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ANTIOXIDANT ACTIVITIES AND QUANTIFICATION OF ANTHOCYANINS IN SOME COLOURED GRAPE HYBRIDS

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ABSTRACT

Anthocyanins, the flavonoid group of polyphenols, is in great demand as an important food constituent, mainly due to its health promoting attributes. The present study was undertaken with 11 newly developed coloured grape hybrids at ICAR-IARI, New Delhi (India). Efforts were made to identify and quantify different anthocyanin fractions. HPLC chromatogram revealed ten different peaks belonging to anthocyanins, which varied significantly from 37.68 to 247.68 µg g^{-1} in terms of C_3G equivalent. The maximum peak height was at peak-5, which represents malvidin-3-glucoside. Maximum anthocyanin content was in grape hybrid 'Hy.16/2A-R_1 P_{18}'. The anti-oxidant activities of hybrids were measured by FRAP assay. The hybrid 'Hy.16/2A-R_1P_{19}' was found the most potent antioxidant rich genotype in terms of FRAP. The potential genotypes identified in terms of high bioactive contents can be helpful in protection against various chronic diseases.

Key words: Antioxidants, anthocyanins, FRAP, grape, HPLC

INTRODUCTION

Grape is an important vine crop bearing nutraceutical and antioxidant-rich berries which have the ability to fight against many deadly chronic diseases (Georgiev *et al*., 2014). This is regarded as a versatile crop with ability to grow in all climatic conditions i.e. tropical, subtropical and temperate. Diverse array of bioactive compounds constitutes the nutraceutical profile of grape berries. In grapes, four classes of flavonoids are commonly detected: flavonols, anthocyanins, flavan-3-ols and their polymeric forms, and condensed tannins (Clarke and Bakker, 2004). The appearance of coloured grapes are associated with the presence of anthocyanin pigments. Anthocyanins are red or purple coloured secondary metabolites present in leaves, stem, flowers and fruits. Generally, anthocyanins are soluble in water and located in grape skins and seeds which appear as red, purple or blue according to their pH (Khoo *et al*., 2017). The word "anthocyanin" has been derived from Greek words '*anthos'* meaning flower and '*kianos'* meaning blue, which forms the important constituent of vascular plant pigments (Pazmino-Duran *et al*., 2001). Anthocyanins possess a wide range of biological roles such as protection against solar exposure and ultraviolet radiation, free radical scavenging and anti-oxidant activity, defense against many different pathogens as well as new proposed modulation of signaling cascades (Schaefer *et al*., 2004; Takahama, 2004).

Anthocyanins in grapes comprise of cyanidin, petunidin, delphinidin, peonidin and malvidin-3 monoglucosides (Wrolstad, 2000), with last one as the most abundant form (Liang *et al*., 2008). Reports reveal that higher consumption of fruits reduces the incidence of chronic diseases such as

arthritis, cardiovascular diseases and ageing (He and Giusti, 2010) and shows anticancer, anti-diabetic and antiobesity effects (Tsuda, 2012). Anthocyanins may also serve as a potential source of natural food colorants to replace harmful synthetic colorants in food sector. The quality of wine, juice and table crops depends on its skin colour i.e. the anthocyanins content. The composition of anthocyanins in grape berries is primarily governed by genetic factor and the relative content of one anthocyanin is stable in skin of a particular cultivar (Guidoni *et al*., 2008; Di Vittori *et al*., 2018). The present study was aimed to evaluate 11 newly developed coloured grape hybrid germplasm for relative anthocyanin content and also to assess the antioxidant activities exhibited by these berries.

MATERIALS AND METHODS

The present study was conducted in the year 2016-2017 at the Division of Fruits & Horticultural Technology and Division of Agricultural Chemicals, ICAR-IARI, New Delhi, India. The vineyard of IARI is situated at an altitude of 228 m amsl with 28° 40'N latitude and 77° 13'E longitudinal geographical coordinates. It has sub-tropical climate. The soils are alluvial, clay loam in texture and low in organic matter with slightly alkaline pH.

Grape hybrids

Eleven grape hybrids, developed at IARI, New Delhi, with parents [(male x female) in brackets] used in the present study were: Hy. 16/2A-R_1P_2 (Ruby Red x Madeleine Angevine), Hy.16/2A-R_1P_7 (Ruby Red x Madeleine Angevine), Hy.16/2A-R_1P_{18} (Beauty Seedless x Banqui Abyad), Hy.16/2A-R_1P_{19} (Beauty Seedless x Banqui Abyad), Hy.16/2A-R_4P_{13} (Beauty Seedless x Banqui Abyad), Hy.16/2A-R_3P_{12} (Beauty Seedless x Black Muscat), Hy.ER-R_1P_{19} (Beauty Seedless x Pearl of Csaba), Hy.ER-R_2P_{36} (Beauty Seedless x Pearl of Csaba), Hy.ER-R_2P_{19} (Beauty Seedless x Pearl of Csaba), Hy.16/2A-R_1P_{14} (Beauty Seedless x Cardinal) and Hy.16/2A-R_1P_8 (A-5 x Hur)

Harvesting and sampling of grape berries

Fully matured grape bunches were randomly harvested from the vineyard. Five uniform bunches were selected from each replicated genotypes. Bunches were sorted out, packed in polythene bag and subsequently transported to laboratory for recording observation on various parameters.

Sample preparations

Randomly selected 100 berries from each genotype were chosen for anthocyanin evaluation. Four replicates for each genotype were used for analytical purpose. One to two berries were homogenized and from this homogenate, 2.0 mL berry was weighed, crushed with 80% ethanol and 10 mL sample volume was made. The mixtures were then centrifuged at 10000 rpm for 10 min at 4°C. For analytical work, the supernatant was collected and used for total anthocyanins estimation.

Total monomeric anthocyanins

The quantity of the total monomeric anthocyanin was measured by UV-VIS spectrophotometer (UV 5704SS, ECIL, India) by pH-differential method (Wrolstad *et al.,* 2005). In this method, two buffers of i) potassium chloride buffer, pH 1 (0.025 M) and ii) sodium acetate buffer, pH 4.5 (0.4 M) were used. The samples were diluted in both the buffers of pH 1.0 and pH 4.5, and absorbance measured at 510 and 700 nm. The total monomeric anthocyanin content was expressed as cynadin-3-glucoside kg^{-1} fresh weight and was calculated by following formulae:

$$\text{Monomeric anthocyanin content (mg kg}^{-1}) = A \times MW \times DF \times 1000/ (\varepsilon \times 1)$$

Where: A (Absorbance) = ($A_{510\ nm}$- $A_{700\ nm}$) pH 1.0 - ($A_{510\ nm}$- $A_{700\ nm}$) pH 4.5; MW (molecular weight) = 493.5 g mol^{-1} of malvidin-3-o-glucoside; DF = dilution factor; ε 28,000 molar extinction coefficient in L mol^{-1} cm^{-1} for malvidin-3-o-glucoside; l = path length in cm; 1000 = conversion from g to mg.

High performance liquid chromatography (HPLC) for anthocyanin quantification

Extraction of anthocyanins: Anthocyanin rich fruit skin and pulp were carefully removed (5 g) and taken in amber colour flask and extracted with 500 mL acidified methanol (0.1% HCl). The content was sonicated in the dark for 15 min on an ultrasonicator (Misonix, NY, USA). The combined extract was concentrated under vaccum (35±1°C) in a rotary evaporator (Heidolph, Germany) for complete removal of methanol.

Anthocyanins detection: The purity of anthocyanin powder concentrate was checked by HPLC (Alliance, Waters Corp., Milford, Mass., USA) equipped with e2695 quaternary pump, autoinjector (20 µL loop), a 2998 photodiode array detector and an "Empower 2" software programme. A C-18 column (Thermo, USA) 25 cm × 4.6 mm × 5 µL was used for anthocyanin separation using a mobile phase comprising of a gradient mixer of solvent A: water (0.1% TFA) and solvent B: water: ACN: TFA (53:46:1 v/v) at a flow rate of 0.6 ml min^{-1}. The gradient mobile phase was: A:B (%), 80:20 for 1 min, A:B (%), 40:60 for 26 min, A:B (%), 80:20 for 30 min. A:B (%) 80:20 for 40 min. Chromatogram was acquired at 520 nm and peak assignments were made based on MS fragmentation patterns and published literature (UV-Vis spectra and elution order).

Characterization of anthocyanins: The purity of anthocyanin powder concentrate was checked by HPLC using a mobile phase comprising of a gradient mixer of solvent A: water (0.1% TFA) and solvent B: water: ACN: TFA (53:46:1 v/v) at a flow rate of 0.6 mL min^{-1}. The gradient mobile phase was A: 80% for 0 min, 40% in next 26 min, 80% for 14 min, total run time was 40 min. Chromatogram was acquired at 520 nm after injection of 20 µL. Standard cyanidin-3-glucoside were also run as per above mentioned flow rate. UV spectra was recorded for each peak found in HPLC analysis. Concentration of individual anthocyanins was calculated based on C_3G equivalent.

Antioxidant activities determination by FRAP assay

The ferric reducing antioxidant power assay was initially developed to measure ferric reducing ability of blood plasma by Benzie and Strain (1996). The FRAP assay takes advantage of electron transfer reactions, wherein a ferric salt, Fe (TPTZ) $_2$ III is used as an oxidant under acidic conditions, pH 3.6. The assay given by Benzie and Strain (1996) for measuring FRAP was performed with some modification. The FRAP value were obtained by comparing the absorbance at 593 nm in sample and reference containing the ferrous ion. The aqueous solutions of known ferrous ion concentration in the range 100-1000 µL ($FeSO_4.7H_2O$) were used for calibration purpose. The reagent was prepared freshly by mixing 300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ in 10:1:1 (v:v:v) ratio. FRAP reagent (3 mL) was mixed with 100 µL sample extract in a test tube and vortexed. Absorbance readings were recorded after 4 min of sample reagent mixing at a wavelength of 593 nm. Results were expressed in terms of Trolox equivalents.

Statistical analysis

The data were analyzed through statistical tool univariate analysis of variances. The means were compared using Duncan's multiple range test (Duncan, 1955). The statistical analysis software SPSS 12 was used. In some cases, the values of different parameters were expressed as mean ± standard error. A difference was considered statistically significant when the *p*-value was less than 0.05 ($p<0.05$).

RESULTS AND DISCUSSION

Total monomeric anthocyanins (TMA)

In present study grape genotypes were evaluated under subtropical climatic conditions of New Delhi (India) which receives very limited growing days (70-80 days from date of full bloom) which adversely affects the berry development. Therefore, it was essential to assess the total anthocyanin

content in grape genotypes grown under shorter growing period. The analytical TMA content of 11 coloured genotypes were examined. The TMA content in coloured grape genotypes ranged from 179.13 ('Hy. ER-R_2P_{36}') to 640.72 mg kg^{-1} fw ('16/2A R_1P_{18}') [Fig. 1]. At 5% level of significance TMA content was significantly higher in '16/2AR_1P_{18}' (640.72), '16/2AR_4P_{13}' (553.63) and 'ER-R_1P_{19}' (438.79 mg kg^{1}) as compared to the other genotypes studied. Kallithraka *et al*. (2005) reported TMA content of 85.7 (Liatiko) to 1914.0 mg kg^{-1} fw (Vapsa) in 19 grape cultivars evaluated. However, the average value was 731.7 mg kg^{-1} grape when grown under long growing days (120-130 days) in Mediterranean climate of Greece, which has hot dry summers and mild wet winters favouring the colour development. Under subtropical climatic conditions the accumulation of total monomeric anthocyanins appears lower than under Mediter ranean climate, presumably being affected by growing days as well as mean summer temperature i.e. 35-45°C under subtropics of Delhi and 20-35°C in Athens. The anthocyanin content varies greatly in coloured grapes genotypes as these are affected by many factors like genotype, berry maturity, climatic, seasonal variations, crop size, etc. (Guidoni *et al*., 2008; Di Vittori *et al*., 2018).

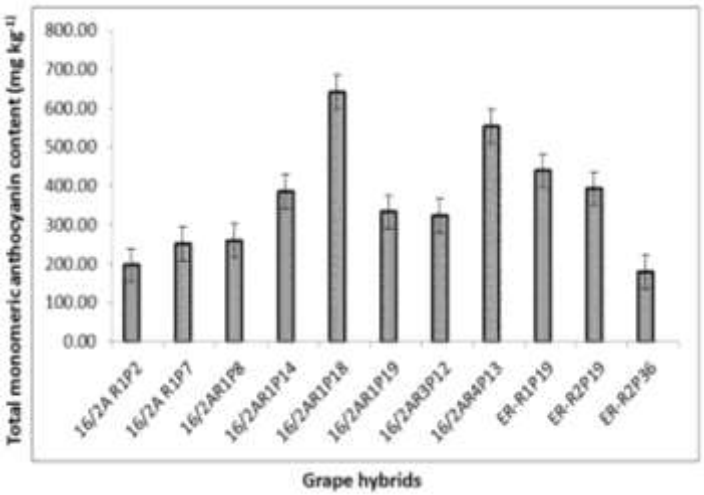

Fig. 1: Toatal monomaric anthocyanin content of eleven grape hybrids

High performance liquid chromatography for anthocyanins

HPLC chromatogram revealed ten peaks separated base to base, except one or two (Table 1 and 2; Fig. 1). All the peaks belonged to anthocyanin group that can be seen from UV-Vis spectrum of each compound (Fig. 1). Characteristic peak at around 517 nm showed typical absorbance of anthocyanin. Table 2 represents cyanidin-3-glucoside (C_3G) equivalent content of individual anthocyanins. As standards for all the anthocyanins were not available, therefore this procedure was followed to quantify the peaks. Ten peaks were detected in all cultivars corresponding to different kinds of anthocyanin fractions. C_3G was taken as standard to quantify anthocyanins which were expressed in terms of C_3G equivalents.

Table 1: Anthocyanins content of grape hybrids measured at various peaks through HPLC

Hybrid	Anthocyanin (µg g^{-1} C_3G eq.) at various peaks											
	Peak1	Peak2	Peak3	Peak4	Peak5	Peak6	Peak7	Peak8	Peak9	Peak10	Min.	Max.
Hy.16/2A-R_1P_2	5.19	11.42	7.22	32.82	41.35	7.05	19.79	1.28	5.41	-	1.28	41.35
Hy.16/2A-R_1P_7	18.25	3.78	8.63	6.57	29.14	0.25	18.41	21.39	-	-	0.25	29.14
Hy.16/2A-R_1P_8	15.14	34.73	15.15	1.29	78.91	49.26	1.52	3.14	4.61	43.93	1.52	78.91
Hy.16/2A-R_1P_{14}	23.70	8.47	7.67	7.06	18.71	11.78	0.50	0.39	19.21	-	0.39	23.70
Hy.16/2A-R_1P_{18}	1.50	0.94	0.23	1.69	1.20	10.80	2.74	9.85	17.04	-	0.23	17.04
Hy.16/2A-R_1P_{19}	4.40	26.53	4.04	1.85	78.74	17.65	1.31	1.61	4.20	5.80	1.31	78.74
Hy.16/2A-R_3P_{12}	2.46	1.68	4.84	4.08	40.56	5.39	7.79	-	-	-	1.68	40.56
Hy.16/2A-R_4P_{13}	0.37	9.70	1.55	17.44	24.29	0.13	0.18	0.41	6.01	-	0.13	24.29
Hy.ER-R_1P_{19}	6.52	2.02	10.45	17.12	72.03	4.14	5.00	1.17	16.03	-	1.17	72.03
Hy.ER-R_2P_{19}	1.97	1.50	2.98	8.16	23.41	2.36	15.31	1.27	7.23	-	1.27	23.41
Hy.ER-R_2P_{36}	2.94	0.73	3.60	1.09	13.19	26.25	0.86	1.28	-	-	0.73	26.25

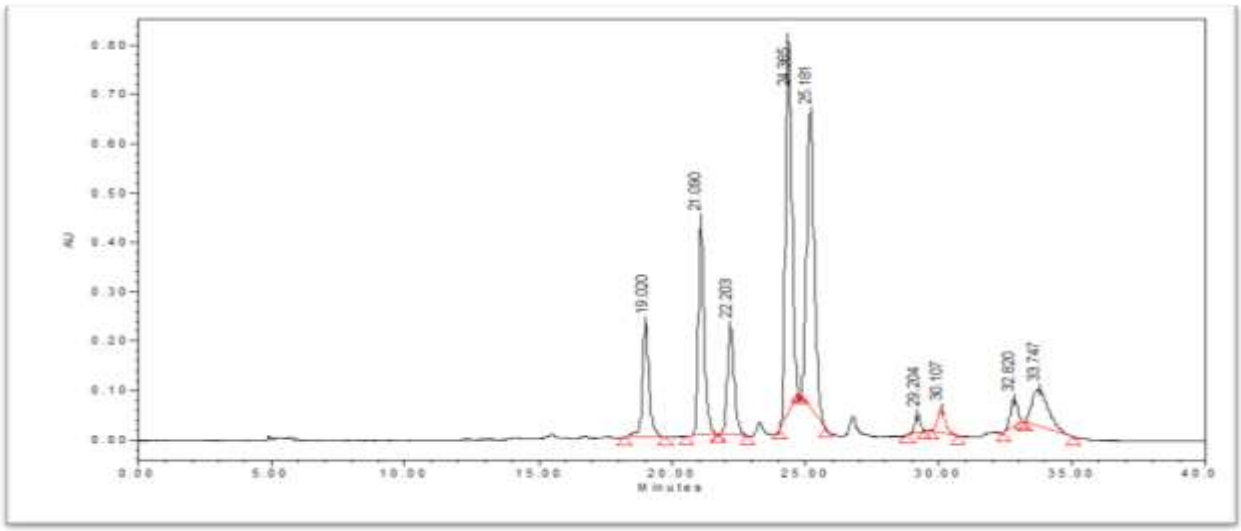

Fig. 1: HPLC chromatogram of anthocyanins in grape hybrids identified at 517 nm

In HPLC analysis, individual content of different anthocyanin fractions corresponding to different peaks were estimated. The highest anthocyanin content was identified at peak 5 in almost maximum genotypes studied. The anthocyanin content at peak 5 ranged from 23.41 µg g^{-1} C_3G eq. in 'Hy. ER-R_1P_{19}' to 41.35 µg g^{-1} C_3G eq. in 'Hy. 16/2A-R_1P_2', which were their corresponding maximum values among all other peaks identified.

Malvidin-3-O-glucoside was the main Mv-derivatives found in most of the *V. vinifera* group of grapes. Some cultivars also had abundant cyanidin, delphinidin and petunidin-derivatives; although the average of these three derivatives was low in *V. vinifera* grapes. The content of Cy-derivatives has been reported high in 'Shiyaira', '96-2' and 'Red Alexandria' having 89.6, 52.8 and 57.3%, respectively (Liang *et al*., 2008). Moreover, abundant Dp-derivatives (61.2%) and Pt-derivatives (20.4%) were found in 'Fenniu'. Recently, pelargonidin-3-O-glucoside and its derivatives have been found in grapes (He and Giousti., 2010). Each anthocyanin fraction gave a particular type of hue. Among anthocyanins, delphinidin derivatives gave blueness while cyanidin derivatives were reddish. Malvidin and petunidin derivatives are associated with blue/dark blue while peonidin were purple. Further, the maximum area percentage was observed at peak 5 in most of the genotypes studied. At peak 4 the corresponding maximum area percentage were 31.44, 27.39, 31.86, 53.88, 60.72, 40.42, 53.56 and 37.86% in hybrids 16/2A R1P2, 16/2A R1P7, 16/2AR1P8, 16/2AR1P19, 16/2AR3P12, 16/2AR4P13, ER-R1P19 and ER-R2P19, respectively. In terms of peak area, peak 5 and 6 were major in nature and the variations across the cultivar have been presented in Table 2.

Table 2: Percentage area of anthocyanin contents in grape hybrids measure by HPLC at various peaks

Hybrids	Peak1	Peak2	Peak3	Peak4	Peak5	Peak6	Peak7	Peak8	Peak9	Peak10
Hy.16/2A-R_1P_2	3.95	8.68	5.49	24.95	31.44	5.36	15.05	0.97	4.11	-
Hy.16/2A-R_1P_7	17.15	3.55	8.11	6.17	27.39	0.24	17.30	20.10	-	-
Hy.16/2A-R_1P_8	6.11	14.02	6.12	0.52	31.86	19.89	0.61	1.27	1.86	17.74
Hy.16/2A-R_1P_{14}	24.31	8.69	7.87	7.25	19.19	12.08	0.51	0.4	19.70	-
Hy.16/2A-R_1P_{18}	3.25	2.03	0.50	3.68	2.62	23.50	5.95	21.42	37.05	-
Hy.16/2A-R_1P_{19}	3.01	18.15	2.77	1.26	53.88	12.08	0.90	1.10	2.88	3.97
Hy.16/2A-R_3P_{12}	3.68	2.51	7.25	6.10	60.72	8.07	11.66	-	-	-
Hy.16/2A-R_4P_{13}	0.62	16.15	2.58	29.03	40.42	0.22	0.30	0.68	9.99	-
Hy.ER-R_1P_{19}	4.85	1.50	7.77	12.73	53.56	3.08	3.72	0.87	11.92	-
Hy.ER-R_2P_{19}	0.58	15.12	2.42	27.19	37.86	0.21	0.28	0.64	9.36	-
Hy.ER-R_2P_{36}	5.88	1.46	7.20	2.18	26.41	52.58	1.73	2.56	-	-

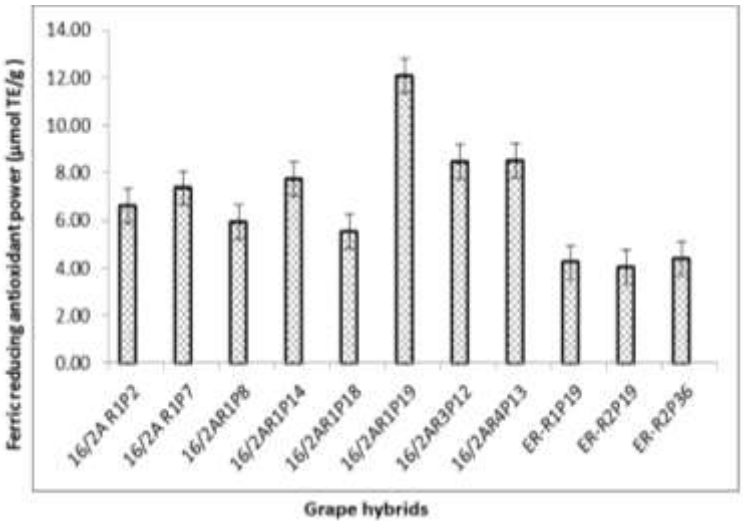

Fig 3: Antioxidant activities of eleven grape hybrids in terms of FRAP

Ferric reducing antioxidant power (FRAP)

Fruits are natural source of powerful antioxidants; hence attract the attention of consumers. Among the processed products, grape juice is in top 50 foods considered rich in antioxidant activities (Halvorsen *et al*., 2006). The ferric reducing antioxidant power (FRAP) varies in grape hybrids. The difference in antioxidant activities are mainly governed by the genotype, irrespective of other factors (Garrido *et al*., 2011). FRAP assay is based on an increase of absorbance at 593 nm due to the formation of tripyridyl (mmol 100 g^{-1} wet weight). In present study significant differences were observed in FRAP activity in genotypes and the values in decreasing order were as: 'Hy.16/2A R_1P_{19}' (12.07)> 'Hy.16/2A R_4P_{13}' (8.53)> 'Hy.16/2A R_3P_{12}' (8.48)> 'Hy.16/2A R_1P_{14}' (7.73)> 'Hy.16/2A R_1P_7' (7.36 µmol TE g^{-1}). These findings are comparable with Jiang and Zhang (2001), Rockenbach *et al*. (2011), Pomar (2005) and Meng *et al*. (2012).

Conclusion: Breeding crops for nutraceutical traits has been drawing the attention of researchers due to increasing health awareness in public. The potentially identified grape hybrids rich in antioxidant properties and anthocyanin content can directly be exploited to fight against some of the important chronic diseases. The highest anthocyanins were found in hybrid 16/2A-R_1P_{18}. However, anthocyanins profile of grape largely varied with the genetic constitution as well as the environmental conditions under which the vineyard grows. Therefore, growers should adopt optimum management practices to accumulate higher anthocyanins in berries.

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