table 4: Effect of extraction methods on total phenols (mg GAE ml^{-1}) during refrigerated (4°C) storage condition

Treatments		T_0	30DAS	60DAS	90DAS	Loss (%)
T_1	Ethanol acidified with 1.5N HCl (85:15)	12.84	10.21	7.09	5.56	56.69
T_2	Ethanol with 2% citric acid	6.99	6.42	4.78	4.07	41.77
T_3	Ethanol with 2% acetic acid	8.11	6.79	5.35	4.41	45.62
T_4	Distilled water with 0.2% pectinase	6.04	5.34	4.03	3.53	41.55
T_5	Fermentation of calyce	9.88	4.67	3.42	3.09	68.72
T_6	Hot water extraction	2.29	1.83	1.45	1.27	44.54
S.Em±		0.34	0.17	0.02	0.01	-
CD at 5%		1.02	0.53	0.07	0.05	-

DAS: Days after storage T_0: Time zero

Total soluble solids (TSS)

The perusual data present in the table 5 and 6 showed the increase in TSS from time zero of storage towards 90 days after storage (DAS).

The highest (28.4°B) TSS was found in treatment ethanol acidified with 1.5N HCl (T_1) which increased to 29.3 °B and 28.93 °B at 90 DAS in ambient and refrigerated storage condition respectively. Under both storage condition least was noticed in treatment of hot water extraction (T_6). The TSS increased gradually throughout storage, this might be due to hydrolysis of polysaccharides into monosaccharides and oligosaccharides (Bhardwaj and Pandey, 2011). The increase in TSS is gradual in refrigerated condition, may be due slow conversion of polysaccharides. Similar trend of increased TSS with storage time was observed in mango-sea buckthorn blended juice stored for 90 days (Khan *et al.,* 2012) and pomegranate kokum mango blends stored for 150 days (Waskar and Gaikwad, 2004) [20] and roselle blend with mango juice (Kilima *et al.,* 2015) [9].

Table 5: Effect of extraction methods on total soluble solids (°B) during ambient storage conditions

Treatments		T_0	30 DAS	60 DAS	90 DAS
T_1	Ethanol acidified with 1.5N HCl (85:15)	28.40	28.50	28.73	29.30
T_2	Ethanol with 2% citric acid	26.75	26.75	27.38	27.60
T_3	Ethanol with 2% acetic acid	25.82	27.15	27.43	27.60
T_4	Distilled water with 0.2% pectinase	18.80	19.55	19.48	19.95
T_5	Fermentation of calyce	24.97	25.73	26.38	26.90
T_6	Hot water extraction	5.65	5.93	6.10	6.25
S.Em±		0.49	0.34	0.26	0.41
CD at 5%		1.46	1.01	0.79	1.22

DAS: Days after storage T_0: Time zero

Table 6: Effect of extraction methods on total soluble solids (°B) during refrigerated (4°C) storage condition

Treatments		T_0	30DAS	60DAS	90DAS
T_1	Ethanol acidified with 1.5N HCl (85:15)	28.40	28.45	28.70	28.93
T_2	Ethanol with 2% citric acid	26.75	26.73	27.13	27.25
T_3	Ethanol with 2% acetic acid	25.82	26.80	27.10	27.35
T_4	Distilled water with 0.2% pectinase	18.80	19.13	19.36	19.93
T_5	Fermentation of calyce	24.97	25.13	24.95	25.08
T_6	Hot water extraction	5.65	5.88	5.90	5.95
S.Em±		0.49	0.25	0.27	0.29
CD at 5%		1.46	0.74	0.80	0.88

DAS: Days after storage T_0: Time zero

Water activity (a_w)

The changes in the water activity during storage of extracted pigment varied significantly among the treatments throughout the storage period, which can be depicted from the Table 7 and 8 in ambient and refrigerated condition respectively.

The water activity was found to decrease from time zero of storage towards the end of storage period, in ambient condition. Extractant from the treatment hot water extraction showed highest water activity and lowest was found in the treatment ethanol with 2 per cent citric acid in ambient and refrigerated storage condition. The greater decrease in a_w at ambient storage compare to refrigerated storage (4°C) may be due to higher storage temperature resulted in rapid water loss and caused the increase in TSS and the rapid drop in titratable acidity (Chundawat *et al.,* 1978; Roongruangsri *et al.,* 2013) [5, 16].

Table 7: Effect of extraction methods on water activity (a_w) during ambient storage condition

Treatments		T_0	30DAS	60DAS	90DAS
T_1	Ethanol acidified with 1.5N HCl (85:15)	0.80	0.79	0.78	0.78
T_2	Ethanol with 2% citric acid	0.81	0.79	0.77	0.77
T_3	Ethanol with 2% acetic acid	0.82	0.80	0.78	0.77
T_4	Distilled water with 0.2% pectinase	0.92	0.92	0.91	0.90
T_5	Fermentation of calyce	0.92	0.92	0.89	0.88
T_6	Hot water extraction	0.94	0.93	0.92	0.91
S.Em±		0.004	0.002	0.002	0.004
CD at 5%		0.010	0.008	0.006	0.010

DAS: Days after storage T_0: Time zero

Table 8: Effect of extraction methods on water activity (a_w) during refrigerated (4°C) storage condition

Treatments		T_0	30DAS	60DAS	90DAS
T_1	Ethanol acidified with 1.5N HCl (85:15)	0.80	0.80	0.78	0.78
T_2	Ethanol with 2% citric acid	0.81	0.79	0.79	0.78
T_3	Ethanol with 2% acetic acid	0.82	0.79	0.80	0.79
T_4	Distilled water with 0.2% pectinase	0.92	0.92	0.92	0.91
T_5	Fermentation of calyces	0.92	0.91	0.89	0.90
T_6	Hot water extraction	0.94	0.94	0.93	0.92
S.Em±		0.004	0.001	0.001	0.001
CD at 5%		0.014	0.005	0.004	0.004

DAS: Days after storage T_0: Time zero

Correlation studies

Correlation studies on effect of extraction method on different parameter in relation to anthocyanin in roselle extract during storage are presented in the Table 9. Correlation is a measure of association between more than one character and it operates the relationship between dependent and independent characters.

In the present study it was observed that, positive relation was higher than negative correlation, the dependent variable was anthocyanin and it was related to many different independent parameters. Anthocyanin exhibited positive and significant association with total phenols (0.77), total soluble solids (0.66). While, anthocyanin showed negative and significant correlation with water activity (-0.51). This is in confirmation with the findings of Olaya *et al.* (2009).

Table 9: Correlation studies on effect of extraction methods on water activity, TSS, Total in relation to anthocyanin content in roselle extract during storage

Parameters		1	2	3	4
1	Water activity	1			
2	Total soluble solids (TSS)	-0.74*	1		
3	Total phenols	-0.34*	0.46*	1	
4	Anthocyanin	-0.51*	0.66*	0.77*	1

* Correlation analysis is significant at 5% level

Conclusion

Extracted pigment showed very good storage stability in the refrigerated condition compare to ambient condition. By storing the pigment in the refrigerated condition, we can reduce quality loss. From the present investigation, the treatment of ethanol acidified with 1.5 N HCl was found to be the best with highest anthocyanin retention, total phenols, with less water activity changes compare to all other treatments which can used for large scale extraction of biocolour from roselle calyces.

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