

भारतीय गुलाब की किस्मों से पौष्टिक-औषधीय पदार्थों के संभावित स्रोत के रूप में एन्थोसयानिन्स वर्णको (पिगमेंट्स) का पृथक्करण और उनका निरूपण

**Isolation and Characterization of Anthocyanin Pigments  
from Indian Rose Varieties as a Potential Source of  
Nutraceuticals**

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# **Isolation and Characterization of Anthocyanin Pigments from Indian Rose Varieties as a Potential Source of Nutraceuticals**

**By**

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### CERTIFICATE

This is to certify that the thesis entitled **“Isolation and Characterization of Anthocyanin Pigments from Indian Rose Varieties as a potential source of nutraceuticals”** submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the degree of **Doctor of Philosophy in Horticulture-Floriculture and Landscape Architecture** by **Ms. Poonam Kumari, Roll No. 10296** embodies the results of the bonafide research work carried out by her under my guidance and supervision. No part of the study reported here has so far been submitted anywhere for the publication or for any other degree or diploma.

It is further certified that the assistance and help received during the course of the investigation has been duly acknowledged by her.

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## 1. INTRODUCTION

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Globally, today there is an upsurge of interest in phytochemicals as new sources of nutraceuticals. A nutraceutical is any substance that is a food or part of a food, that provides medical or health benefits, including the prevention and treatment of disease. Nutraceuticals may be isolated nutrients, dietary supplements, specific diets, designer foods, herbal products, processed foods or processed beverages (Morris, 2003). The source of nutraceutical compounds in human diet is exclusively provided by fruits and vegetables (Cavaiuolo *et al.* 2013). However, flowers are becoming important sources of several bioactive compounds that can be added in the diet as food. Although flowers were already used as food in ancient Greece and Rome (Melillo, 1994), they have only recently sparked off nutraceutical research (Mlcek and Rop, 2011), focusing on new agronomic and economic horizons (Kelley and Biernbaum, 2000). Their rich pigmentation, which evolved to attract pollinators (Grotewold, 2006), suggests a high antioxidant activity that is of interest for human nutrition. Nowadays, several metabolomics studies revealed the chemical compositions of ornamental flowers, showing the presence of important bioactive molecules (Cavaiuolo *et al.* 2013).

*Rosa hybrida* L., of the family Rosaceae, is one of the most important commercial flower crop with over 150 species and more than 24,000 cultivars that are widely distributed in Europe, Asia, Middle East, and North America (Ogata *et al.* 2005) with colour spectre ranging from subtle whites, yellows and pinks to intense purple, orange and red tones. The colour of various plant tissues, such as flower petals (Mikanagi *et al.* 1995) and leaves (Schmitzer *et al.* 2009), can be attributed to anthocyanins and other phenolics, for example quercetins, acting as co-pigments (Eugster and Fischer, 1991).

Rose petals have been consumed for many years in teas, cakes and flavour extracts, as well as folk medicine to treat blood circulation disorders and control cancer growth (Shafei *et al.* 2010; Rezaie-Tavirani *et al.* 2013). Moreover, rose has been used as a food additive and traditional medicinal therapy of colds and other infections. In the food industry, organic cultured roses are known as edible flowers and phenolic compounds extracted from this plant have been used to make tea and

functional beverages that exert beneficial effects on human health (Vinokur *et al.* 2006). Recent studies by Lee *et al.* (2011) indicated that anthocyanins from red petals of Korean edible rose exhibited potent antioxidant, anticancer and antiallergic properties that can be used as nutraceuticals. Hence food safety concerns are minimal in this crop. Many investigations have also revealed that roses contain a wide diversity of phenolic compounds such as gallic acid, catechin, epicatechin, kaempferol, rutin, myricetin and quercetin that not only possess antioxidant activities but also exert antiallergic, antiinflammatory, antiatopic, antibacterial, antiviral, antifungal, antidepressant and antistress effects (Ulusoy *et al.* 2009; Talib and Mahasneh, 2010; Boskabady *et al.* 2011).

Pigments are chemical compounds that absorb light in the wavelength range of the visible region. Pigments responsible for the appearance of colours in higher plants are classified into several groups such as chlorophylls, carotenoids, flavonoids and betalains (Mlodzinska, 2009). These natural pigments are of great interest in the food industry, due to their attractive colours and beneficial health effects, including antiatherogenic, anticancer, antidiabetic, antiinflammatory and antioxidant activities (Castañeda-Ovando *et al.* 2009; Prior and Wu, 2006; Wang *et al.* 1997). Humans ingest a considerable amount of anthocyanins from natural sources in daily life. The average intake of anthocyanins by USA citizens has been estimated at up to 180–215 mg/day, which is higher than that of other phenolic compounds (Clifford, 2000). For this reason, food and medical industries are increasingly interested in natural sources with high anthocyanin contents for manufacture of supplements with therapeutic and nutritional uses.

Anthocyanins, a major constituent of natural food colourants, are a group of water-soluble pigments that are composed of an aglycone anthocyanidin and sugar moieties (Huang *et al.* 2009). They belong to flavonoid family and are basically responsible for the brilliant colours of numerous fruits, flowers and vegetables. Their specific colour depends on co-pigments, metal ions and pH (Tanaka *et al.* 2008). To date, there have been more than 635 anthocyanins identified in nature, featuring six common aglycones [cyanidin (Cy), peonidin (Pn), pelargonidin (Pg), malvidin (Mv), delphinidin (Dp), and petunidin (Pt)] and various types of glycosylations and acylations. Although there are close to 25 different aglycones that have been identified, differing in the patterns of hydroxylation and methylation on the different

positions of the rings, only six of them are commonly found in nature and approximately 95% of all anthocyanins are derived from these six anthocyanidins (Kong *et al.* 2003, He and Giusti, 2010 and Bueno *et al.* 2012). In the structure of anthocyanins, 17 anthocyanidins have been distinguished, the most common of which are cyanidin, delphinidin, pelargonidin, peonidin, malvidin and petunidin (Mlodzinska, 2009). Previous studies have revealed that anthocyanin compounds have a high free radical scavenging capacity and play an essential role in the prevention of cardiovascular disease, obesity, cancer, diabetes and other diseases (Prior and Wu, 2006). The high radical scavenging ability of anthocyanins is mainly due to the phenolic hydroxyl groups in molecule. The phenolic hydroxyl groups can prevent peroxidation effectively by providing hydrogen atoms which can remove free radicals and consequently cut off the chain reaction of oxidation (Qin and Xiaojun, 2013). Anthocyanins were also found to have many times more activity than common antioxidants such as ascorbate (Wang *et al.* 1997). According Siger *et al.* (2012) the interest in natural antioxidants has increased considerably in recent years due to their beneficial effects in preventing and reducing the risk of various diseases. Huge potential as natural colourants and their significant health benefitting activity led to increase in research interest on anthocyanins (Jimenez-Aguilar *et al.* 2011).

The production of secondary metabolites in plant cell cultures may be of interest for obtaining compounds that are difficult to synthesize or highly unstable, which is usually associated with high economic value of the substances, but may also be useful to help elucidating the metabolic pathways involved in the synthesis of such compounds. Regarding *in vitro* production of secondary metabolites, a description of the effect of changes in growing conditions, addition of precursors, use of growth regulators, and of the utilization of elicitors and stressors on the production of these compounds is done (Gomez-Zeledon and Jimenez, 2011). Research on induction of anthocyanin pigments by plant cell and tissue cultures is increasing to find an effective system, such as food biotechnology (Koda *et al.* 1992) or some disease treatments including anticancer and cardio protective bioassays (Jackman *et al.* 1987; Wang and Jiao, 2000; Gantet and Memelink, 2002; Hou, 2003; Lila, 2004). Pigment recovery from the fresh materials involves such limitations as variability and seasonal availability of raw materials, fresh material losses and pigment degradation caused by

storage and the extraction process. This method has added advantage of producing substances that are difficult to synthesize by alternative chemical methods.

Various strategies are used to enhance the pigment production, which may contribute to scale up and commercialization of plant cell cultures for the production of pigment based food colorants. The protocol, thus developed would be helpful to upgrade the technology towards the pilot scale to produce the pigments under *in vitro* conditions throughout the year in a highly controlled manner. Therefore, there is a need to develop a system in order to freely express such nutraceutical pigments at will in controlled conditions and to harvest them without seasonal boundaries.

In recent years, research on anthocyanins attracted more and more attention, especially their isolation and purification in some of the fruit and vegetable crops. In contrast, there have been few studies on flower crops which are well known for the presence of a variety of pigments with myriad colours. Similarly work on the antioxidant property of anthocyanins from Indian rose varieties is limited. However, to the best of our knowledge, previous studies have not extensively investigated the exact anthocyanin component and antioxidant properties of Indian rose varieties.

Rose breeding at IARI was initiated by Dr. B.P. Pal during 1950s. His efforts resulted in the development of 105 rose varieties of varied colours. Subsequently more than 80 varieties were developed by the Division of Floriculture and Landscaping at ICAR- IARI. These varieties possess varied colours contributed by anthocyanins and carotenoids. The potential of the red pigment from rose petals, however, is not completely excavated. It is essential to decipher the pigment profile of Indian rose varieties in order to explore the possibilities of utilizing the pigments as potential nutraceuticals.

Keeping this in view, the present investigation was undertaken with an aim to develop anthocyanin profile and establish their antioxidant property in some of the Indian rose varieties with the following objectives:

1. Isolation and quantification of total anthocyanin pigments from Indian rose varieties.
2. Identification and characterization of anthocyanin pigments from selected rose varieties.
3. *In vitro* induction of nutraceutical pigments in promising rose variety.

## 2. REVIEW OF LITERATURE

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Anthocyanins are the largest and most diverse group of plant pigments derived from the phenylpropanoid pathway, ranging in colour from red to violet and blue (Tunen and Mol, 1991). They are water-soluble phenolic compounds and part of a large and widespread group of plant flavonoids. Anthocyanin pigments are important to food quality because of their contribution to colour and appearance. There is increasing interest in the anthocyanin content of foods and nutraceuticals because of possible health benefits. Anthocyanin pigment content can also be a useful criterion in quality control and purchase specifications of fruit juices, nutraceuticals and natural colourants. Anthocyanin pigments are responsible for various colours of rose. The attractive colours and quality of a product play an important role in attracting consumer (Azeredo, 2009; Downham and Collins, 2000). Nowadays, pigments produced synthetically are very toxic to human health and are not eco-friendly. Therefore, researchers from all over world are involved in extraction and utilization of natural pigments from plants.

Scanty information is available on the *in vitro* production of anthocyanins in rose especially on Indian varieties. There are various reports on natural pigments extracted from horticultural crops such as fruits, vegetables etc. However few reports on pigments from flower crops are available (Heuer *et al.* 1994; Vaillant *et al.* 2005; Feugang *et al.* 2006). Therefore, in this chapter, an attempt has been made to present a background of research available on the anthocyanin pigment isolation, their characterization, antioxidant activities and anthocyanin pigment production through *in vitro* technology. The literature is compiled and presented under the following heads.

- 2.1 Variability of anthocyanin pigments
- 2.2 Variation of antioxidant activity and total phenolic content
- 2.3 Characterization of anthocyanin pigments using HPLC (High Performance Liquid Chromatography)
- 2.4 Characterization of phenolic compounds using HPLC (High Performance Liquid Chromatography)
- 2.5 *In vitro* induction of anthocyanin pigments

## 2.1 Variability of anthocyanin pigments

Biolley and Jay (1993) investigated anthocyanins content of fifteen rose (*Rosa hybrida* L.) varieties using spectrophotometer. Depending on the variety, the observed colour variations were based on a more or less complex mixture of cyanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, quercetin and kaempferol glycosides. The total anthocyanin content ranged from 4 to 109 mg/g petal dry weight, while the total amount of flavonol glycosides was never less than 8 mg/g and could reach 136 mg/g petal dry weight.

Wrolstad *et al.* (2005) described the spectroscopic methods for easy measurement of total anthocyanin pigments and indices for polymeric colour and browning. They modified the existing methods and developed a number of methods for monitoring colour and pigment changes that have been effective for several research projects and also be suitable for many quality control applications in the industry.

Kallithrakaa *et al.* (2005) determined the major anthocyanin pigments in Hellenic native grape varieties (*Vitis vinifera*). Six prevalent anthocyanins were determined in 13 Hellenic native grapes. Anthocyanin content varied from 85.7 to 1914.0 mg/kg fresh berry weight.

The red and blue shades of 15 flowers (*Canna indica*, *Clitoria ternatea*, *Ixora chinensis*, *Mirabilis jalapa*, *Jatropha integerrima*, *Impatiens balsamina*, *Lagerstroemia indica*, *Nerium oleander*, *Portulaca graniflora*, *Delonix regia*, *Rosa indica*, *Ruellia tuberosa*, *Thunbergia erecta*, *Hibiscus mutabilis* and *Quisqualis indica*) were extracted with 1% acidic methanol and these extracts were then screened for total anthocyanin content (TAC) using the pH method. Cyanidin-3-glucoside was the predominant anthocyanin in most of the chosen flowers (Vankar and Srivastava, 2010).

Gantait *et al.* (2010) evaluated the spray chrysanthemum cultivars under polyhouse and open field conditions to determine the anthocyanin content. They observed that the cultivars Tata Red (19.60 mg/100g), Red Gold (18.56 mg/100g), Aarti (16.83 mg/100g), Apsara Violet (16.34 mg/100g) and Jaya (14.64 mg/100g) have high anthocyanin content.

Gary *et al.* (2012) evaluated the rhubarb (*Rheum* sp.) varieties for anthocyanin content and observed that anthocyanin content ranged from  $19.8 \pm 1.5$  (Crimson Red) to  $341.1 \pm 41.6$  mg/100g DW (Valentine). The percentage of two main anthocyanins in rhubarb, cyanidin 3-glucoside and cyanidin 3-rutinoside varied from 66.07:33.93 in Valentine and 9.36:90.64 in *Rheum officinale*, respectively.

Zhao *et al.* (2013) analysed individual anthocyanin pigment content of three red pomegranate cultivars (Lvbaoshi, Hongbaoshi, Moshiliu). Large amounts of Cy3G and Dp3G were found in dark red cv. Moshiliu, exhibiting a similar pattern over time. The main concentration of Cy3G was 53.52 mg/100g, which was 35-fold and 12-fold higher compared to Lvbaoshi and Hongbaoshi cultivar.

Tan *et al.* (2014) investigated the main pigments in the petals of six orchid cultivars spectrophotometrically, and the values ranged from 0 mg/g (in *Dendrobium* Shavin White) to 2.128 mg/g (in *Mokara Aranda*). Highest anthocyanin content was found in *Mokara Aranda*.

The anthocyanin content of 23 different cultivars of chrysanthemum flower was analysed by Park *et al.* (2015). The cultivar 'Magic' showed greatest accumulation of total and individual anthocyanins including cyanidin 3-glucoside (C3g) and cyanidin 3-(3"-malonoyl) glucoside (C3mg). Magic ( $18.00 \pm 0.84$  mg/g), Angel ( $13.86 \pm 0.17$  mg/g) and Relance ( $13.00 \pm 0.33$  mg/g) had high amounts of anthocyanins and showed a wide range of red and purple colours in their petals.

Benvenuti *et al.* (2016) investigated the antioxidant activity and anthocyanin content of 12 ornamental species. The antioxidant power in the edible flowers ranged from 3.6 m mol FeSO<sub>4</sub> per 100g fresh weight for *Calendula officinalis* to 70.4 m mol FeSO<sub>4</sub>100 g<sup>-1</sup> fresh weight for *Tagetes erecta*. In the red varieties of *Viola* × *wittrockiana*, *Dianthus* × *barbatus*, *Pelargonium peltatum* the high anthocyanin content (12.4, 13.3, 12.5 mg cyn-3-glu eq. per 100g fresh weight, respectively) was associated to a higher antioxidant activity.

### **2.1.1 Effect of seasonal variation on anthocyanin content**

Temperature is one of the main external factors affecting anthocyanin accumulation in plant tissues: low temperatures increase and elevated temperatures decrease anthocyanin concentration (Zhong, and Yoshida, 1993). One of the causes for the lower anthocyanin concentration in plants at high temperatures is due to

decreased rate of synthesis of anthocyanin pigments (Shvarts *et al.* 1997). The expression of anthocyanin genes is strongly affected by temperature, with low temperatures causing a several-fold increase in the transcript levels of genes whose products are involved in pigmentation changes. These include key enzymes in the general phenylpropanoid pathway, such as phenylalanine ammonia lyase (PAL), and enzymes that catalyze reactions committed to flavonoid and anthocyanin biosynthesis, such as chalcone synthase (CHS), chalcone isomerase (CHI) and dihydroflavonol reductase (DFR) (Christie *et al.* 1994; Leyva *et al.* 1995 and Shvarts, *et al.* 1997).

Anthocyanin concentration in foliage is tightly dependent on environmental conditions such as light quality, light intensity and growth temperature. Foliar anthocyanin accumulation is induced at high light intensities, in particular high UV light, and low growth temperatures (Chalker-Scott, 1999). This process is often reversible, with greening of red foliage and stems when growth conditions change to low light intensity and warmer temperatures. High temperature often reduces the quality of anthocyanin colour in flowers, which is a problem in the commercial production of ornamental plants. Anthocyanins are the predominant pigments in flowers of the rose.

The dark red pigmentation of both the young and mature leaves of the garden plant *Cotinus coggygria* 'Royal Purple', occurs only with both low temperatures ( $\leq 17^{\circ}\text{C}/9^{\circ}\text{C}$  day/night respectively) and high UV irradiation. Anthocyanins of red *Cotinus* sp., grown at low temperatures, are degraded and their foliage turns green when covered with a UV screen. Similarly, anthocyanin degradation occurs in red-pigmented plants when the temperature is elevated but UV radiation remains high (Oren-Shamir and Levi-Nissim, 1997).

Dela *et al.* (2003) described the impact of transient high temperature on the concentration and composition of anthocyanins in 'Jaguar' rose flowers. They found that a 1-day heat treatment of  $39/18^{\circ}\text{C}$  day/night has no effect on pigmentation but a 3-day treatment has a substantial effect. The effect on anthocyanin concentration was most pronounced when the high-temperature conditions were applied at stages 3 and 4 of bud development and the effect was long term. The transcript levels of chalcone synthase (CHS) and dihydroflavonol reductase (DFR), two key enzymes in the anthocyanin synthesis pathway, decreased by 50% after heat treatment, suggesting that the decreased anthocyanin concentration is due in part to reduced transcription.

Anthocyanin pigmentation of fruits also fluctuates in response to environmental conditions such as growth temperature and light intensity. An increase in growth temperatures causes degradation of anthocyanins in apple and pear (Steyn *et al.* 2004).

The dependence of anthocyanin concentration on environmental conditions was also demonstrated in *Arabidopsis thaliana* plants transformed to over-express the transcription factor PAP1 (Production of Anthocyanin Pigments 1) (Rowan *et al.* 2009). The mutated arabidopsis plants were dark red under room temperature and high light conditions (22°C, 440  $\mu\text{mol}/\text{m}^2/\text{s}$ ), but when transferred to conditions of high temperature and low light conditions (30°C, 150  $\mu\text{mol}/\text{m}^2/\text{s}$ ) the plants turned green. The change in colour was due to the simultaneous down-regulation of synthesis and degradation of the anthocyanins.

Lai *et al.* (2011) investigated the effect of elevated temperature on anthocyanin accumulation in the lily tepals of a pink-flowered cultivar Marrero. The elevated temperature at 35°C caused poor colouration in the tepals at stage (St) 2 and 3. At 20°C, anthocyanin accumulation began at St 2 and anthocyanin content increased at St 3.

## **2.2 Variation of antioxidant activity and total phenolic content**

Tsai *et al.* (2002) investigated the relation between antioxidant activity and anthocyanin content in Roselle (*Hibiscus sabdariffa* L.) petals and showed that the antioxidant capacity of Roselle extract increased when extraction time or weight of petals increased. The FRAP assay showed a linear relationship with anthocyanin content. Comparisons between FRAP and ORAC or FRAP and TAS assays gave a linear relation.

Olsson *et al.* (2005) analysed fruits of 10 different *Rosa* taxa for their total phenolics content and carotenoids. The results show a wide range in the content of the total carotenoids depending on species/cultivars, whereas there was less variation in the content of total phenolics.

Vinokur *et al.* (2006) investigated antioxidant activity, total phenols, and total anthocyanins contents of hot water infusions (teas) of air-dried petals of 12 cultivars of rose. Rose petal teas from different cultivars exhibited scavenging capacity toward 2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonate cation radical (ABTS<sup>+</sup>) ranging

between 712.7 and 1770.7  $\mu\text{M}$  trolox equivalents (TE) per gram of dry petals, as compared with 1227.6  $\mu\text{M}$  TE/g dry weight in the green tea. The range of total phenols content in rose teas was 50.7 to 119.5 mg gallic acid equivalents (GAE) per gram of dry matter.

Wojdyło *et al.* (2007) investigated total equivalent antioxidant capacities (TEAC) and phenolic contents of 32 spices extracts. The TEAC values of the spices ranged from 1.76 to 346  $\mu\text{M}$  trolox/100g dw, from 7.34 to 2021  $\mu\text{M}$  trolox/100g dw, and 13.8 to 2133  $\mu\text{M}$  trolox/100g dw for ABTS<sup>+</sup>, DPPH<sup>•</sup> and FRAP, respectively. The total phenolic content ranged from 0.07 to 15.2 mg of gallic acid equivalents (GAE)/100g dw. The herbs with the highest TEAC values were *Syzygium aromaticum*, *Epilobium hirsutum* and the species belonging to the Labiatae and Compositae family.

Garzon and Wrolstad (2009) investigated the antioxidant activity of major anthocyanins in nasturtium flowers (*Trapaeolum majus*). The total phenolic content was 406 mg GAE/100g FW. The radical scavenging activities against ABTS and DPPH radicals were 458 and 91.87  $\mu\text{M}$  trolox eq/g fresh weight, respectively.

Cerezo *et al.* (2010) studied the bioactive composition of strawberry (Cultivar Camarosa) and revealed its anthocyanin composition and the antioxidant activity of isolated pigments. Pelargonidin-3-glucoside was the major compound followed by pelargonidin-3-rutinoside and 11 pelargonidin and cyanidin derivatives. They isolated two different fractions with pelargonidin-3-glucoside and pelargonidin-3-rutinoside at 90% and 92% purity, respectively, using CCC, ORAC and FRAP assays and showed that pelargonidin-3-glucoside is more active than 3-rutinoside.

Burin *et al.* (2010) evaluated the colour, phenolic content and antioxidant activity of grape juice. There was a strong positive correlation ( $r = 0.9566$ ) between the antioxidant activity and total phenolic content for the commercial juice. The antioxidant capacity evaluated using the DPPH method ranged from 2.51 to 11.05 mM for all juices.

Ozgen *et al.* (2010) analysed fourteen purple-black American elderberry accessions (*Sambucus canadensis* L.) for total monomeric anthocyanin contents and for their antioxidant capacity by the ferric reducing antioxidant power (FRAP) and DPPH radical scavenging assays. Variability among accessions was greatest for total

anthocyanin content (CV 37.5%); individuals ranged nearly threefold from 1308 to 4004  $\mu\text{g cy3-GE/g}$  on a fresh weight basis. Total anthocyanin content is highly correlated to antioxidant capacity values ( $r = 0.70-0.85$ ).

Jaime *et al.* (2010) determined antioxidant capacity, total anthocyanins, and total phenols of wild and cultivated berries. Percentages of DPPH discoloration of different berries studied were between 67.8% and 95.3% for red sarsaparilla and rosehip, respectively. Maqui berries showed a significantly higher anthocyanin content (2240.2 and 1445.3 mg/l cyanidin 3-glucoside) than other berries, and a mean for all berries of 335.5 mg/l. Higher phenol content levels were obtained in two cultivars of saskatoon (773.9 and 1001.9 mg/l gallic acid) and wild rosehip (1457.0 and 1140.4 mg/l gallic acid).

Lee *et al.* (2011) investigated the antioxidant activity of Korean roses and found that cyanidin 3,5-di-O-glucoside exhibited good scavenging activity against DPPH radical with IC<sub>50</sub> value of 55.2  $\mu\text{g/mL}$ .

Gonzalez *et al.* (2012) evaluated the antioxidant activity of liquid extracts of dried Roselle. The antioxidant capacity ranged from  $3.11 \pm 0.50$  to  $8.0 \pm 0.2$  mmoles of trolox/100g of calyces.

Sayed *et al.* (2012) investigated the antioxidant activity of taif rose flowers. The antioxidant activity was chemically determined using three methods; 1,1-diphenyl picrylhydrazyl (DPPH.) free radical scavenging activity, phosphomolybdenum method and reducing power activity. The methanol extract showed high antiradical activity with SC<sub>50</sub>=49.44  $\mu\text{g/ml}$ .

Baydar and Baydar (2013) analysed total phenolic content and antioxidant activity in fresh and spent flowers and green leaves of oil-bearing-rose (*Rosa damascena* Mill.). The highest values of total phenolics were obtained from the hot and cold methanolic extractions of leaf with 478.34 and 530.40 mg GAE/g. The strongest antioxidant properties measured with the FRAP assay had leaf cold extractions as 1.43 and 1.72 g/ml at the concentrations of 100 and 150 g/ml, respectively.

Ginova *et al.* (2013) studied the antioxidant capacity of *Rosa damascena* petals and leaves in Bulgaria, representing different climatic conditions, during two consecutive growing seasons (2009 and 2010). Highest total polyphenolic contents of

rose petals were observed in the year 2009 for all plantations, the total anthocyanin concentrations were significantly smaller (except for Mirkovo sample). Positive correlation was found between antioxidant activity and phenolic content of rose petals and leaves.

Roman *et al.* (2013) determined the bioactive compounds and antioxidant activity of *Rosa canina* L. biotypes from spontaneous flora of Transylvania. The total polyphenolic content varied from 575 mg/100g frozen pulp to 326 mg/100g frozen pulp. The antioxidant activity investigated using DPPH method revealed a good correlation with total polyphenols.

Antioxidant activities of the six endemic species of Chilean Edible Berry were analysed by Brito *et al.* (2014). Calafate fruits showed the highest antioxidant activity ( $2.33 \pm 0.21$   $\mu\text{g/mL}$  in the DPPH assay), followed by blueberry ( $3.32 \pm 0.18$   $\mu\text{g/mL}$ ), and arrayán ( $5.88 \pm 0.21$ ), respectively.

Liu *et al.* (2014) studied the antioxidant activity of *Trifolium repens* L. The results showed that *Trifolium repens* had strong scavenging activities for DPPH, ABTS cation radical and superoxide anion radical. The radical scavenging IC<sub>50</sub> values of ethanol extract of *Trifolium repens* was  $276 \pm 14$   $\mu\text{g/ml}$  and  $54 \pm 2$   $\mu\text{g/ml}$  for DPPH and ABTS, respectively.

Zeng *et al.* (2014) evaluated the extracts from 19 Chinese edible flowers for antioxidant extracts. The results showed the extracts of *Paeonia suffruticosa* Andr., *Paeonia lactiflora* Pall., and *Rosa rugosa* Thunb. possessed stronger DPPH FRSA ( $94.221 \pm 0.102$ ;  $93.739 \pm 0.424$  and  $94.244 \pm 0.163\%$ , respectively and polyphenolic contents ( $96.208 \pm 0.689$ ;  $87.938 \pm 1.187$  and  $92.164 \pm 0.799$  mg CE/g, respectively) that were superior or comparable to black and green teas.

Lin *et al.* (2014) determined antioxidant components and antioxidant activities of methanolic extracts of four different rose cultivars (Macarena, Onnuri, Oklahoma, Colorado). Results showed that cv. Oklahoma had the highest polyphenol ( $129.8 \pm 7.3$  mg/1g RMEs), flavonoids ( $23.7 \pm 0.2$  mg/1g RMEs) and anthocyanins ( $18.699 \pm 0.354$  mg/1g RMEs) contents, Oklahoma also exhibited the highest DPPH, ABTS radical scavenging activity, reducing power, and inhibitory activity of lipid peroxidation except metal chelating effect.

Lim *et al.* (2014) screened different varieties of *Portulaca grandiflora* for antioxidant activity and total phenolic content. The total phenolic content ranged from 46.39-82.19 mg GAE/100 g, 36.72-56.45 mg GAE/100 g, and 41.46-85.70 mg GAE/100 g for methanolic, acetone and ethanolic extract, respectively. The antioxidant activity of plant extracts as determined by DPPH scavenging assay were ranging from 0.69-2.14 mg/mL, 1.40-4.38 mg gallic acid/g and 7.32-29.21 mg ascorbic acid/g when expressed as IC<sub>50</sub>, GEAC and AEAC, respectively.

The total phenolic content and antioxidant activity of aqueous and methanol extracts of *Rosa damascena* flower petals was determined by Tatke *et al.* (2015). The IC<sub>50</sub> values of aqueous and methanol extract were 8.30 µg/ml and 4.85 µg/ml respectively by DPPH method and 70.90 µg/ml and 47.18 µg/ml respectively by Griess assay method.

Fartosy *et al.* (2015) studied the antioxidant activity of anthocyanins extracted from *Iresine herbstii* L. flowers after drying and freezing. The results showed that fresh samples (AEFF) had the highest amount of total anthocyanin content ( $8.31 \pm 0.23$  mg/g dry matter, expressed as cyaniding 3-glucoside equivalents). The anthocyanins extracts showed remarkable scavenging activity on superoxide anion radicals, hydroxyl radicals, hydrogen peroxide, nitric oxide radicals and deoxyribose degradation.

Petrova *et al.* (2016) investigated the total phenolic content, total flavonoid content and antioxidant activity of five edible flowers (*Tagetes erecta* L., *Calendula officinalis* L., *Geranium macrorrhizum* L., *Bougainvillea spectabilis* Willd. and *Helianthus tuberosus* L.). 95% ethanolic extracts of geranium were evaluated as the richest source of total phenols 19.79 mg GAE/g fresh weight, while 80% methanol extracts of *Helianthus tuberosus* L. showed the highest values of total flavonoids content 8.89 mg QE/g fresh weight. The highest antioxidant capacities using two methods, DPPH and FRAP assays were obtained for 95% ethanol geranium (*Geranium macrorrhizum* L.) extracts 242.9 and 106 mM TE/g fresh weight, respectively.

Oprica *et al.* (2016) analysed hips of different rose species for the composition of polyphenol, flavonoids, anthocyanins contents and antioxidant activity. The smallest content of total polyphenols was recorded in *Rosa nitidula*, both in seeds

(3.11 mg GAE/g dry weight) and pulp (23.99 mg GAE/g dry weight). The maximum concentration of total polyphenols was found in *R. pendulina*, both in seeds (17.68 mg GAE/g dry weight) and pulp (71.48 mg GAE/g dry weight). The results revealed that antioxidant activity of pulp extract from *R. pendulina* showed the largest scavenging activity while the lowest scavenging capacity was recorded for *R. nitidula*.

Bitis *et al.* (2017) determined antioxidant activities and total phenolic content of various extracts from *Rosa sempervirens* leaves. The ethyl acetate extract (E) had significant antioxidant activity with half maximal inhibitory concentration (IC50) values of 3.96 in the DPPH and 2.92 mg/ml in the ABTS assay. The total phenolic (203.8 mg gallic acid equivalents/g extract) and total flavonoid (95.81 mg catechin equivalents/g extract). Quercetin 3-xyloside, quercitrin and hyperoside were isolated from the E extract and quercetin, from the chloroform extract and quercetin and hyperoside were identified for the first time in this species. Quercitrin was found to be a major compound in the ethyl acetate extract of *Rosa sempervirens*.

### **2.3 Characterization of anthocyanin pigments using HPLC (High Performance Liquid Chromatography)**

Biolley *et al.* (1994) characterized anthocyanin pigments of more than 100 cyanic cultivars of *Rosa hybrida* by means of spectrophotometric and HPLC techniques. Total anthocyanin of 60 mg/g dry weight consisting of various mixtures of cyanidin 3,5-diglucoside and pelargonidin 3,5-diglucoside were observed, while only pure cyanidin 3,5-diglucoside was found to accumulate in amounts above 60 mg/g dry weight. A small amount of 3-monoglucosides was detected and paeonidin 3,5-diglucoside was rarely present.

The flowers of 20 taxa of petunia were analysed by HPLC for the occurrence of anthocyanins (Ando *et al.* 1999). The investigation revealed the presence of 24 anthocyanins in petunia flowers, of which 18 known anthocyanins isolated from the flowers of *Petunia exserta*, *P. guarapuavensis*, *P. integrifolia*, *P. occidentalis*, and *P. reitzii* were identified as 3-glucoside of delphinidin; 3-rutinosides of cyanidin, delphinidin, and petunidin; 3-rutinoside-5-glucosides, 3-trans and -cis-p-coumaroylrutinoside-5-glucosides, and 3-trans-caffeoylrutinoside-5-glucosides of delphinidin, petunidin, and malvidin; and 3-transcaffeoylglucosyl-trans-(cafeoyl or p-coumaroyl) rutinoside-5-glucosides of malvidin.

Mikanagi *et al.* (2000) studied 44 taxa of three sections (*Cinnamomeae* (*Rosa* 26, *Chinenses* 8 and *Gallicanae* 10) and eight modern garden roses in the genus *Rosa* for their floral anthocyanins. Eleven anthocyanins: 3-glucosides and 3,5-diglucosides of cyanidin (Cy), pelargonidin (Pg) and peonidin (Pn), 3-rutinosides and 3-o-coumaroylglucoside-5-glucosides of Cy and Pn, and Cy 3-sophoroside, were isolated from flowers of these taxa and identified by chemical and spectroscopic techniques. Four anthocyanins: Cy 3-rutinoside, Pn 3-rutinoside, Pn 3-o-coumaroylglucoside-5-glucoside and Cy 3-sophoroside were found for the first time in *Rosa* flowers.

Fukui *et al.* (2006) isolated rosacyanins from *Rosa hybrida* cv. 'Mme Violet'. Rosacyanins A1 (1) and A2 (2) had a common chromophore, which contained cyanidin with a galloyl group link between positions 4 and 5 of the hydroxy group of the flavylum nucleus and tellimagrandin 1 and 2, respectively, at C-3 of cyanidin nucleus. Researchers found that mauve roses containing a small amount of blue pigments co-existing with a red anthocyanin, cyanidin 3,5-diglucoside, and determined that the coexistence of a small quantity of the blue pigments (rosacyanins) is the reason that the roses look bluish.

Kelebek *et al.* (2007) investigated the anthocyanin composition of rose wine made from cv. Okuzgozu, a variety of grape. High-performance liquid chromatography with diode-array detection (HPLC-DAD) and mass spectrometry (MS) were used for anthocyanin analysis. Thirteen different anthocyanins, including five glucosides, five acetyl glucosides and three coumaroyl glucosides were identified and quantified.

Truong *et al.* (2010) identified 17 anthocyanins in Stokes Purple and NC 415 varieties of sweet potato with five major compounds: cyanidin 3-caffeoylsophoroside-5-glucoside, peonidin 3-caffeoylsophoroside-5-glucoside, cyanidin 3-caffeoyl-p-hydroxybenzoylsophoroside-5-glucoside, peonidin 3-caffeoyl-p-hydroxybenzoylsophoroside-5-glucoside and peonidin-caffeoyl-feruloylsophoroside-5-glucoside using HPLC-DAD/ESI-MS/MS.

Lee *et al.* (2011) characterized anthocyanins from rose as cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-O-glucoside using reversed-phase C18 column chromatography, NMR spectroscopy and HPLC-DAD-ESI/MS analysis. Cyanidin

3,5-di-O-glucoside was the predominant constituent (375 mg/100 g), representing about 85% of total content.

Liu *et al.* (2012) identified anthocyanin components from fruit skin of *Rhodomyrtus tomentosa* using High Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (HPLC-ESI-MS) analysis. The results revealed the presence of five anthocyanin components, which were identified as delphinidin-3-glucoside, cyanidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside.

Qin and Xiaojun (2013) detected 3 kinds of anthocyanins in rose and found that Cy-3,5-diglucoside was the predominant constituent which accounted for approximately 94.9% of total anthocyanins according to the analysis results of high performance liquid chromatography-photodiode array detection (HPLC-PAD).

Chun *et al.* (2013) identified three anthocyanins (cyanidin 3-diglucoside, cyanidin 3-sambubioside and cyanidin 3-glucoside) in the flower and bulb of *Lycoris radiata* using high-performance liquid chromatography coupled with UV detection and electrospray ionization mass spectrometry.

Assous *et al.* (2014) characterized anthocyanin pigments derived from purple carrots using HPLC. Purple carrots contain 168.7 mg anthocyanin/100 g on fresh weight basis, where the major constituents were cyanidin-3-xylosyl-galactoside acylated with ferulic acid (33.65%) followed by cyanidin-3-xylosyl-galactoside acylated with coumaric acid (29.85%) and cyanidin-3-xylosyl-galactoside (28.70%).

Brito *et al.* (2014) identified 31 anthocyanins in Chilean edible berry extracts. The major ones were quantified by HPLC-DAD, mostly branched 3-O-glycosides of delphinidin, cyanidin, petunidin, peonidin and malvidin. Three phenolic acids (feruloylquinic acid, chlorogenic acid and neochlorogenic acid) and five flavonols (hyperoside, isoquercitrin, quercetin, rutin, myricetin and isorhamnetin) were also identified.

Skowrya *et al.* (2014) identified fourteen constituents by HPLC coupled with diode-array detection (DAD) and mass spectrometry (ESI-MS) in three varieties of *Viola × wittrockiana* (yellow, red and violet), ten of them were flavonoids and four

anthocyanins. The major compounds found in violet pansy were rutin, violanthin and violanin.

Ludmila *et al.* (2015) investigated the anthocyanin composition of ‘Tornado’ Floribunda rose cultivar using HPLC diode array (DAD) and MS (6130 Quadrupole LC/MS) detectors. The main components of the ‘Tornado’ flower anthocyanin were found to be 3,5-diglucosides of cyanidin (80.9 %) and peonidin (13.9 %). While in flower petals of ‘Shock Versilia’, ‘New Fashion’ and ‘Black Magic’ Hybrid Tea rose cultivars cyanidin-3,5-diglucoside was also the main anthocyanin, that of “Corvette” cultivar is a proper source to get the same pelargonidin derivative (68.4 %).

Grajeda-Iglesias *et al.* (2016) isolated and characterized the hibiscus anthocyanins (delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside) using NMR and HPLC-ESIMS. Delphinidin-3-O-sambubioside was the most abundant anthocyanin identified in hibiscus.

#### **2.4 Characterization of phenolic compounds using HPLC (High Performance Liquid Chromatography)**

Flowers of *Rosa species* were found to contain 4'-glucosides, 3-rhamnosides, 3-sophorosides and 3-glucosides of kaempferol, ellagic acid, cyanadin and quercetin derivatives (Sumere *et al.* 1993).

Hvattum (2002) determined the phenols in rose hip (*Rosa canina*) extract using liquid chromatography coupled with negative and positive electrospray ionisation (ESI) tandem mass spectrometry (MS/MS) and diode-array detection (DAD). This combined approach allowed the identification in rose hip extract of an anthocyanin, *i.e.* cyanidin-3-O-glucoside, several glycosides of quercetin and glycosides of taxifolin and eriodictyol. Phloridzin was identified and several conjugates of methyl gallate were also found. Catechin and quercetin were found as the aglycones in the extract.

Nowak (2006) identified phenolic acids from fourteen species of wild growing roses using HPLC. Eleven major phenolic acids (gallic, protocatechuic, gentisic, p-hydroxybenzoic, vanillic, caffeic, syringic, p-coumaric, ferulic, phydroxyphenylacetic, salicylic) were quantitatively investigated. The amount of individual compounds ranged from 0.2 mg/g to 303.2 mg/g of dry material. Conjugated forms of phenolic acids were predominated in the fruits and they were

hydrolyzed mainly to gallic acid (93-303 mg/g in dry plant material). The total amount of phenolic acid after hydrolyses was from 186.4 mg/g (*Rosa inodora*) to 466 mg/g (*Rosa rugosa*) of dry weight of plant material.

Kumar *et al.* (2008) developed a rapid, simple, sensitive, robust and improved HPLC method and validated for determination of 10 polyphenols, namely gallic acid, catechin, epicatechin, rutin, m-coumaric acid, quercitrin, myricetin, quercetin, apigenin, and kaempferol in fresh flowers of *Rosa bourboniana* and *R. brunonii* and in both fresh flowers and marc (left after industrial distillation of rose oil) of *R. damascena*.

Schmitzer *et al.* (2009) reported that rose buds contained significantly more quercetins (quercetin-3-O-rutinoside, quercetin-3-O-glucoside and quercetin-3-O-rhamnoside), catechin, and phenolic acids (gallic acid, protocatechulic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid) than flowers of subsequent developmental stages.

Barros *et al.* (2013) characterized phenolic compounds of *Castanea sativa*, *Filipendula ulmaria* and *Rosa micrantha* flowers by HPLC–DAD–ESI/MS. *Castanea sativa* recorded highest amount of phenolic compounds ( $18973 \pm 40 \mu\text{g/g}$ , fw) and hydrolysable tannins ( $14873 \pm 110 \mu\text{g/g}$ ). The highest amounts of phenolic acids ( $569 \pm 20 \mu\text{g/g}$ ) and flavonoids ( $6090 \pm 253 \mu\text{g/g}$ ) were obtained in *Filipendula ulmaria* and *Rosa micrantha* respectively. Hydrolysable tannins were the main group of phenolic compounds in *Castanea sativa* and *Filipendula ulmaria* samples, while flavonoids (catechin and procyanidin dimers and trimers) were the most abundant group in *Rosa micrantha*.

Stănilă *et al.* (2015) used a qualitative high-performance liquid chromatography coupled with electrospray ionization mass spectrometric (ESI-MS) detection in positive ion mode to identify phenolic compounds from (*Rosa canina* L.) rose hip crude extract. The chromatograms revealed the presence of a large number of compounds (19), identified and grouped as phenolic acids and flavones/ols, flavan- 3-ols and also anthocyanins.

Sulaiman and Balachandran (2017) analysed the extract of *Garcinia gummi-gutta* by LC/MS in order to identify phenolic compounds. Phenolic compounds such as luteolin 7-O-glucuronide, kaempferol 3-O-(6-O-acetyl) glycoside, dicaffeoylquinic

acid, apigenein-6-C-pentosyl-8-C-hexoside and p-coumarylquinic acid were identified from hydro alcoholic extract of *Garcinia gummi-gutta*.

## **2.5 *In vitro* induction of anthocyanin pigments**

### **2.5.1 Callus induction**

Matkowski (2004) found that callus initiation and growth were best on medium containing 5 or 25  $\mu\text{mol/L}$  2, 4-D and 0.5 or 5  $\mu\text{mol/L}$  BA, respectively. The presence of both types of hormones was beneficial for callus induction in the roots, leaves and stem segment explants which were the best sources of callus tissue in *Pueraria lobata*.

Lim *et al.* (2009) induced the callus from leaf explants of *Ocimum sanctum* on MS medium supplemented with different concentrations and combinations of 2, 4-dichlorophenoxyacetic acid (2,4-D), picloram, and indole-3-butyric acid (IBA). Highest callusing was observed when MS medium supplemented with picloram (3 mg/ L).

Yang *et al.* (2009) reported maximum frequency of callus induction *Euphorbia helioscopia* cultures when cultured on MS medium supplemented with 3.0 mg/L 2,4-D.

Ram *et al.* (2011) cultured two explants (petal and leaf disc) under different conditions (light and dark); leaf disc was most suitable tissue for callus initiation in *Rosa hybrida* L. Early and prolific callus induction was observed when cultured under total dark conditions on semi-solid MS medium supplemented with 2,4-D.

Dube *et al.* (2011) cultured leaf explants of *Rosa chinensis* cv. 'Dutch' on MS basal medium supplemented with different combination and concentration of plant growth regulators i.e. NAA, IBA and 2,4-D (1-3 mg/L) along with BAP (0.5 mg/L). They found that 2, 4-D (3 mg/L) along with BAP (0.5 mg/L) proved to be the best medium for optimum callus induction. At this concentration callus obtained was light green, compact, hard and nodular with white patches after 35 days of inoculation.

Jala (2014) cultured rose stem node with axillary bud on MS medium supplemented with different concentrations of (0.1, 0.2, 0.5, 2, 5, 10 mg/L) 2,4-D. It was found that MS medium supplemented with 0.5 mg/ L 2,4-D gave the highest number of green callus.

### 2.5.2 Medium optimization

Hirasuna *et al.* (1991) showed that low levels of 2,4-D (0.5 mg/L) were most effective in producing anthocyanins compared with other auxins. Cytokinins reduced anthocyanin production. At an early stage of culture, high anthocyanin production was achieved with low concentration of 2,4-D, probably due to stress in plant cells from growth limitation.

Nishiyama and Yamakawa (2004) studied the anthocyanins accumulation in hairy root culture of *Ipomoea batatas* cv. Ayamurasaki. High sucrose concentration resulted in high accumulation of anthocyanins in hairy roots. The highest anthocyanins were reported when hairy roots were cultured on PRL-4C medium containing 3% sucrose.

Hennayake *et al.* (2006) investigated the effect of different conditions for proficient production of anthocyanins in suspension culture of *Rosa hybrida* cv. Charleston. Anthocyanins yield was enhanced by culturing cells in the EM medium with high sucrose concentration under additional UV-B radiation to white light in suspension cultured cell line.

Mathur *et al.* (2010) reported that the root derived callus line of *Panax sikkimensis* accumulated anthocyanins. The selected line showed anthocyanins content of 2.76 mg/g FW (7.076% DW) in 50 to 60 days of growth on a modified MS medium containing 4.5  $\mu$ M 2,4-dichlorophenoxy acetic acid and 1.2  $\mu$ M kinetin under 16/8 h light and dark photoperiodic conditions.

The maximum biomass was observed in the stem derived callus cultures of *Cleome rosea* on medium supplemented with 0.90 mM 2,4-D and reddish-pink regions were observed on callus surface 6–7 months after culture. Anthocyanin production was enhanced by reducing temperature and increasing light irradiation. Pigmented calluses transferred to half-strength MS medium with a 1:4 ratio  $\text{NH}_4^+$ :  $\text{NO}_3^-$ , 70 g/L sucrose and supplementation with 0.90 mM 2,4-D maintained a high biomass accumulation and showed an increase of 150% on anthocyanin production as compared with the initial culture conditions (Simões *et al.* 2009).

### 2.5.3 Effect of sucrose on *in vitro* induction of anthocyanin pigments

Anthocyanins accumulation was regulated positively by sugar and light signaling and negatively by ethylene signaling in *Arabidopsis thaliana* (Jeong *et al.*

2010). Sugars are involved in many metabolic and signalling pathways in plants (Moghaddam and den Ende, 2012). Sugars act as signaling molecules, whose signal transduction pathways may lead to the activation or inactivation of gene expression. Whole genome transcript profiling reveals that the flavonoid and anthocyanins biosynthetic pathways are strongly up-regulated following sucrose treatment and it affected both flavonoid and anthocyanin contents in the *Arabidopsis thaliana* (Solfanelli *et al.* 2006).

Nagira and Ozeki (2004) developed a system in which anthocyanins synthesis can be induced under defined conditions in regenerated *Torenia fournieri* shoots. The sugars (glucose, fructose, galactose or maltose) actively induced anthocyanin pigments, but in medium containing more than 7% glucose, fructose, or galactose, the growth of shoots was strongly suppressed.

Teng *et al.* (2005) observed that sucrose is the most effective inducer of anthocyanins biosynthesis in *Arabidopsis thaliana* seedlings and the other sugars and osmotic controls were either less effective or ineffective. In pigmented cells of *Vitis vinifera* suspension cultures, best accumulation of anthocyanins was obtained when nitrate concentration was reduced from 25 mM to 6.25 mM and when sucrose concentration was increased from 88 to 132 mM. The high sucrose and low nitrate concentrations can be one of the important culture factors in controlling of anthocyanin production by cell cultures (Do and Cormier, 1991).

High sucrose (30 g/ L) and nitrogen (100% N of standard MS) concentrations and low temperature (15°C) significantly promoted anthocyanin accumulation in cultured shoots of *Clematis pitcheri*. Reduction of nitrogen compound level in the medium to 50% and lowering sucrose concentration to 10 g/L leads to decrease of anthocyanin accumulation in the shoots cultured at 15 and 20°C (Kawa-Miszczak *et al.* 2009).

Ram *et al.* (2011) observed early pigment initiation, enhancement and maximum anthocyanin production from calluses cultured on *Euphorbia millii* (EM) medium supplemented with high sucrose concentration and under 16/8 h (light/dark) photoperiod regime.

## 2.4 Effect of plant growth regulators on *in vitro* induction of anthocyanin pigments

Mazukami *et al.* (1988) observed that the callus growth and anthocyanins synthesis were stimulated by 2, 4-D in roselle (*Hibiscus sabdariffa* L.). The highest anthocyanins yield was observed when 1.0  $\mu\text{M}$  2,4-D in combination with 0.1–1.0  $\mu\text{M}$  kinetin was used.

Gagné *et al.* (2011) demonstrated that exogenous ABA supplementation in the culture medium promoted anthocyanin production only after 4 days in grape cell culture.

Sreenivas *et al.* (2011) reported that Murashige and Skoog (MS) medium fortified with 21.48  $\mu\text{M}$   $\alpha$ -naphthaleneacetic acid was superior for callus growth of *Bridelia stipularis* derived from leaf and internode explants. The calluses incubated in light on MS medium with 4% glucose containing 2.22  $\mu\text{M}$  N<sup>6</sup>-benzyladenine (BA) and 2.26  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid (2,4-D) at pH 3.5 yielded the highest amount (a mean of 0.42 mg/g callus) of anthocyanins.

Chaudhary and Mukhopadhyay (2012) produced pigmented callus from *in vitro* grown seedlings of purple brinjal as explant. Various combinations of phytohormones were added to the culture media and the induction of pigmented callus was studied. The best results were recorded when Naphthalene Acetic Acid (NAA) at 1 mg/L was used with Kinetin at 0.25 and 0.1 mg/L, at a light intensity of 4000 lux. The total anthocyanin accumulation in the pigmented callus was calculated as 70  $\mu\text{g/g}$ .

Tarrahi and Rezanejad (2013) obtained highest callogenesis on MS medium containing ratios of 2 and 3 mg/L of 2,4-dichlorophenoxyacetic acid to 1 mg/L of 6-benzylamino purine in *Rosa gallica* and *Rosa hybrida*. The highest anthocyanin and chlorophyll yield was produced in vegetative calluses of *Rosa gallica*. In *Rosa hybrida*, the highest value of pigment was observed in callus from leaf and stem.

Ram *et al.* (2013) investigated the effects of salicylic acid (SA) and methyl jasmonate (MeJA) on anthocyanin induction, biomass accumulation, and colour value (CV) indices for both pigment content (PC) and pigment production (PP) in callus cultures of *Rosa hybrida* cv. Pusa Ajay. A concentration-dependent response was exhibited by cultures on SA and MeJA at different concentrations individually or in

combinations to *Euphorbia millii* medium supplemented with 204.5 mM sucrose, 2.45 mM indole butyric acid and 2.33 mM kinetin. Treatment with 0.5  $\mu$ M MeJA was most effective in inducing anthocyanin biosynthesis in callus cultures.

Usha *et al.* (2014) studied the effect of different concentration of Abscisic acid (ABA) on anthocyanin induction in callus culture of *Petunia hybrida*. ABA when applied at 30  $\mu$ M concentration resulted in a synergistic increase in anthocyanin production. The colour value index (CV) was observed maximum with the addition of ABA 30  $\mu$ M ( $1.58 \pm 0.08$  CV per gram fresh cell weight) and maximum response for pigment production ( $3.22 \pm 0.12$  CV per test tube).

Largado-Valler (2016) determined the anthocyanin content in callus cultures of *Sesbania grandiflora* L. (Red Katuray) using petal explants. Earliest callus initiation was observed in 2.5 ppm to 5.0 ppm 2,4-D without BA and 2.5 ppm 2,4-D with 2.5 ppm BA. Maximum weight of callus was registered at 2.5 ppm 2,4-D without BA. Highest anthocyanin content was found in 2.5 ppm to 5.0 ppm 2,4-D without or with 2.5 ppm BA, with 2.5 and 5.0 ppm BA and with 5.0 ppm 2,4-D and 2.5 ppm BA. Positive correlation was observed between callus formation and anthocyanin production.

### 3. MATERIALS AND METHODS

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The present investigation entitled “**Isolation and Characterization of Anthocyanin Pigments from Indian Rose Varieties as a Potential Source of Nutraceuticals**” was carried out at the Division of Floriculture and Landscaping, Division of Agricultural Chemicals and Central Tissue Culture Laboratory, ICAR-Indian Agricultural Research Institute, New Delhi-110012, during 2014-2016. The materials and methods followed for conducting the present study are as follows:

#### 3.1 Materials

##### 3.1.1. Plant material

The plant material utilized for conducting the experiment consisted of 50 rose varieties which were grown and maintained at the research farm of the Division of Floriculture and Landscaping, ICAR-Indian Agricultural Research Institute, New Delhi-110012.

**Rose varieties utilized:** The plant material used in the present study included 49 Indian varieties *viz.* Pusa Arun, Raktima, Nehru Centenary, Pusa Bahadur, Lalima, Bhim, Ashwini, Dr. S.S. Bhatnagar, Jantar Mantar, Jawani, Raktagandha, Pusa Mohit, Pusa Ajay, Pusa Muskan, Pusa Barahmasi, Pusa Virangana, Pusa Priya, Dr. Bharat Ram, Dr. M.S. Randhawa, Priyadarshini, Dulhan, Dr. B.P. Pal, Anurag, Arjun, Haseena, Raja Surendra Singh of Nalagarh, Sadabahar, Nurjehan, Pink Montezuma, Surekha, Dr. Benjamin Pal, Pusa Ranjana, Arunima, Manasi, Rose Sherbet, Pusa Garima, Pusa Gaurav, Suryakiran, Krishna, Shola, Pusa Pitamber, Lahar, Ganga, Raja Ram Mohan Roy, Pusa Abhishek , Mridula, Shabnam, Chingari, Surkhab and one exotic variety *viz.* Iceberg (Plate 3.1).

##### 3.1.2 Chemicals and glasswares

The glasswares, *viz.* volumetric flasks, measuring cylinder, beakers, pipettes, test tubes, petri dishes, vials, Erlenmeyer flasks, glass jar, vials with caps and bottles, etc. were procured from Borosil Glassworks Limited and used for conducting experiments. Chemicals used in the study were of analytical grade (AR) and details of procurement is given in Table 3.1.

**Table 3.1 List of chemicals, standards, equipments, instruments and reagents used in experiments**

<b>Name of chemicals/standards/reagents</b>	<b>Source of procurement</b>
<b>Standards</b>	
Cyanidin 3,5 di-o-glucosides(90%)	Sigma–Aldrich Fine Chemicals
Pelargonidin chloride	Sigma–Aldrich Fine Chemicals
Catechin	Sigma–Aldrich Fine Chemicals
Rutin	Sigma–Aldrich Fine Chemicals
Epicatechin	Sigma–Aldrich Fine Chemicals
Quercetin	Sigma–Aldrich Fine Chemicals
3- hydroxyl cinnamic acid	Sigma–Aldrich Fine Chemicals
TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine)	Sigma–Aldrich Fine Chemicals
DPPH (2,2-Diphenyl-1-picrylhydrazyl)	Sigma–Aldrich Fine Chemicals
Gallic acid	Sigma–Aldrich Fine Chemicals
Ferrous Sulphate	Sigma–Aldrich Fine Chemicals
<b>Chemicals/Reagents</b>	
Ethanol	CDH
Folin & ciocalteu’s phenol reagent	Merck Pvt. Ltd
Sodium carbonate	Merck Pvt. Ltd
Copper chloride	Merck Pvt. Ltd
Ammonium acetate	Merck Pvt. Ltd
Sodium acetate trihydrate	Merck Pvt. Ltd
Acetic acid glacial	Merck Pvt. Ltd
Hydrochloric acid	Merck Pvt. Ltd
Iron (III) chloride anhydrous	Merck Pvt. Ltd
Sodium nitrite	Merck Pvt. Ltd
Aluminium chloride anhydrous	Merck Pvt. Ltd
Sodium hydroxide	Merck Pvt. Ltd
Methanol	Hi-Media Laboratories Pvt. Ltd, Mumbai,
HPLC grade Methanol	Sigma–Aldrich Fine Chemicals
Sucrose	Titan
MS medium	Titan
Agar Agar Type I	Titan
Abscisic acid	Titan
Inorganic salts, 8-hydroxyquinoline citrate	Qualigens Fine Chemicals
Vitamins, plant growth regulators (PGRs)	Hi-Media Laboratories Pvt.Ltd, Mumbai
Carbendazim	BASF India Ltd., Mumbai
Mancozeb	Rallis India Ltd., Mumbai

## **3.2 Methodology**

Rose varieties were grown following the recommended cultural practices at the Research Farm of the Division of Floriculture and Landscaping, ICAR- Indian Agricultural Research Institute, New Delhi. The farm is situated at 77° 12' E longitude, 28°40' latitude and at an altitude of 228.16 m above mean sea level.

### **3.2.1 Isolation and quantification of total anthocyanin pigments from rose varieties**

Rose varieties shown in Plate 3.1 viz. Pusa Arun, Raktima, Nehru Centenary, Pusa Bahadur, Lalima, Bhim, Ashwini, Dr. S.S. Bhatnagar, Jantar Mantar, Jawani, Raktagandha, Pusa Mohit, Pusa Ajay, Pusa Muskan, Pusa Barahmasi, Pusa Virangana, Pusa Priya, Dr. Bharat Ram, Dr. M.S. Randhawa, Priyadarshini, Dulhan, Dr. B.P. Pal, Anurag, Arjun, Haseena, Raja Surendra Singh of Nalagarh, Sadabahar, Nurjehan, Pink Montezuma, Surekha, Dr. Benjamin Pal, Pusa Ranjana, Arunima, Manasi, Rose Sherbet, Pusa Garima, Pusa Gaurav, Suryakiran, Krishna, Shola, Pusa Pitamber, Lahar, Ganga, Raja Ram Mohan Roy, Mridula, Shabnam, Chingari, Surkhab, Iceberg and Pusa Abhishek were evaluated for total anthocyanin, antioxidant activity and phenolic content on fresh weight basis.

### **Observations recorded**

Observations on the following characters were recorded in each treatment, variety and replication on fresh weight basis.

#### **3.2.1.1 Extraction and quantification of total anthocyanins**

The anthocyanins were extracted according to the methodology of (Wrolstad *et al.* 2005). Petals and leaves (0.5 g) of 50 varieties were extracted with acidified methanol (methanol + 1% HCl) in ultra sonicator water bath for 30 minutes. The extracts were centrifuged at 10,000 rpm for 10 min by using refrigerated centrifuge. Final volume was prepared by adding the acidified methanol (methanol + 1% HCl) to the supernatant. Then final samples were prepared by taking the 5 ml volume of sample and dilutes separately in potassium chloride buffer 0.025 M (pH 1.0) and sodium acetate buffer 0.4 M (pH 4.5) (5ml each). After 15 min of incubation at room temperature, absorption was measured at 520 and 700 nm. Absorbance readings were made at room temperature against distilled water as blank. A Jasco V 530 UV-Vis spectrophotometer was used for measurements.

Absorbance was calculated as  $A = [(A_{510} - A_{700}) \text{ at pH } 1.0] - [(A_{510} - A_{700}) \text{ at pH } 4.5]$  with a molar extinction coefficient of 26,900 for anthocyanin.

The total anthocyanin content was calculated as cyanidin-3-glucoside equivalents as:

$$\text{Anthocyanin content (mg/g)} = (A \times \text{MW} \times \text{DF} \times V \times 10^3) / (\epsilon \times L \times m)$$

where A is absorbance, MW is the molecular weight of cyanidin-3-glucoside (449.2 Da), DF is the dilution factor, V is the final volume (mL),  $10^3$  is the factor for conversion from g to mg,  $\epsilon$  is the cyanidin-3-glucoside molar absorbance (26,900), L is the cell path length (1 cm), and m is the petal weight (g).

### 3.2.1.2 Quantification of phenolic compounds

#### 3.2.1.2.1 Sample extraction

The phenolic compounds from rose petals were extracted using a modification of the procedure described by Dragović-Uzelac *et al.* (2010). Each 0.5 g sample was extracted with 20 ml 80% methanol. The extract was then centrifuged at 10,000 rpm at 4°C for 20 minutes. The supernatant was taken for determination of total phenolic content.

#### 3.2.1.2.2 Total phenolics content (TPC)

Total phenols were estimated according to the procedure given by Singleton and Rossi (1965). A 0.5 g sample was extracted with 20 ml 80% methanol. The aliquot (1 ml) were taken in the test tubes and were added with 2.9 ml of Folin and Ciocalteu's Phenol Reagent (1N). To that, 0.5 ml of distilled water was added and all the tubes were shaken well. Then, 2 ml of sodium carbonate (20%) solution was added to all the tubes and kept for incubation at room temperature for 30 minutes. The colour developed was read in spectrophotometer at 750 nm wavelength.

Standard curve was drawn using gallic acid as standard. Different concentrations of gallic acid were prepared and O.D was read at 700 nm wavelength. The concentration of samples was calculated based on the standard curve.

#### Calculation:

$$\text{mg gallic acid equivalence per gram} = \frac{\text{O.D} \times \text{standard curve factor} \times \text{volume made up} \times \text{Dilution}}{\text{Aliquot taken} \times \text{weight of sample}}$$

**Plate 3.1: Rose varieties used for the estimation of total anthocyanins, phenolic content and antioxidant activities**



**Anurag**



**Arjun**



**Arunima**



**Bhim**



**Chingari**



**Dr. Benjamin Pal**



**Dr. Bharat Ram**



**Dr. M.S. Randhawa**



**Dulhan**



**Ganga**



**Haseena**



**Jantar Mantar**

Plate 3.1 contd....



**Jawani**



**Krishna**



**Lahar**



**Lalima**



**Manasi**



**Mridula**



**Nehru Centenary**



**Priyadarshini**



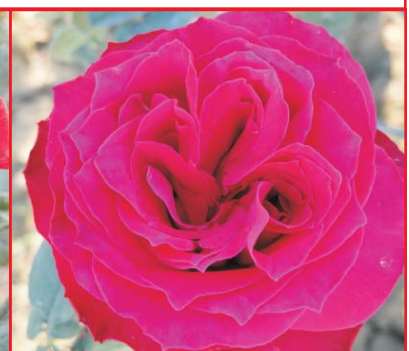
**Pusa Abhishek**



**Pusa Ajay**



**Pusa Arun**



**Pusa Bahadur**

Plate 3.1 contd....



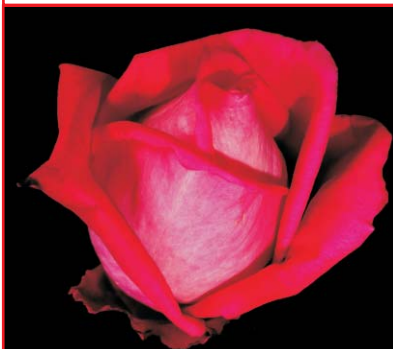
**Pusa Barahmasi**



**Pusa Garima**



**Pusa Gaurav**



**Pusa Mohit**



**Pusa Muskan**



**Pusa Pitamber**



**Pusa Priya**



**Pusa Ranjana**



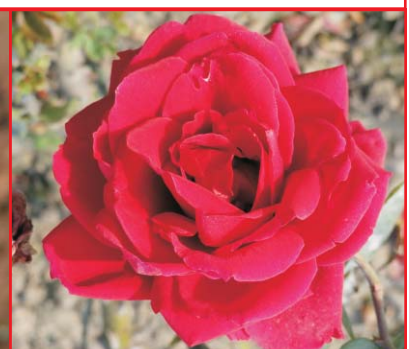
**Pusa Virangana**



**Raja Ram Mohan Roy**



**R.S.S. of Nalagarh**



**Raktagandha**

Plate 3.1 contd....



**Raktima**



**Rose Sherbet**



**Dr. S.S. Bhatnagar**



**Sadabahar**



**Surekha**



**Surkhab**



**Suryakiran**



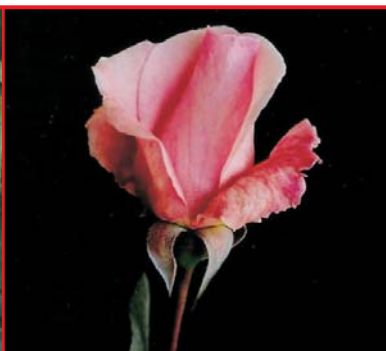
**Ashwini**



**Iceberg**



**Shabnam**



**Pink Montezuma**



**Dr. B.P. Pal**

### 3.2.1.3 Total chlorophyll content from leaves

Chlorophyll content was estimated as per the method described by Hiscox and Israelstam (1979). The procedure for estimation of chlorophyll content in plants is based on the absorption of light by chlorophyll extracts prepared by incubating the leaf tissues in DMSO (dimethyl sulfoxide). DMSO renders plasmalemma permeable thereby, causing the leaching of the pigments (Hiscox and Israelstam, 1979). The absorbance of the known volume of solution containing known quantity of leaf tissue at two respective wavelengths (663 and 645) was determined for chlorophyll content. Chlorophyll a, chlorophyll b and total chlorophyll content were estimated using the formula given by Arnon (1949). 50 mg fresh leaf samples were added to the test tubes containing 4 ml DMSO. Tubes were kept in dark for 4 h at 65°C. Then the samples were taken out cooled at room temperature and the absorbance was recorded at 663 and 645 nm using DMSO as blank and was expressed as mg/g dry wt.

$$\text{Chlorophyll 'a'} = (12.7 \times A_{663} - 2.69 \times A_{645}) \times V/W \times 1000$$

$$\text{Chlorophyll 'b'} = (22.9 \times A_{645} - 4.68 \times A_{663}) \times V/W \times 1000$$

$$\text{Total chlorophyll} = (20.2 \times A_{645} + 8.02 \times A_{663}) \times V/W \times 1000$$

Where,

$A_{663}$  = Absorbance values at 663 nm

$A_{645}$  = Absorbance values at 645 nm

W = Weight of the sample in g

V = Volume of the solvent used (ml)

### 3.2.2 Determination of antioxidant activities of 50 rose varieties.

#### 3.2.2.1 Sample extraction

Each 0.5 g sample was extracted with 20 ml 80% ethanol. The extract was then centrifuged at 10000 rpm at 4°C for 20 minutes. The supernatant was taken for determinations including total antioxidant activity by CUPRAC, FRAP and DPPH.

#### 3.2.2.2 Cupric reducing antioxidant capacity (CUPRAC)

Antioxidant activity was determined by following CUPRAC method, which was standardized by Apak *et al.* (2004). It measures the copper (II) or cupric ion

reducing ability of polyphenols. This is a simple and widely applicable antioxidant capacity index for dietary polyphenols, vitamins C and E. It makes use of the copper (II)-neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidizing agent. For determining antioxidant activity, copper (II) chloride solution, a neocuproine alcoholic solution and an ammonium aqueous buffer (pH-7) was mixed and then measurements of the developed colour were taken after 30 minutes in a spectrophotometer at absorbance at 450 nm.

To a test tube, 1ml each of copper (II) chloride solution ( $10^{-2}$  M), Neocuproine solution (Nc) of  $7.5 \times 10^{-3}$  M and ammonium acetate (NH<sub>4</sub>Ac) buffer (pH-7) solutions were added. Antioxidant sample (or standard) solution (0.1 ml) and H<sub>2</sub>O (1.0 ml) were added to the initial mixture so as to make the final volume 4.1 ml. The tubes were then capped and after one hour, the absorbance at 450 nm was recorded against a reagent blank. The standard calibration curve of each antioxidant compound was constructed as absorbance *versus* concentration. The molar absorptivity of the CUPRAC method for each antioxidant was found from the slope of the calibration line concerned and the antioxidant activity was expressed as  $\mu\text{mol Trolox/g}$ .

#### Calculations:

$$\text{Antioxidant capacity } (\mu\text{mol Trolox/g}) = (A / \epsilon_{\text{TR}}) (V_f / V_s) r (V_t / m)$$

Where

A= absorbance

$\epsilon_{\text{TR}}$  = Molar absorptivity of Trolox=  $1.67 \times 10^4$

$V_f$  = Final volume made=4.1 ml

$V_s$  = Sample volume taken from diluted extract (ml)

$V_t$  =Total volume (ml)

R= Dilution factor

m= Weight of sample taken (g)

#### 3.2.2.3 Ferric Reducing Antioxidant Potential (FRAP)

Antioxidant activity of rose petals was estimated using FRAP (Ferric Reducing Antioxidant Potential) method as described by Benzie and Strain (1996).

### Preparation of working FRAP reagent

Acetate buffer 300 mM pH 3.6, TPTZ (2, 4, 6-tripyridyl-*s*- triazine) 10 mM in 40mM HCl and ferric chloride (FeCl<sub>3</sub>.6H<sub>2</sub>O) 20 mM in 10:1:1 ratio were freshly prepared on the same day. The ethanolic extract (0.1 ml) of the sample was taken in test tubes and 3 ml of working FRAP reagent was added. Then, the tubes were kept for incubation at room temperature for 4-5 minutes. The blue colour so developed was read in a spectrophotometer at 593 nm using the FRAP reagent as a blank and expressed as Trolox equivalents. Standard curve was prepared using FeSO<sub>4</sub> as standard. Different concentrations of FeSO<sub>4</sub> were prepared and O.D was read at 593 nm. The concentration of samples was calculated based on the standard curve.

#### Calculation:

$$\text{FRAP } (\mu\text{mol FeSO}_4/\text{g}) = \frac{\text{O.D x Factor x volume made up}}{\text{Aliquot taken x weight of sample}}$$

#### 3.2.2.4 DPPH free radical scavenging activity

The DPPH assay method is based on the reduction of DPPH, a stable free radical. The antioxidant activity of rose petal extracts was estimated using DPPH assay described by Braca *et al.* (2001). Aqueous extract 0.1 ml was added to 3.9 ml of 0.0025 M DPPH (2, 2-Diphenyl-1- picrylhydrazyl) in methanol (70%). The mixture was shaken and kept for 30 min. in dark at room temperature. Absorbance was recorded at 517 nm in spectrophotometer. The per cent inhibition of activity was calculated by the following formula:

$$\text{Per cent inhibition (\%)} = [(A_o - A_e) / A_o] \times 100$$

(A<sub>o</sub> = absorbance without extract; A<sub>e</sub> = absorbance with extract)

#### 3.2.3 Identification and characterization of anthocyanin pigments from selected rose varieties using HPLC (High Performance Liquid Chromatography)

The petals from fresh flowers of promising varieties of rose were utilized for characterization of anthocyanin pigments.

**Observations recorded:****Anthocyanin pigments**

Cyanidin 3,5-di-O-glucoside

Pelargonidin 3,5-di-O-glucoside

**3.2.3.1 Estimation of Cyanidin 3,5-di-O-glucoside and Pelargonidin 3,5-di-O-glucoside**

Purity of anthocyanin powder concentrate was checked by HPLC instrument (Alliance, Waters Corp., Milford, Mass., U.S.A.) equipped with e2695 quaternary pump, auto injector (20 $\mu$ L loop), a 2998 photodiode array detector and an “Empower 2” software programme. A C-18 column (Thermo, USA) 25 cm  $\times$  4.6 mm  $\times$  5 $\mu$  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<sup>-1</sup>. The gradient mobile phase is as follows:

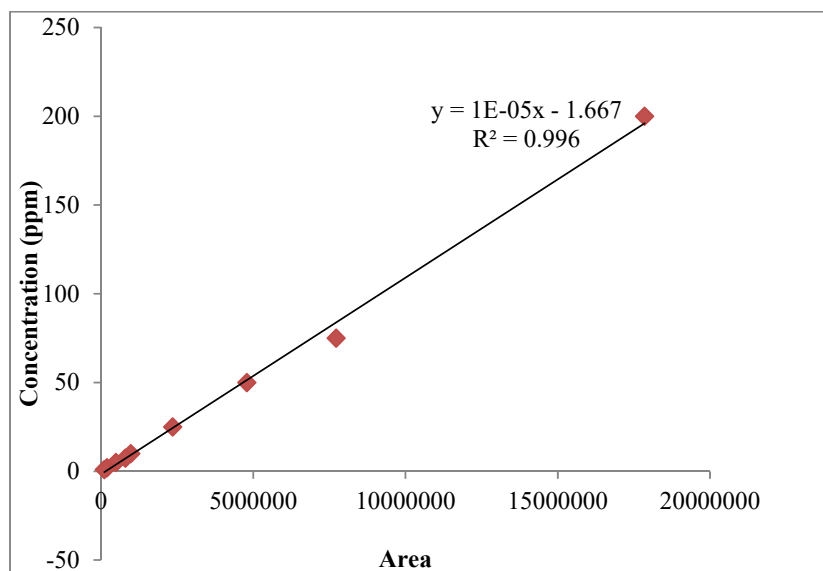
<b>Time</b>	<b>Flow</b>	<b>%A</b>	<b>%B</b>
1 min	0.60	80.0	20.0
26 min	0.60	40.0	60.0
30 min	0.60	80.0	20.0
40 min	0.60	80.0	20.0

Chromatogram was acquired at 520 nm and peak assignments were made based on by comparison with authentic standards and published literature (UV-Vis spectra, retention time and elution order).

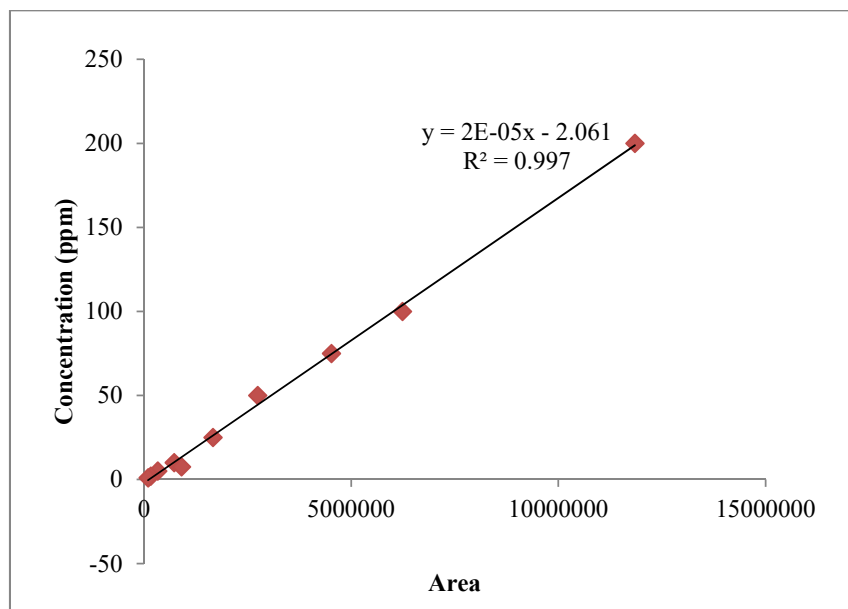
**3.2.3.2 Extraction of anthocyanins**

Anthocyanin rich petals were carefully removed (2 g) and taken in the amber colour flask and extracted with 500 ml of acidified methanol (0.1% HCl). The content was sonicated in the dark for 30 min on an ultrasonicator (Misonix, NY, U.S.A). The combined extract was concentrated under vacuum (35 $\pm$ 1 $^{\circ}$ C) in a rotary evaporator (Heidolph, Germany) for complete removal of methanol. HPLC grade methanol was added to the dried sample. The supernatant was filtered through a 0.45- $\mu$ m syringe filter (Whatman Inc., Maidstone, UK) prior to HPLC analysis.

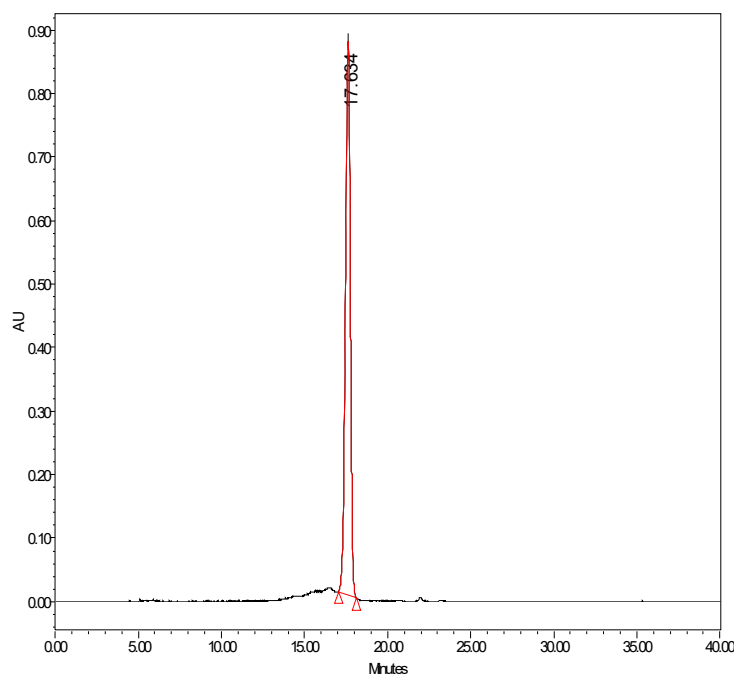
**Figure 3.1 Calibration curve for standard cyanidin 3,5-di-O-glucoside**



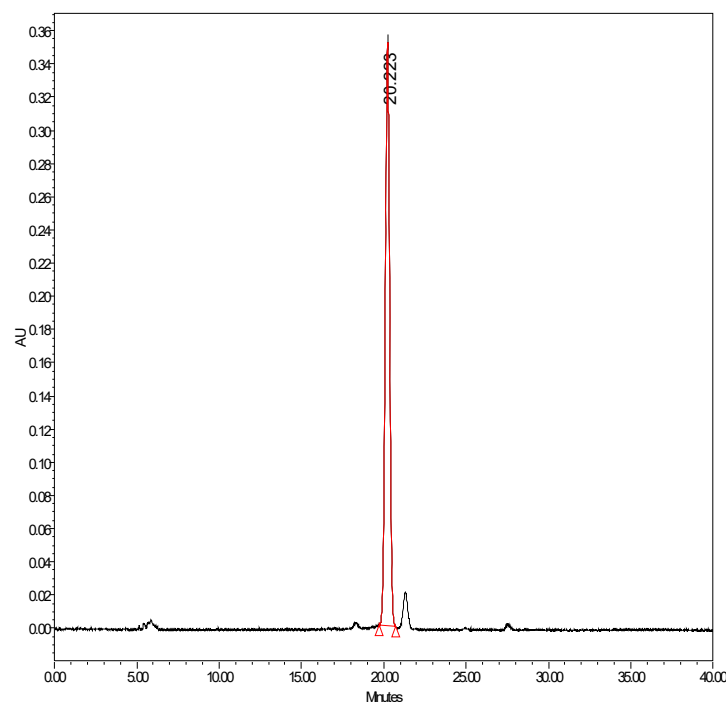
**Figure 3.2 Calibration curve for standard pelargonidin 3,5-di-O-glucoside**



**Figure 3.3 Chromatogram of standard cyanidin 3,5-di-O-glucoside**



**Figure 3.4 Chromatogram of standard pelargonidin 3,5-di-O-glucoside**



### 3.2.3.3 Preparation of calibration curve for anthocyanins quantification

The anthocyanin content was quantified based on the modified procedure described by Zheng *et al* (2011). The peak area of the anthocyanin was integrated from the HPLC chromatogram at 520 nm using the Agilent ChemStation software and plotted against concentration. The stock solutions were made with 1% TFA (v/v) in methanol to give a 1 µg/ml concentration. Calibration curve was prepared with Cyanidin-3,5 di-O-glucoside at 10 different concentrations (1, 2, 5, 7.5, 10.0, 25.0, 50.0, 75.0, 100.0, 200.0 µg/ml) and each absorbance was measured at 520 nm so as to get the following linear equation:  $Y$  (absorbance) =  $1E-05X-1.667$  ( $r = 0.9967$ ) (Figure 3.1). Similarly, calibration curve was obtained with Pelargonidin-3,5 di-O-glucoside at 10 different concentrations (1, 2, 5, 7.5, 10.0, 25.0, 50.0, 75.0, 100.0, 200.0 µg/ml) and each absorbance was measured at 520 nm so as to get the following linear equation:  $Y$  (absorbance) =  $2E-05X-2.061$  ( $r = 0.997$ ) (Figure 3.2). The sample was appropriately diluted with acidic methanol solution until the absorbance was in the range of 0.200 to 0.800 at 520 nm. The anthocyanins content was finally calculated according to the standard curve and expressed as mg/100g fresh weight.

### 3.2.3.4 Characterization of anthocyanin pigments

Purity of anthocyanin powder concentrate was checked by HPLC instrument 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<sup>-1</sup>. 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-3,5 di-O-glucoside and Pelargonidin-3,5 di-O-glucoside were also run according to the above mentioned flow rate. UV spectra also recorded for each peak found in HPLC analysis. Concentration of individual anthocyanins were calculated based on Cyanidin-3,5 di-O-glucoside and Pelargonidin-3,5 di-O-glucoside equivalent.

## 3.2.4 Identification and characterization of phenolic compounds from selected rose varieties using HPLC (High Performance Liquid Chromatography)

### 3.2.4.1 Preparation of Standard and Sample Solutions

Five standard stock solutions, catechin (500 µg/ml), rutin (500 µg/ml), quercetin (500 µg/ml), epicatechin and 3-hydroxy cinnamic acid (500 µg/ml), were

prepared in HPLC grade solvent mixture of acetonitrile/formic acid (1:1; v/v) and filtered using membrane disc filter (0.45  $\mu\text{m}$ ). Solution (5 mg/ml) of all samples was prepared in HPLC grade solvent mixture of acetonitrile/formic acid (1:1; v/v) and filtered using membrane disc filter (0.45  $\mu\text{m}$ ).

HPLC instrument (Alliance, Waters Corp., Milford, Mass., U.S.A.) equipped with e2695 quaternary pump, auto injector (20 $\mu\text{L}$  loop), a 2998 photodiode array detector and an “Empower 2” software programme was used for characterization of phenolic compounds. A C-18 column (Thermo, USA) 25 cm  $\times$  4.6 mm  $\times$  5 $\mu$  was used for separation of phenolic compounds using a mobile phase comprising of gradient elution of solvent A: water (0.1% Formic Acid) and solvent B: acetonitrile (0.1% Formic Acid) at a flow rate of 0.5 ml/min. The elution profile was 0 min 100 % A, then the solvent B was increased first to 20% in 20 min, thereafter to 30% in 10 min followed by 50% in 10 more min and finally to 100% for 10 min. The total run time was 50 min. The injected volume was 20  $\mu\text{l}$ . For detection and quantification of phenolics, the photodiode array detector was set at 280 nm.

### **3.2.5 *In vitro* induction of anthocyanin pigments in promising rose variety**

Rose variety with high amount of anthocyanin pigments and with high antioxidant activities was subjected to *in vitro* induction of pigments.

#### **3.2.5.1. Sterilization of glassware and equipment**

All the glassware were washed with laboratory detergent (Teepol® @ 0.1 per cent) followed by sufficient washing with running tap water to remove the detergent residues and rinsed with double-distilled water. Forceps and scalpels wrapped in aluminium foil were sterilized in a vertical autoclave at 121.6°C for 20 min at 15 psi. The entire culture vessels used for media preparation were dried in hot air oven at 160°C for 5-6 hrs, cooled. Aseptic operations were conducted in horizontal laminar air-flow chamber fitted with high efficiency particulate air filter (HEPA, 0.22  $\mu\text{m}$ ) and a gas burner. Ultraviolet light of the laminar airflow chamber was switched on for 30-40 min after that working table of the chamber was surface sterilized with absolute ethyl alcohol. Forceps, scalpels, and carbon steel blades were flame-sterilized on a gas burner before each inoculation. The hands were swabbed with ethyl alcohol (70%, v/v) before any operation on laminar air-flow hood.

### **3.2.5.2. Preparation and storage of stock solutions**

Stock solutions for macronutrients, micronutrients, iron, calcium, vitamins, 2,4-D and ABA were prepared separately. All the stock solutions of the salts were prepared by dissolving the required amount of chemicals in double-distilled water and stored in reagent bottles in a refrigerator at 4-7°C until use. Stock solutions of 2,4-D and ABA were prepared by dissolving them in a small volume of 1N NaOH and final volume was made by adding double-distilled water.

### **3.2.5.3 Preparation and sterilization of culture media**

Double-distilled water was used to prepare culture media. Required amounts of stock solutions were pipetted out, followed by mixing. Sucrose and other supplements were added as per treatments. Required amount of PGRs and elicitors were added to the culture medium as per the finalized concentrations for the experiment. Using 1 N HCl or KOH, pH of the culture media was adjusted to 5.8. Mixture was heated after addition of 5.5 g/l agar-agar (w/v) powder. Prepared media were poured in test tubes containing 20 ml medium followed by plugging. The test tubes were then autoclaved at 121°C for 16 min at 15 psi. After autoclaving media was allowed to cool and solidify. Inoculation was done three days later to ensure that the test tubes were free from any microbial contamination. The composition of Murashige and Skoog (1962) and *Euphorbia millii* (Yamamoto *et al.* 1989) media are given in Table 3.2.

**Table 3.2: Composition of MS (Murashige and Skoog, 1962) and EM (Yamamoto *et al.* 1989) media**

Stock solution	Ingredients	MS medium (mg/ L)	EM medium (mg/ L)
<b>INORGANICS</b>			
Macro-nutrients			
A (50x)	NH <sub>4</sub> NO <sub>3</sub>	1650	141
	KNO <sub>3</sub>	1900	2648
	MgSO <sub>4</sub> .7H <sub>2</sub> O	370	500
	KH <sub>2</sub> PO <sub>4</sub>	170	–
	NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	-	150
	KCl	-	264
B (50x)	CaCl <sub>2</sub> .2H <sub>2</sub> O	440	150
Micro-nutrients			
C (1000x)	H <sub>3</sub> BO <sub>3</sub>	6.2	3
	MnSO <sub>4</sub> .4H <sub>2</sub> O	22.3	10
	ZnSO <sub>4</sub> .7H <sub>2</sub> O	8.6	2
	KI	0.83	0.75
	Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.25	0.22
D (1000x)	CuSO <sub>4</sub> .5H <sub>2</sub> O	0.025	0.025
	CoCl <sub>2</sub> .6H <sub>2</sub> O	0.025	0.025
E (100x)	FeSO <sub>4</sub> .7H <sub>2</sub> O	27.80	13.9
	Na <sub>2</sub> EDTA.2H <sub>2</sub> O	37.30	18.7
<b>ORGANICS</b>			
F (100x)	Thiamine-HCl	0.1	0.1
	Pyridoxine-HCl	0.5	0.5
	Nicotinic acid	0.5	0.5
	Glycine	2.0	2.0
G (100x)	Myo-inositol	100	100
CARBON SOURCE (without stock)	Sucrose	30,000	50,000

#### 3.2.5.4 Preparation and culture of leaf and petal explants

Young leaves and rose buds were collected from the field during morning hours. Individual petals were separated from bud. After washing in running tap water for 10 min leaves and petals were dipped in 0.1% Teepol® solution for 5 min. and then again washed with tap water for 10-15 min. Petals and leaves were then treated with different combination of pre-treatments and set aside on a horizontal shaker (120

rpm) for 3 hours. The petals and leaves were then rinsed in sterile double-distilled water at least 4 times and were subsequently air-dried on sterile blotting paper in a laminar air-flow hood. Leaf and petal segments were prepared by cutting leaf and petals (5 × 5 mm size). For callusing petal and leaf segments were inoculated on the culture medium. Sub culturing of induced callus was done at 21 days interval. For callus induction and multiplication, the culture vessels were kept in an insulated room at a constant temperature of 24 ± 1°C in dark. Further induced calli were subjected to different treatments for pigment induction.

### **3.2.5.5 Establishment of aseptic cultures from petal explants**

#### **3.2.5.5.1 Standardization of pre-treatments for petal explants**

Well prepared explants were washed with 0.1 per cent Teepol® solution for 5 min. followed by thorough washing in running tap water for 10-15 min to remove any residue of the detergent. Afterward explants were subjected to different pre-treatments and surface sterilization before inoculation to minimize the microbial contamination. Pre-treatment comprising of carbendazim and mancozeb-45 of different concentration for different duration were used followed by surface sterilization with HgCl<sub>2</sub> @ 0.1 per cent.

#### **3.2.5.5.2 Standardization of culture medium for callus induction and multiplication**

Murashige and Skoog's medium was supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction and multiplication from rose petal explant.

### **Observations recorded**

#### **a. Survival percentage of explants**

Survival of explants was calculated using the given formula:

$$\text{Survival (\%)} = \frac{\text{Total number of survived explants}}{\text{Total number of cultured explants}} \times 100$$

### **b. Microbial contamination percentage**

Microbial contamination in cultures was calculated using the given formula:

$$\text{Microbial contamination (\%)} = \frac{\text{Total number of contaminated explants}}{\text{Total number of cultured explants}} \times 100$$

### **c. Induction coefficient of callusing**

Per cent callusing of explants was calculated using the given formula:

$$\text{Induction coefficient} = \frac{\text{Total number of induced calli}}{\text{Total number of cultured explants}} \times 100$$

### **d. Days required for callus induction**

Cultures were observed daily for callus initiation and observations were recorded on the number of days taken by inoculated explants for callus induction.

### **e. Visual observations**

Visual observations were judged for the following parameters:

- Site of callus initiation — on cut surface, on cut surface and punctured place
- Status of callus — compact, friable, semi-friable
- Colour of callus — white, creamish-white, yellowish-white, greenish-white

### **f. Measurement of callus growth**

After 42 days of culture growth of callus was determined on the basis of fresh- (FCW) and dry (DCW) cell weight. Dry mass of callus was measured after drying at 45°C for the first 24 h and 55°C for the next 24 h. Fresh cell weight: dry cell weight ratio (FCW: DCW R) was calculated to measure the actual gain in callus biomass accumulation.

### **g. Classification of callus growth status**

Callus growth status was classified according to score as described by Matkowski (2004):

I = Weak callus initiation and poor growth.

II = Good induction of callus but poor growth.

III = Good initiation and moderate growth.

IV = Best induction and vigorous growth of callus.

### 3.2.5.6 Standardization of different concentrations of sucrose and ABA in the culture media for pigment induction

Freshly initiated calluses were transferred onto the solid EM medium supplemented with the given treatments for anthocyanin pigment induction.

**Details of treatment for anthocyanin induction:** *Euphorbia millii* medium (Yamamoto *et al.*1989) supplemented with following sucrose and abscissic acid (ABA) concentrations was used for anthocyanin induction.

1. EM + 0 ( control)
2. EM + sucrose (50 g/l)
3. EM + sucrose (60 g/l)
4. EM + sucrose (70 g/l)
5. EM + sucrose (80 g/l)
6. EM + ABA (20  $\mu$ M)
7. EM + ABA (30  $\mu$ M)
8. EM + ABA (40  $\mu$ M)
9. EM + ABA (50  $\mu$ M)
10. EM + sucrose (50 g/l) + ABA (50  $\mu$ M)
11. EM + sucrose (60 g/l) + ABA (40  $\mu$ M)
12. EM + sucrose (70 g/l) + ABA (30  $\mu$ M)
13. EM + sucrose (80 g/l) + ABA (20  $\mu$ M)

#### Observations recorded

##### a. Response coefficient

Percent of cultures showing response were calculated using the given formula.

$$\text{Response coefficient (\%)} = \frac{\text{Total number of cultures showing pigmentation}}{\text{Total number of cultures}} \times 100$$

**b. Number of days taken for pigment initiation**

It was recorded on the basis of number of days taken for anthocyanin pigment initiation in callus cultures from each treatment.

**c. Number of days taken for pigment intensification**

It was recorded on the basis of number of days taken for colour intensification in callus cultures from each treatment.

**d. Growth index of pigmented callus (FW)**

Callus biomass accumulation was estimated by taking the fresh weight of callus cultures after 15 days of cultures.

**e. Microscopic study of pigmented callus**

Digital images of the cultures were captured with a Nikon D 40X camera (Nikon Corporation, Japan). Microscopic images of the experiments were visualized with a Carl Zeiss Discovery.v8 Stereo microscope (Carl Zeiss MicroImaging GmbH, Berlin, Germany) and images were captured with a Carl Zeiss Axiovision digital camera (software version: Axiovision 4.8.2).

**f. Determination of anthocyanin content**

**Total monomeric anthocyanins:** As explained in experiment 3.2.1.1

**3.3 Reproducibility of the results and statistical analysis**

All the experiments were laid out in completely randomized design (CRD). The data presented in the results are the means  $\pm$  standard error obtained from at least three independent determinates. All the data were subjected to Turkey's honestly significant difference (HSD) test for the comparison of means and significance for the test was assumed if  $P \leq 0.05$ . The analyses were carried out through the statistical software SPSS 20.0 (SPSS Inc., Chicago, USA).

## 4. RESULTS

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To meet the objectives of the research work entitled “**Isolation and Characterization of Anthocyanin Pigments from Indian Rose Varieties as a Potential Source of Nutraceuticals**” a series of experiments was designed. The data obtained from each of the experiment was subjected to statistical analysis. Results of the present investigations are presented under the following headings:

- 4.1 Estimation of total anthocyanin content, total phenolic content and antioxidant activities in rose petals
- 4.2 Estimation of total anthocyanin content, phenolic content and chlorophyll content of rose leaves
- 4.3 Characterization of anthocyanin pigments of promising rose varieties using HPLC
- 4.4 Characterization of phenolic compounds of promising rose varieties using HPLC
- 4.5 *In vitro* induction of anthocyanin pigments in a promising rose variety

### 4.1 Estimation of total anthocyanin content, total phenol content and antioxidant activities in rose petals

#### 4.1.1. Total anthocyanin content

The total anthocyanin content of rose varieties in two consecutive growing seasons (December 2014 and March 2015) is presented in Table 4.1. Data presented showed a significant variation for anthocyanin content of all the varieties in both the seasons. Higher anthocyanin content was observed for all the varieties in December 2014 as compared to March 2015. The results presented in Table 4.1 indicated that there was a significant variation among the rose varieties for the total anthocyanins. The total anthocyanins ranged from  $0.24 \pm 0.02$  to  $578.10 \pm 6.76$  mg/100g on fresh weight basis. In the present study, rose variety Ashwini, a dark red coloured variety recorded highest anthocyanin content ( $578.10 \pm 6.76$  mg/100g fresh weight) followed by Dr. S.S. Bhatnagar ( $369.86 \pm 0.16$  mg/100g fresh weight) and Nehru Centenary ( $288.15 \pm 1.29$  mg/100g fresh weight). The minimum anthocyanin content was recorded in white coloured varieties *i.e.*, Iceberg, Mridula and Shabnam ( $0.24 \pm 0.02$  mg/100g,  $0.53 \pm 0.03$  mg/100g and  $1.38 \pm 0.13$  mg/100g fresh weight). Higher

anthocyanin content was observed in dark red flowers followed by deep pink, orange, pink, yellow and white (Table 4.1).

#### **4.1.2 Total phenolic content (TPC)**

The total phenolic content varied from  $5.21 \pm 0.39$  mg GAE/100g fresh weight to  $427.59 \pm 3.47$  mg GAE/100g fresh weight of petals among various varieties of rose taken into the study (Table 4.2). Variety Ashwini ( $427.59 \pm 3.47$  mg GAE/100g fresh weight) showed highest phenolic content followed by Dr. S.S. Bhatnagar ( $379.24 \pm 2.26$  mg GAE/100g fresh weight), Nehru Centenary ( $342.67 \pm 3.05$  mg GAE/100g fresh weight), Bhim ( $333.20 \pm 1.43$  mg GAE/100g fresh weight) and Pusa Arun ( $306.78 \pm 2.46$  mg GAE/100g fresh weight). Variety Iceberg showed lowest phenolic content ( $5.21 \pm 0.39$  mg GAE/100g fresh weight).

#### **4.1.3 Antioxidant activity**

##### **4.1.3.1 CUPRAC assay**

The CUPRAC values of the rose petals of different varieties are shown in Table 4.2. The CUPRAC values varied significantly among different varieties. The CUPRAC value among rose varieties ranged from  $13.93 \pm 0.55$   $\mu\text{mol trolox/g}$  fresh weight to  $512.71 \pm 2.01$   $\mu\text{mol trolox/g}$  fresh weight. The highest reducing power was observed in variety Ashwini ( $512.71 \pm 2.01$   $\mu\text{mol trolox/g}$  fresh weight) followed by Dr. S.S. Bhatnagar ( $432.76 \pm 1.96$   $\mu\text{mol trolox/g}$  fresh weight), Nehru Centenary ( $408.77 \pm 0.80$   $\mu\text{mol trolox/g}$  fresh weight), Pusa Arun ( $379.95 \pm 0.43$   $\mu\text{mol trolox/g}$  fresh weight) and Bhim ( $351.99 \pm 3.03$   $\mu\text{mol trolox/g}$  fresh weight). However, variety Iceberg exhibited lowest reducing power ( $13.93 \pm 0.55$   $\mu\text{mol trolox/g}$  fresh weight) which was statistically on par with Mridula ( $16.36 \pm 0.50$   $\mu\text{mol trolox/g}$  fresh weight).

##### **4.1.3.2 FRAP assay**

Highly significant variation for FRAP values were recorded among different varieties of rose (Table 4.2). The FRAP values among rose varieties ranged from  $8.92 \pm 0.36$   $\mu\text{mol trolox/g}$  fresh weight to  $397.15 \pm 0.82$   $\mu\text{mol trolox/g}$  fresh weight. The rose variety Iceberg recorded the lowest reducing power ( $8.92 \pm 0.36$   $\mu\text{mol trolox/g}$  fresh weight) which was on par with variety Mridula ( $11.77 \pm 0.47$   $\mu\text{mol trolox/g}$  fresh weight). The maximum reducing power was observed in Ashwini ( $397.15 \pm 0.82$   $\mu\text{mol trolox/g}$  fresh weight) followed by Dr. S.S. Bhatnagar ( $286.97 \pm 1.37$   $\mu\text{mol trolox/g}$  fresh weight), Nehru Centenary ( $268.31 \pm 1.82$   $\mu\text{mol trolox/g}$  fresh weight), Pusa Arun

**Table 4.1 Estimation of total anthocyanin content of rose varieties**

Varieties	Colour	Anthocyanin content in December 2014 (mg/100g)	Anthocyanin content in March 2015 (mg/100g)	Mean of both seasons (mg/100g)
Pusa Arun	Dark red	317.17±0.98	248.03±4.97	282.60±2.74
Raktima	Red	210.67±2.63	194.59±2.16	202.63±0.76
Nehru Centenary	Dark red	300.82±0.92	275.47±2.33	288.15±1.29
Pusa Bahadur	Red	134.40±1.54	115.34±1.30	124.87±0.65
Lalima	Red	116.85±0.86	107.33±0.78	112.09±0.53
Bhim	Red	246.96±0.90	225.43±1.49	236.20±0.40
Ashwini	Dark red	667.46±12.28	488.73±1.78	578.10±6.76
Dr. S.S. Bhatnagar	Dark red	401.24±0.92	338.48±1.19	369.86±0.16
Jantar Mantar	Red	123.43±0.85	116.70±0.99	120.07±0.88
Jawani	Red	75.44±0.51	67.30±1.64	71.37±0.58
Raktagandha	Red	188.78±0.56	176.37±0.87	182.58±0.71
Pusa Mohit	Pink	87.85±1.06	61.81±0.44	74.83±0.50
Pusa Ajay	Pink	40.23±0.22	35.13±1.38	37.68±0.80
Pusa Muskan	Colour blend (Cream with pink edges)	47.94±0.64	20.48±0.52	34.21±0.53
Pusa Barahmasi	Pink	110.96±1.31	64.89±1.86	87.92±1.27
Pusa Virangana	Red	235.22±4.41	125.78±0.99	180.50±2.60
Pusa Priya	Pink	12.25±0.77	9.26±0.19	10.76±0.47
Dr. Bharat Ram	Pink	7.45±0.46	5.27±0.61	6.36±0.47
Dr. M.S. Randhawa	Colour blend (White and pink)	14.87±0.56	13.43±0.48	14.15±0.06
Priyadarshini	Colour blend (White and pink)	14.58±1.04	9.96±0.53	12.27±0.69
Dulhan	Red	76.80±0.81	64.56±1.15	70.68±0.87
Dr. B.P. Pal	Pink	66.82±0.62	61.40±0.37	64.11±0.39
Anurag	Pink	27.48±0.72	19.49±0.48	23.49±0.16
Arjun	Pink	56.83±1.32	48.89±0.21	52.86±0.72
Haseena	Pink	82.57±1.01	74.74±1.45	78.66±0.25
R.S.S. of Nalagarh	Pink	44.76±0.63	40.51±0.35	42.63±0.49
Sadabahar	Pink	30.84±0.73	25.82±0.42	28.33±0.33
Nurjehan	Pink	29.21±0.92	26.24±0.31	27.73±0.35
Pink Montezuma	Pink	11.99±0.55	9.23±0.51	10.61±0.53

Surekha	Pink	21.01±1.25	18.26±0.60	19.64±0.63
Dr. Benjamin Pal	Pink	14.15±0.64	11.83±0.31	12.99±0.42
Pusa Ranjana	Deep pink	109.33±1.34	99.65±0.61	104.49±0.92
Arunima	Pink	28.20±0.90	22.83±1.05	25.52±0.33
Manasi	Pinkish white	3.88±0.16	2.73±0.08	3.31±0.07
Rose Sherbet	Deep pink	143.68±1.12	100.29±0.3	121.98±.68
Pusa Garima	Pink	50.66±1.29	41.97±0.98	46.32±1.12
Pusa Gaurav	Deep pink	102.99±1.90	92.23±1.09	97.61±1.40
Suryakiran	Orange	108.38±0.72	85.00±0.48	96.69±0.48
Krishna	Orange	32.29±0.29	28.28±0.69	30.29±0.21
Shola	Orange	55.94±0.69	51.59±1.13	53.76±0.55
Pusa Pitamber	Yellow	4.24±0.16	3.66±0.23	3.95±0.09
Lahar	Yellow	5.13±0.40	3.95±0.56	4.54±0.39
Ganga	Pale yellow	2.14±0.07	1.69±0.16	1.92±0.09
Raja Ram Mohan Roy	Yellow	4.33±0.32	3.29±0.20	3.81±0.26
Iceberg	White	0.27±0.02	0.21±0.03	0.24±0.02
Mridula	White	0.64±0.04	0.42±0.03	0.53±0.03
Shabnam	White	1.62±0.09	1.13±0.17	1.38±0.13
Chingari	Bicolored (Yellow+ red)	55.10±0.13	28.83±0.58	41.97±0.31
Surkhab	Bicolored (red+white)	85.14±0.91	79.19±1.36	82.17±0.99
Pusa Abhishek	Striped (Pink with white stripes)	66.29±0.75	62.10±1.11	64.20±0.85
SEm±		2.05	1.19	1.25
CD ( P≤ 0.05)		5.76	3.33	3.52

**Table 4.2 Estimation of antioxidant activities and phenolic content of rose varieties**

Varieties	CUPRAC ( $\mu\text{mol Trolox/g}$ )	FRAP ( $\mu\text{mol Trolox/g}$ )	DPPH (%)	Total phenolic content (mgGAE/100g)
Pusa Arun	379.95 $\pm$ 0.43	237.145 $\pm$ 0.84	87.43 $\pm$ 0.0.10 (69.20)	306.78 $\pm$ 2.46
Raktima	266.65 $\pm$ 1.59	192.1467 $\pm$ 1.38	83.88 $\pm$ 0.05 (66.30)	251.71 $\pm$ 1.56
Nehru Centenary	408.77 $\pm$ 0.80	268.31 $\pm$ 1.82	89.67 $\pm$ 0.16 (71.22)	342.67 $\pm$ 3.05
Pusa Bahadur	229.84 $\pm$ 0.62	178.08 $\pm$ 0.91	75.62 $\pm$ 0.18 (60.39)	219.11 $\pm$ 1.67
Lalima	214.39 $\pm$ 2.84	169.83 $\pm$ 0.79	71.58 $\pm$ 0.16 (57.76)	195.45 $\pm$ 3.79
Bhim	351.99 $\pm$ 3.03	216.89 $\pm$ 0.78	85.77 $\pm$ 0.14 (67.81)	333.20 $\pm$ 1.43
Ashwini	512.71 $\pm$ 2.01	397.15 $\pm$ 0.82	93.47 $\pm$ 0.19 (75.17)	427.59 $\pm$ 3.47
Dr. S.S. Bhatnagar	432.76 $\pm$ 1.96	286.97 $\pm$ 1.37	91.36 $\pm$ 0.05 (72.88)	379.24 $\pm$ 2.26
Jantar Mantar	215.59 $\pm$ 6.79	174.22 $\pm$ 1.09	72.62 $\pm$ 0.17 (58.43)	209.12 $\pm$ 0.86
Jawani	144.68 $\pm$ 0.99	102.27 $\pm$ 1.08	62.73 $\pm$ 0.08 (52.34)	132.38 $\pm$ 3.75
Raktagandha	274.71 $\pm$ 4.15	193.32 $\pm$ 4.31	81.80 $\pm$ 0.10 (64.72)	217.99 $\pm$ 3.84
Pusa Mohit	150.27 $\pm$ 0.34	106.22 $\pm$ 0.81	64.35 $\pm$ 0.16 (53.32)	124.21 $\pm$ 2.05
Pusa Ajay	121.67 $\pm$ 0.99	86.76 $\pm$ 1.70	35.71 $\pm$ 0.18 (36.68)	101.03 $\pm$ 2.32
Pusa Muskan	78.12 $\pm$ 0.46	64.68 $\pm$ 1.69	33.61 $\pm$ 0.17 (35.40)	67.18 $\pm$ 1.95
Pusa Barahmasi	152.06 $\pm$ 1.76	110.81 $\pm$ 0.96	66.44 $\pm$ 0.12(54.58)	116.45 $\pm$ 1.76
Pusa Virangana	234.52 $\pm$ 0.89	183.93 $\pm$ 1.28	80.67 $\pm$ 0.08(63.90)	201.22 $\pm$ 1.37
Pusa Priya	65.06 $\pm$ 0.34	51.11 $\pm$ 1.09	20.74 $\pm$ 0.05 (27.08)	44.77 $\pm$ 2.68
Dr. Bharat Ram	41.53 $\pm$ 0.50	29.59 $\pm$ 0.62	15.74 $\pm$ 0.16 (23.36)	21.77 $\pm$ 0.59
Dr. M.S. Randhawa	79.17 $\pm$ 0.54	65.37 $\pm$ 0.86	23.88 $\pm$ 0.00 (29.24)	55.78 $\pm$ 2.65
Priyadarshini	70.85 $\pm$ 0.46	61.23 $\pm$ 1.12	22.63 $\pm$ 0.10 (28.40)	55.17 $\pm$ 2.43
Dulhan	147.99 $\pm$ 1.17	114.32 $\pm$ 2.66	58.49 $\pm$ 0.09 (49.87)	124.10 $\pm$ 2.85
Dr. B.P. Pal	153.98 $\pm$ 0.67	116.07 $\pm$ 1.32	59.45 $\pm$ 0.10 (50.43)	128.45 $\pm$ 1.39
Anurag	86.13 $\pm$ 0.51	72.35 $\pm$ 0.71	28.52 $\pm$ 0.08 (32.27)	64.44 $\pm$ 0.90
Arjun	155.10 $\pm$ 0.86	121.78 $\pm$ 00.73	57.42 $\pm$ 0.06 (49.25)	131.06 $\pm$ 0.90
Haseena	194.42 $\pm$ 0.92	142.60 $\pm$ 1.18	60.41 $\pm$ 0.08 (50.99)	163.04 $\pm$ 1.96
R.S.S. of Nalagarh	148.07 $\pm$ 1.36	113.97 $\pm$ 1.99	46.41 $\pm$ 0.12 (42.93)	129.69 $\pm$ 2.15
Sadabahar	114.22 $\pm$ 0.22	96.05 $\pm$ 0.71	31.47 $\pm$ 0.13 (34.11)	94.56 $\pm$ 1.22
Nurjehan	111.26 $\pm$ 0.65	92.25 $\pm$ 0.54	30.69 $\pm$ 0.09 (33.63)	90.71 $\pm$ 0.61

Pink Montezuma	75.80±0.51	65.49±0.82	21.52±0.15 (27.63)	54.74±2.12
Surekha	90.16±0.58	77.31±0.59	25.86±0.05 (30.56)	70.68±0.54
Dr. Benjamin Pal	79.70±0.14	63.94±0.50	22.76±0.11(28.49)	61.76±1.78
Pusa Ranjana	228.18±1.38	179.70±1.92	70.47±0.12 (57.06)	199.61±0.65
Arunima	100.40±0.47	83.10±1.03	28.48±0.13 (32.24)	69.94±3.32
Manasi	30.88±0.25	21.92±0.89	10.74±0.10 (19.12)	12.21±0.97
Rose Sherbet	242.88±1.84	186.23±1.37	72.40±0.11 (58.28)	205.05±3.15
Pusa Garima	156.77±0.68	114.75±0.77	48.55±0.09 (44.15)	136.02±1.16
Pusa Gaurav	209.22±0.54	171.02±0.81	63.47±0.13 (52.79)	174.74±0.64
Suryakiran	204.34±0.88	167.93±0.89	62.31±0.11 (52.10)	153.48±2.08
Krishna	109.91±0.69	87.96±0.99	32.74±0.09 (34.89)	78.30±0.85
Shola	175.08±0.42	132.86±1.15	49.55±0.14 (44.73)	143.08±1.76
Pusa Pitamber	35.94±0.42	23.51±0.47	11.59±0.18 (19.89)	17.63±1.54
Lahar	40.02±0.25	27.47±0.62	12.77±0.05 (20.93)	21.92±0.86
Ganga	24.68±0.15	20.61±0.49	8.51±0.27 (16.95)	11.32±0.59
Raja Ram Mohan Roy	37.39±0.37	25.43±1.33	11.62±0.16 (19.92)	18.91±0.38
Iceberg	13.93±0.55	8.92±0.36	4.45±0.30 (12.17)	5.21±0.39
Mridula	16.36±0.50	11.77±0.47	5.36±0.32 (13.38)	7.53±0.79
Shabnam	22.14±0.13	17.81±1.04	8.23±0.09 (16.66)	11.11±0.93
Chingari	123.73±0.29	99.77±0.46	44.66±0.14 (41.92)	92.40±0.64
Surkhab	198.87±0.52	161.40±1.15	61.68±0.09 (51.73)	164.41±0.74
Pusa Abhishek	170.77±0.69	129.10±1.05	58.40±0.07 (49.82)	130.60±0.98
SEm±	1.52	1.26	0.14	1.96
CD ( P≤0.05)	4.26	3.54	0.38	5.50

\*Values in parenthesis are arc sin transformed values

**Table 4.3 Linear correlation coefficients (*r*) between total anthocyanin content, antioxidant assays (CUPRAC, FRAP, DPPH) and total phenolic content in petals of 50 rose varieties obtained by Pearson's analysis**

Parameters	TAC	CUPRAC	FRAP	DPPH	TPC
TAC	1	0.945**	0.934**	0.796**	0.932**
PC		1	0.988**	0.920**	0.994**
CUPRAC			1	0.921**	0.982**
FRAP				1	0.929**
DPPH					1

\*\* Correlation is significant at the 0.01 level (2-tailed)

**Table 4.4 Grouping of rose varieties based on anthocyanin content in petals**

Low anthocyanin content (0.20-40mg/100g)	Medium anthocyanin content (41-120mg/100g)	High anthocyanin content (121-580 mg/100g)
<ol style="list-style-type: none"> <li>1. Raja Ram Mohan Roy</li> <li>2. Shabnam</li> <li>3. Pink Montezuma</li> <li>4. Surekha</li> <li>5. Dr. Benjamin Pal</li> <li>6. Krishna</li> <li>7. Arunima</li> <li>8. Manasi</li> <li>9. Pusa Pitamber</li> <li>10. Lahar</li> <li>11. Ganga</li> <li>12. Pusa Priya</li> <li>13. Dr. Bharat Ram</li> <li>14. Dr. M.S. Randhawa</li> <li>15. Priyadarshini</li> <li>16. Anurag</li> <li>17. Sadabahar</li> <li>18. Nurjehan</li> <li>19. Iceberg</li> <li>20. Pusa Ajay</li> <li>21. Pusa Muskan</li> <li>22. Mridula</li> </ol>	<ol style="list-style-type: none"> <li>1. Chingari</li> <li>2. Surkhab</li> <li>3. Pusa Abhishek</li> <li>4. Pusa Ranjana</li> <li>5. Pusa Garima</li> <li>6. Pusa Gaurav</li> <li>7. Suryakiran</li> <li>8. Shola</li> <li>9. Pusa Barahmasi</li> <li>10. Dulhan</li> <li>11. Dr. B.P. Pal</li> <li>12. Arjun</li> <li>13. Haseena</li> <li>14. Raja Surendra Singh of Nalagarh</li> <li>15. Lalima</li> <li>16. Jantar Mantar</li> <li>17. Jawani</li> <li>18. Pusa Mohit</li> </ol>	<ol style="list-style-type: none"> <li>1. Pusa Virangana</li> <li>2. Rose Sherbet</li> <li>3. Pusa Arun</li> <li>4. Nehru Centenary</li> <li>5. Ashwini</li> <li>6. Dr. S.S. Bhatnagar</li> <li>7. Raktima</li> <li>8. Pusa Bahadur</li> <li>9. Bhim</li> <li>10. Raktagandha</li> </ol>

(237.15±0.84 µmol trolox/g fresh weight) and Bhim (216.89±0.78 µmol trolox/g fresh weight).

#### **4.1.3.3 DPPH radical scavenging activity**

DPPH scavenging activity of rose petals of various rose varieties is presented in Table 4.2. Significant variations for DPPH scavenging activity were observed among different varieties of rose. The DPPH value among rose varieties ranged from 4.45±0.30 to 93.47±0.19%. The highest DPPH radical scavenging was observed in the variety Ashwini (93.47±0.19%) followed by Dr. S.S. Bhatnagar (91.36±0.05%), Nehru Centenary (89.67±0.16%), Pusa Arun (87.43±0.10%) and Bhim (85.77±0.14%) whereas Iceberg exhibited lowest DPPH radical scavenging activity (4.45±0.30%). Jantar Mantar (72.62±0.17%) and Rose Sherbet (72.40±0.11%) were statistically on par. Jawani (62.73±0.08%) and Suryakiran (62.31±0.11%) were statistically on par. Dulhan (58.49±0.09%) and Pusa Abhishek (58.40±0.07%) were statistically on par.

#### **4.1.4 Correlation between total anthocyanin content, antioxidant activities and total phenolic content**

To identify the relationship between total anthocyanin content, antioxidant activity and total phenolic content, Pearson's correlation coefficient was computed. Pearson's correlation coefficient identified a statistically significant positive correlation between total anthocyanin content, antioxidant activity and total phenolic content of rose petals, as shown in Table 4.3. A strong and positive correlation was found between the total antioxidants (between the FRAP, CUPRAC and DPPH) value and total phenolic content as shown in the Table 4.3.

#### **Grouping of rose varieties based on their anthocyanin content**

On the basis of total anthocyanin content, rose varieties were divided into three groups (Table 4.4). The three groups were, low anthocyanin content (0.20-40 mg/100g fresh weight), medium anthocyanin content (41-120 mg/100g fresh weight) and high anthocyanin content (121-580 mg/100g fresh weight). Twenty two varieties namely Iceberg, Raja Ram Mohan Roy, Mridula, Shabnam, Pink Montezuma, Surekha, Dr. Benjamin Pal, Krishna, Arunima, Manasi, Pusa Pitamber, Lahar, Ganga, Pusa Priya, Dr. Bharat Ram, Dr. M.S. Randhawa, Priyadarshini, Anurag, Sadabahar, Nurjehan, Pusa Ajay and Pusa Muskan were present under first group. Medium anthocyanin content (41-120 mg/100g fresh weight) group contained Chingari,

Surkhab, Pusa Abhishek, Pusa Ranjana, Pusa Garima, Pusa Gaurav, Suryakiran, Shola, Pusa Barahmasi, Dulhan, Dr. B.P. Pal, Arjun, Haseena, Raja Surendra Singh of Nalagarh, Lalima, Jantar Mantar, Jawani and Pusa Mohit. Pusa Virangana, Rose Sherbet, Pusa Arun, Nehru Centenary, Ashwini, Dr. S.S. Bhatnagar, Raktima, Pusa Bahadur, Bhim and Raktagandha were present in high anthocyanin group (121-580 mg/100g fresh weight).

#### **4.2 Estimation of total anthocyanin content, total phenol and chlorophyll content of rose leaves**

The data presented in Table 4.5 showed that there were significant differences among the rose varieties for all the characters studied.

##### **4.2.1. Total anthocyanin content**

Among all the rose varieties the total anthocyanin content ranged from 20.94±1.04 to 242.53±4.61 mg/100g fresh weight of leaves. Variety Pusa Priya showed highest total anthocyanin content (242.53±4.61 mg/100g fresh weight) followed by Pusa Ajay (170.36±2.62 mg/100g fresh weight), Pusa Mohit (144.04±2.06 mg/100g fresh weight), Lalima (131.70±6.07 mg/100g fresh weight) and Krishna (129.44±3.28 mg/100g fresh weight). However, lowest total anthocyanin content was obtained by Shabnam, Iceberg and Manasi (20.94±1.04, 25.68±0.92 and 26.95±2.88 mg/100g fresh weight) respectively (Table 4.5).

##### **4.2.2 Total phenolic content (TPC)**

The total phenolic content (Table 4.5) varied from 4.96±0.46 mg GAE/100g fresh weight to 134.32±1.20 mg GAE/100g fresh weight of petals among different varieties of rose taken into the study. Variety Pusa Priya (134.32±1.20 mg GAE/100g fresh weight) showed highest phenolic content followed by Pusa Ajay (73.38±0.52 mg GAE/100g fresh weight), Pusa Mohit (61.40±0.69 mg GAE/100g fresh weight), Lalima (55.97±1.86 mg GAE/100g fresh weight) and Krishna (51.85±0.74 mg GAE/100g fresh weight). Shabnam exhibited lowest phenolic content (4.96±0.46 mg GAE/100g fresh weight).

##### **4.2.3 Total chlorophyll content**

Among the rose genotypes studied, the total chlorophyll content ranged from 0.25±0.01 mg/g to 1.45±0.01 mg/g fresh weight of leaves. Variety Shabnam showed

**Table 4.5 Estimation of total anthocyanin content and phenolic content of rose leaves**

Varieties	Anthocyanin content (mg/100g)	Total phenolic content (mgGAE/100g)	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll (mg/g)
Pusa Arun	69.01±2.30	17.11±1.71	0.69±0.02	0.18±0.02	0.87±0.00
Raktima	81.73±2.35	20.54±0.63	0.57±0.01	0.18±0.02	0.75±0.01
Nehru Centenary	28.05±2.14	7.82±0.97	0.86±0.02	0.16±0.01	1.02±0.02
Pusa Bahadur	31.66±0.46	8.89±0.39	0.86±0.01	0.17±0.01	1.03±0.01
Lalima	131.70±6.07	55.97±1.86	0.32±0.01	0.11±0.01	0.43±0.01
Bhim	30.47±4.33	10.80±0.32	0.85±0.01	0.14±0.01	0.99±0.01
Ashwini	88.68±2.50	26.57±0.49	0.63±0.01	0.13±0.00	0.75±0.00
Dr. S.S. Bhatnagar	121.66±2.08	51.65±0.58	0.32±0.01	0.13±0.01	0.45±0.01
Jantar Mantar	71.03±1.10	22.55±0.58	0.66±0.00	0.19±0.01	0.84±0.01
Jawani	62.33±0.97	19.22±0.33	0.62±0.02	0.16±0.01	0.78±0.01
Raktagandha	27.22±2.82	7.85±0.71	0.86±0.01	0.19±0.01	1.05±0.01
Pusa Mohit	144.04±2.06	61.40±0.69	0.30±0.01	0.10±0.00	0.40±0.01
Pusa Ajay	170.36±2.62	73.38±0.52	0.23±0.01	0.11±0.01	0.34±0.01
Pusa Muskan	94.31±3.69	26.11±0.87	0.60±0.02	0.16±0.01	0.76±0.01
Pusa Barahmasi	28.58±0.64	7.81±0.96	0.81±0.00	0.15±0.01	0.96±0.01
Pusa Virangana	72.25±3.49	20.83±0.65	0.53±0.02	0.16±0.01	0.69±0.01
Pusa Priya	242.53±4.61	134.32±1.20	0.15±0.00	0.10±0.01	0.25±0.01
Dr. Bharat Ram	43.95±1.39	10.43±0.60	0.82±0.00	0.14±0.02	0.96±0.02
Dr. M.S. Randhawa	59.76±5.40	18.46±0.57	0.50±0.01	0.17±0.00	0.67±0.01
Priyadarshini	65.28±4.16	15.31±0.89	0.69±0.01	0.16±0.01	0.85±0.01
Dulhan	38.93±2.04	8.65±0.48	0.78±0.01	0.17±0.00	0.95±0.01
Dr. B.P. Pal	39.82±0.27	8.65±0.46	0.77±0.01	0.17±0.02	0.95±0.02
Anurag	27.51±1.01	7.21±0.24	0.80±0.01	0.16±0.01	0.96±0.02
Arjun	57.40±3.44	14.58±0.58	0.53±0.02	0.15±0.01	0.68±0.01
Haseena	44.98±2.38	10.34±0.72	0.57±0.02	0.17±0.01	0.74±0.01
R.S.S. of Nalagarh	55.44±1.44	14.91±0.92	0.51±0.01	0.16±0.02	0.66±0.01
Sadabahar	44.77±2.73	9.84±1.22	0.69±0.00	0.17±0.01	0.86±0.01
Nurjehan	49.19±0.17	14.49±1.54	0.71±0.01	0.13±0.01	0.84±0.00
Pink Montezuma	48.64±2.12	19.55±1.06	0.52±0.01	0.17±0.01	0.69±0.00
Surekha	126.92±7.09	50.56±0.49	0.24±0.01	0.14±0.00	0.38±0.00

Dr. Benjamin Pal	107.60±3.24	44.02±0.92	0.20±0.01	0.16±0.02	0.36±0.01
Pusa Ranjana	59.82±4.93	20.69±0.57	0.51±0.01	0.16±0.02	0.67±0.01
Arunima	30.63±1.06	8.09±1.16	0.90±0.00	0.16±0.00	1.06±0.01
Manasi	26.95±2.88	5.84±0.64	0.96±0.00	0.15±0.00	1.10±0.01
Rose Sherbet	29.39±3.60	7.75±1.00	0.98±0.01	0.15±0.01	1.13±0.01
Pusa Garima	52.39±2.86	18.25±1.13	0.52±0.02	0.16±0.02	0.68±0.01
Pusa Gaurav	100.67±1.53	41.42±0.88	0.24±0.01	0.16±0.00	0.40±0.01
Suryakiran	89.66±9.34	29.55±3.22	0.55±0.01	0.16±0.00	0.71±0.01
Krishna	129.44±3.28	51.85±0.74	0.20±0.01	0.10±0.00	0.31±0.01
Shola	68.69±4.32	17.27±0.70	0.49±0.02	0.15±0.01	0.63±0.01
Pusa Pitamber	43.28±0.57	17.81±2.38	0.72±0.01	0.14±0.00	0.87±0.00
Lahar	68.22±2.42	22.60±1.30	0.54±0.00	0.17±0.00	0.70±0.01
Ganga	49.01±2.04	13.87±0.85	0.69±0.01	0.15±0.01	0.84±0.01
Raja Ram Mohan Roy	94.92±0.94	40.85±0.90	0.26±0.01	0.16±0.00	0.42±0.01
Iceberg	25.68±0.92	5.74±0.71	1.16±0.01	0.21±0.00	1.35±0.00
Mridula	29.05±0.75	8.73±0.55	1.16±0.02	0.15±0.01	1.30±0.01
Shabnam	20.94±1.04	4.96±0.46	1.22±0.01	0.23±0.01	1.45±0.01
Chingari	31.17±0.90	10.43±0.34	1.11±0.01	0.12±0.01	1.23±0.01
Surkhab	99.62±2.09	35.32±1.21	0.28±0.00	0.14±0.01	0.42±0.01
Pusa Abhishek	71.70±3.62	18.51±1.57	0.57±0.01	0.13±0.01	0.70±0.01
SEm±	3.17	1.04	0.01	0.01	0.01
CD ( P≤ 0.05)	8.89	2.92	0.03	0.03	0.03

**Table 4.6. Linear correlation coefficients (*r*) between total anthocyanin content, total phenolic content (TPC), chlorophyll a content, total chlorophyll b content and total chlorophyll content in leaves of 50 rose varieties obtained by Pearson's analysis.**

<b>Parameters</b>	<b>TAC</b>	<b>TPC</b>	<b>CHL.a</b>	<b>CHL.b</b>	<b>T.CHL</b>
<b>TAC</b>	1	0.971**	-0.821**	-0.604**	-0.838**
<b>TPC</b>		1	-0.748**	-0.606**	-0.768**
<b>CHL.a</b>			1	0.492**	0.997**
<b>CHL.b</b>				1	0.557**
<b>T.CHL</b>					1

\*\* Correlation is significant at the 0.01 level (2-tailed).

highest total chlorophyll ( $1.45 \pm 0.01$  mg/g) followed by Iceberg ( $1.35 \pm 0.00$  mg/g), Mridula ( $1.30 \pm 0.01$  mg/g), Chingari ( $1.23 \pm 0.01$  mg/g) and Rose Sherbet ( $1.13 \pm 0.01$  mg/g). However, lowest total chlorophyll was obtained in variety Pusa Priya ( $0.25 \pm 0.01$  mg/g) and Krishna ( $0.31 \pm 0.01$  mg/g) fresh weight of leaves (Table 4.5).

#### **4.2.4 Correlation between pigments and phenolic content of rose leaves**

To identify the relationship between total anthocyanin content, phenolic content and chlorophyll content, Pearson's correlation coefficient was computed. Pearson's correlation coefficient identified a statistically significant positive correlation ( $r = 0.971$ ) between total anthocyanin content and phenolic content of rose leaves, as shown in Table 4.6. Chlorophyll a, chlorophyll b and total chlorophyll content in leaves showed a statistically significant negative correlation with total anthocyanin content. Total phenol content also exhibited a statistically significant negative correlation with chlorophyll a, chlorophyll b and total chlorophyll content in leaves.

### **4.3 Characterization of anthocyanin pigments of promising rose varieties using HPLC (High Performance Liquid Chromatography)**

High performance liquid chromatography (HPLC) is basically a highly improved form of column liquid chromatography. All chromatographic separations, including HPLC operate under the same basic principle; separation of a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation. It pumps a sample mixture or analyte in a solvent (known as the mobile phase) at high pressure through a column with chromatographic packing material (stationary phase). The time taken for a particular compound to travel through the column to the detector is known as its retention time. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound. Different compounds have different retention times.

Rose varieties which recorded highest total anthocyanin content were selected for HPLC analysis of anthocyanin pigments. HPLC chromatogram indicated several peaks separated base to base except one or two. All the peaks belong to anthocyanin group that can be seen from the UV-Vis spectrum of each compound. Anthocyanins identification was conducted by comparing RT (retention time) and elution order of

sample with those of standards under the same HPLC conditions. Cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-O-glucoside standards were used to identify the anthocyanin compounds. Several peaks were detected in all varieties corresponding to different kinds of anthocyanin fractions. Two major anthocyanins were identified with the help of external standards i.e. cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-O-glucoside. As standards of all anthocyanins were not available, so cyanidin-3-glucoside (C3G) was taken as standard to quantify the unknown peaks, which were expressed in terms of C3G equivalents. The identification of anthocyanins was mainly conducted by comparing RT (retention time) and elution order of sample with those of standards under the same HPLC conditions.

The total anthocyanin content estimated by HPLC ranged from 144.08 mg/100g to 558.81 mg/100g on fresh weight basis (Table 4.7 to Table 4.19). Total anthocyanin content was highest in variety Ashwini (558.81 mg/100g) followed by Pusa Arun (527.91 mg/100g), Nehru Centenary (497.28 mg/100g), Raktagandha (484.18 mg/100g) and Dr. S.S. Bhatnagar (400.20 mg/100g). Lowest anthocyanin content was found in Pusa Bahadur (144.08 mg/100g). In all the red and pink coloured varieties cyanidin 3,5-di-O-glucoside was predominant anthocyanin, whereas in orange colour variety pelargonidin 3,5-di-O-glucoside was predominant anthocyanin. Among all the varieties cyanidin 3,5-di-O-glucoside content ranged from 29.28 to 497.79 mg/100g, whereas pelargonidin 3,5-di-O-glucoside content among all the varieties varied from 0.39 to 185.43 mg/100g. Maximum cyanidin 3,5-di-O-glucoside content was recorded in Ashwini (497.79 mg/100g), whereas minimum cyanidin 3,5-di-O-glucoside content was recorded in Suryakiran (29.28 mg/100g). Similarly, maximum pelargonidin 3,5-di-O-glucoside content was recorded in Suryakiran (185.43 mg/100g), whereas minimum pelargonidin 3,5-di-O-glucoside content was recorded in Pusa Bahadur (0.39 mg/100g).

In the HPLC analysis, individual content of different anthocyanin fractions corresponding to different peaks was estimated. Figure 4.1 represented the HPLC chromatogram of anthocyanins from variety Ashwini. Totally, five kinds of anthocyanins were detected in variety Ashwini. Peak 1 and peak 2 had RT (Retention time) of 17.621 and 19.715 min, respectively, which was in coincidence with standard 1 (cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside) as revealed in Figure 3.3 and 3.4. In the chromatogram of Ashwini peak 1 corresponded

to cyanidin 3,5-di-O-glucoside (497.79 mg/100g), peak 2 corresponded to pelargonidin 3,5-di-O-glucoside (7.60 mg/100g), peak 3, 4 and 5 corresponded to unknown anthocyanins which were expressed as C3G equivalent (17.24, 32.42 and 3.76 mg/100g respectively). Cyanidin 3,5-di-O-glucoside was the predominant constituent which accounted for 89.38% of total anthocyanins according to the analysis results of high performance liquid chromatography-photodiode array detection (Table 4.7).

Figure 4.2 represented the HPLC chromatogram of anthocyanins from variety Pusa Arun. As shown, five anthocyanins were revealed with peak 1 representing about 90.24% of the total peak area according to normalization method. Peak 1 and peak 2 had RT of 18.068 and 19.994 min, respectively, which was in coincidence with standard 1 (Cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside). In the chromatogram of Pusa Arun peak 1 represented cyanidin 3,5-di-O-glucoside (448.54 mg/100g), peak 2 represented pelargonidin 3,5-di-O-glucoside (61.75 mg/100g), peak 3, 4 and 5 corresponded to unknown anthocyanins which were expressed as C3G equivalent (5.88, 10.71 and 1.02 mg/100g respectively). Cyanidin 3,5-di-O-glucoside was the predominant constituent (Table 4.8).

Totally, four kinds of anthocyanins were observed in HPLC analysis of variety Nehru Centenary (Figure 4.3 and Table 4.9). Peak 1 and peak 2 had RT of 17.623 and 19.626 min, respectively, which was in coincidence with standard 1 (cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside). Maximum peak area was found under peak 1 (91.42 %). In the chromatogram of variety Nehru Centenary peak 1 corresponded to cyanidin 3,5-di-O-glucoside (452.12 mg/100g), peak 2 corresponded to pelargonidin 3,5-di-O-glucoside (5.43 mg/100g), peak 3 and 4 corresponded to unknown anthocyanins which were expressed as C3G equivalent (23.10 and 15.74 mg/100g respectively).

From the chromatogram of variety Raktagandha (Figure 4.4 and Table 4.10) five anthocyanins were revealed with peak 1 representing 94.65% of the total peak area. Peak 1 and peak 2 had RT of 17.646 and 19.596 min, respectively, which was in coincidence with standard 1 (cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside). Peak 1 represented cyanidin 3,5-di-O-glucoside (457.66 mg/100g) which was predominant anthocyanin in variety Raktagandha. Peak 2 corresponded to pelargonidin 3,5-di-O-glucoside (1.31 mg/100g), peak 3, 4 and 5

corresponded to unknown anthocyanins which were expressed as C3G equivalent (1.31, 7.48 and 16.42 mg/100g respectively).

Totally, four kinds of anthocyanins were observed in HPLC analysis of variety Raktima (Figure 4.5 and Table 4.11). Peak 1 and peak 2 had RT of 17.639 and 19.628 min, respectively, which was in coincidence with standard 1 (cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside). Maximum peak area was found under peak 2 (89.20 %) which represented cyanidin 3,5-di-O-glucoside (338.91 mg/100g), peak 3 corresponded to pelargonidin 3,5-di-O-glucoside (10.66 mg/100g), peak 3 and 4 corresponded to unknown anthocyanins which were expressed as C3G equivalent (17.33 and 13.77 mg/100g respectively).

From the chromatogram of variety Suryakiran (Figure 4.6 and Table 4.12) six anthocyanins were revealed with peak 2 representing 60.83% of the total peak area. Peak 1 and peak 2 had RT of 17.649 and 19.585 min, respectively, which was in coincidence with standard 1 (cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside). Peak 1 represents cyanidin 3,5-di-O-glucoside (29.28 mg/100g). Peak 2 corresponded to pelargonidin 3,5-di-O-glucoside (185.43 mg/100g) which was predominant anthocyanin in Suryakiran. Peak 3, 4, 5 and 6 corresponded to unknown anthocyanins which were expressed as C3G equivalent (11.60, 1.22, 11.56 and 6.02 mg/100g respectively).

Chromatogram of variety Pusa Bahadur revealed four types of anthocyanin (Figure 4.7 and Table 4.13). Peak 1 and peak 2 had RT of 17.620 and 19.561 min, respectively, which was in coincidence with standard 1 (cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside). Peak 1 corresponded to cyanidin 3,5-di-O-glucoside (137.59 mg/100g) which represented 95.62% of the total peak area. Peak 2 represented to pelargonidin 3,5-di-O-glucoside (0.39 mg/100g). Peak 3 and 4 corresponded to unknown anthocyanins which were expressed as C3G equivalent (2.14 and 3.97 mg/100g respectively).

Totally, four kinds of anthocyanins were observed in HPLC analysis of variety Bhim (Figure 4.8 and Table 4.14). Peak 1 and peak 2 had RT of 17.623 and 19.625 min, respectively, which was in coincidence with standard 1 (cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside). In the chromatogram of Bhim peak 1 corresponded to cyanidin 3,5-di-O-glucoside (175.80 mg/100g) which

occupied 96.72% of total peak area. Peak 2 corresponded to pelargonidin 3,5-di-O-glucoside (2.31 mg/100g), peak 3 and 4 corresponded to unknown anthocyanins which were expressed as C3G equivalent (1.50 and 3.32 mg/100g respectively).

From the chromatogram of variety Dr. S.S. Bhatnagar (Figure 4.9 and Table 4.15) four anthocyanins were revealed with peak 1 representing 90.78 % of the total peak area. Peak 1 and peak 2 had RT of 17.584 and 19.744 min, respectively, which was in coincidence with standard 1 (cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside). Peak 1 represented cyanidin 3,5-di-O-glucoside (362.40 mg/100g), Peak 2 corresponded to pelargonidin 3,5-di-O-glucoside (1.98 mg/100g). Peak 3 and 4 corresponded to unknown anthocyanins which were expressed as C3G equivalent (21.91 and 13.90 mg/100g respectively).

Chromatogram of variety Rose Sherbet revealed five types of anthocyanin (Figure 4.10 and Table 4.16). Peak 1 and peak 2 had RT of 17.574 and 19.565 min, respectively, which was in coincidence with standard 1 (cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside). Peak 1 corresponded to cyanidin 3,5-di-O-glucoside (206.57 mg/100g) which was predominant anthocyanin in Rose Sherbet and represented 86.35% of the total peak area. Peak 2 represented pelargonidin 3,5-di-O-glucoside (0.93 mg/100g). Peak 3, 4 and 5 correspond to unknown anthocyanins which were expressed as C3G equivalent (15.75, 9.08 and 7.36 mg/100g respectively).

Figure 4.11 represented the HPLC chromatogram of anthocyanins from variety Pusa Virangana. As shown in Table 4.17, four anthocyanins were revealed with peak 1 representing about 96.04 % of the total peak area. Peak 1 and peak 2 had RT of 17.566 and 19.541 min, respectively, which was in coincidence with standard 1 (cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside). In the chromatogram of Pusa Virangana peak 1 corresponded to cyanidin 3,5-di-O-glucoside (301.66 mg/100g) which was predominant anthocyanin in Pusa Virangana. Peak 2 corresponded to pelargonidin 3,5-di-O-glucoside (1.30 mg/100g), peak 3 and 4 represented unknown anthocyanin which was expressed as C3G equivalent (2.34 and 9.45 mg/100g respectively).

Totally, four kinds of anthocyanins were observed in HPLC analysis of variety Surkhab (Figure 4.12 and Table 4.18). Peak 1 and peak 2 had RT of 17.955 and

20.079 min, respectively, which was in coincidence with standard 1 (cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside). In the chromatogram of Surkhab peak 1 corresponded to cyanidin 3,5-di-O-glucoside (173.60 mg/100g) which occupied 93.63% of total peak area. Peak 2 corresponded to pelargonidin 3,5-di-O-glucoside (1.58 mg/100g), peak 3 and 4 corresponded to unknown anthocyanins which were expressed as C3G equivalent (2.99 and 8.03 mg/100g respectively).

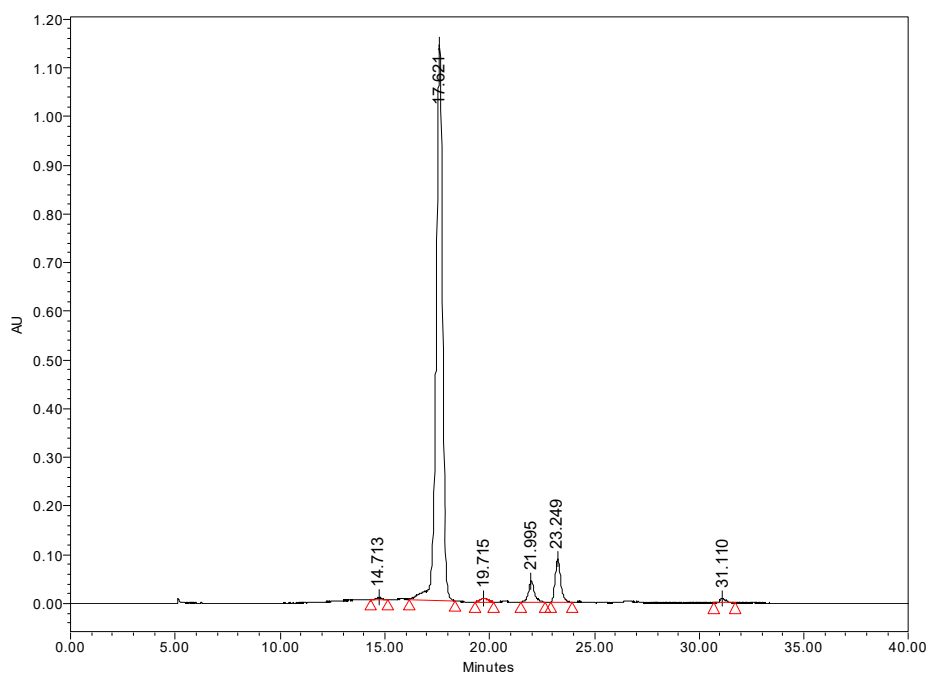
Chromatogram of variety Pusa Ajay revealed four types of anthocyanin (Figure 4.13). Peak 1 and peak 2 had RT of 17.979 and 20.122 min, respectively, which was in coincidence with standard 1 (cyanidin 3,5-di-O-glucoside) and standard 2 (pelargonidin 3,5-di-O-glucoside). Peak 1 corresponded to cyanidin 3,5-di-O-glucoside (190.05 mg/100g) which represented 94.08 % of the total peak area. Peak 2 represented pelargonidin 3,5-di-O-glucoside (2.13 mg/100g). Peak 3, 4 corresponded to unknown anthocyanins which were expressed as C3G equivalent (2.58 and 8.31 mg/100g respectively). Cyanidin 3,5-di-O-glucoside was predominant anthocyanin in Pusa Ajay (Table 4.19).

#### **4.4 Characterization of phenolic compounds of promising rose varieties using HPLC**

HPLC chromatogram indicated several peaks; all the peaks belong to phenolic group that can be seen from the UV-Vis spectrum of each compound. Figure 4.14 illustrated the phenolic separation by HPLC in different rose varieties. Characteristic peaks show the typical absorbance of phenols. Phenolic identification was conducted by comparing RT (retention time) and elution order of sample with those of standards under the same HPLC conditions. Catechin, Epicatechin, Rutin, Quercetin and 3-hydroxy cinnamic acid standards were used to identify the phenolic compounds. Several peaks were detected in all varieties corresponding to different kinds of phenolic fractions. Five types of phenolic compounds were identified with the help of external standards (Table 4.21).

From the Tables 4.21 it is evident that quercetin was present in all the varieties. Quercetin content ranged from 2.38 to 603.88 mg/100g. Maximum quercetin content was found in variety Nehru Centenary (603.88 mg/100g) followed by Surkhab (139.00 mg/100g), Raktagandha (130.89 mg/100g) and Pusa Bahadur (86.91

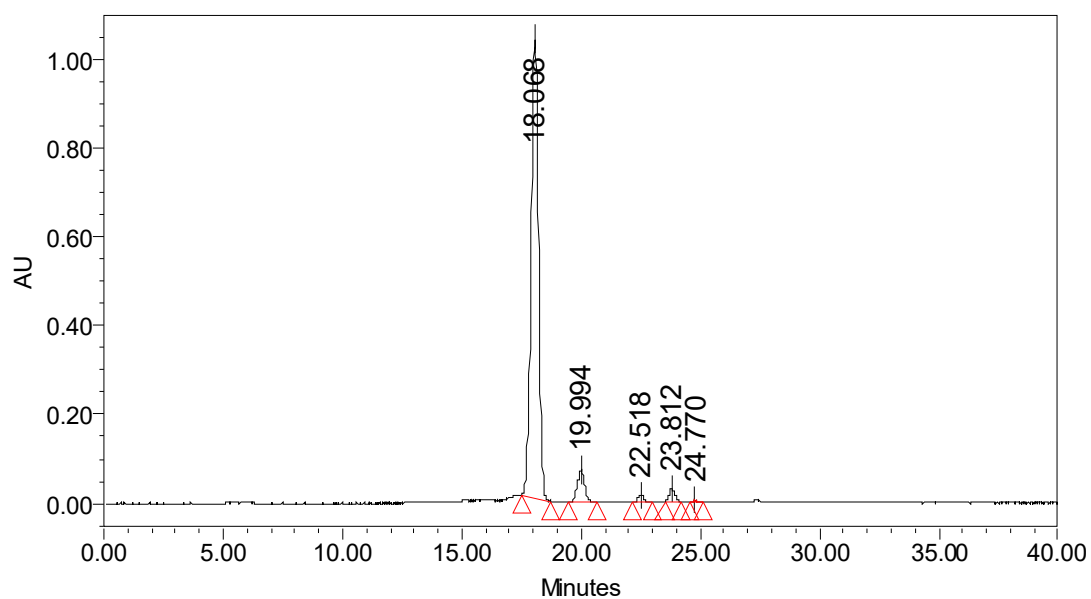
**Figure 4.1 Chromatogram of anthocyanin pigments of variety Ashwini**



**Table 4.7 Chromatographic and spectral characteristics of anthocyanins in variety Ashwini**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	17.621	89.38	Cyanidin 3,5-di-O-glucoside	497.79
2.	19.715	0.68	Pelargonidin 3,5-di-O-glucoside	7.60
3.	21.995	3.10	Unknown	17.24
4.	23.249	5.82	Unknown	32.42
5.	31.110	0.68	Unknown	3.76
Total				558.81

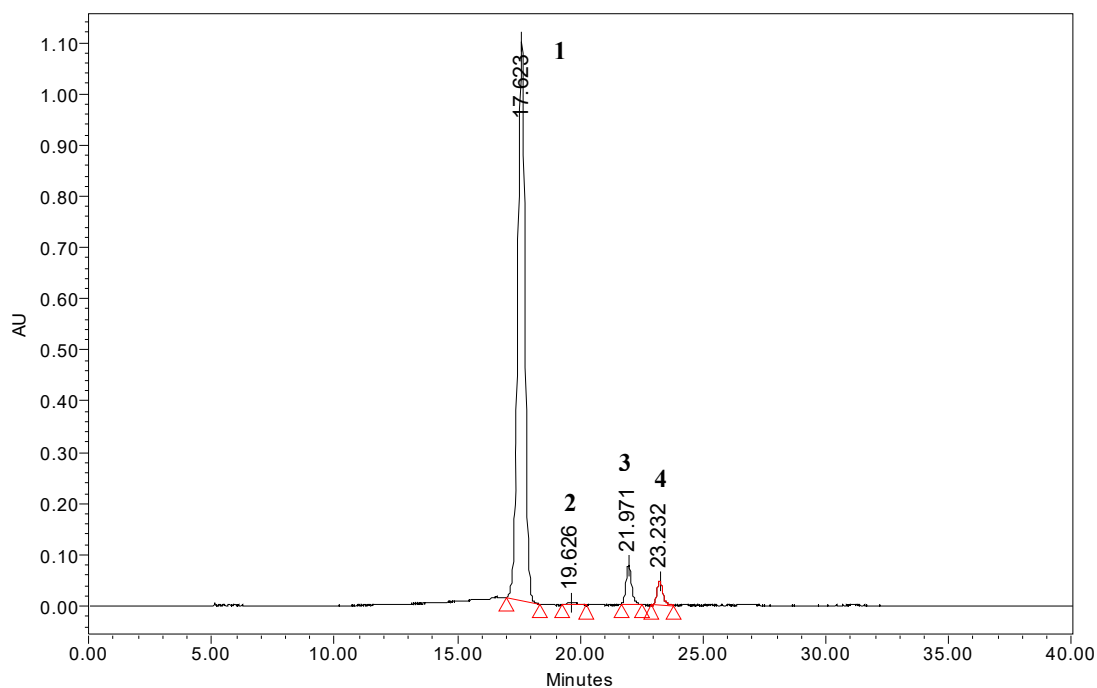
**Figure 4.2 Chromatogram of anthocyanin pigments of variety Pusa Arun**



**Table 4.8 Chromatographic and spectral characteristics of anthocyanins in variety Pusa Arun**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	18.068	90.24	Cyanidin 3,5-di-O-glucoside	448.54
2.	19.994	6.21	Pelargonidin 3,5-di-O-glucoside	61.75
3.	22.518	1.18	Unknown	5.88
4.	23.812	2.16	Unknown	10.71
5.	24.770	0.20	Unknown	1.02
Total				527.91

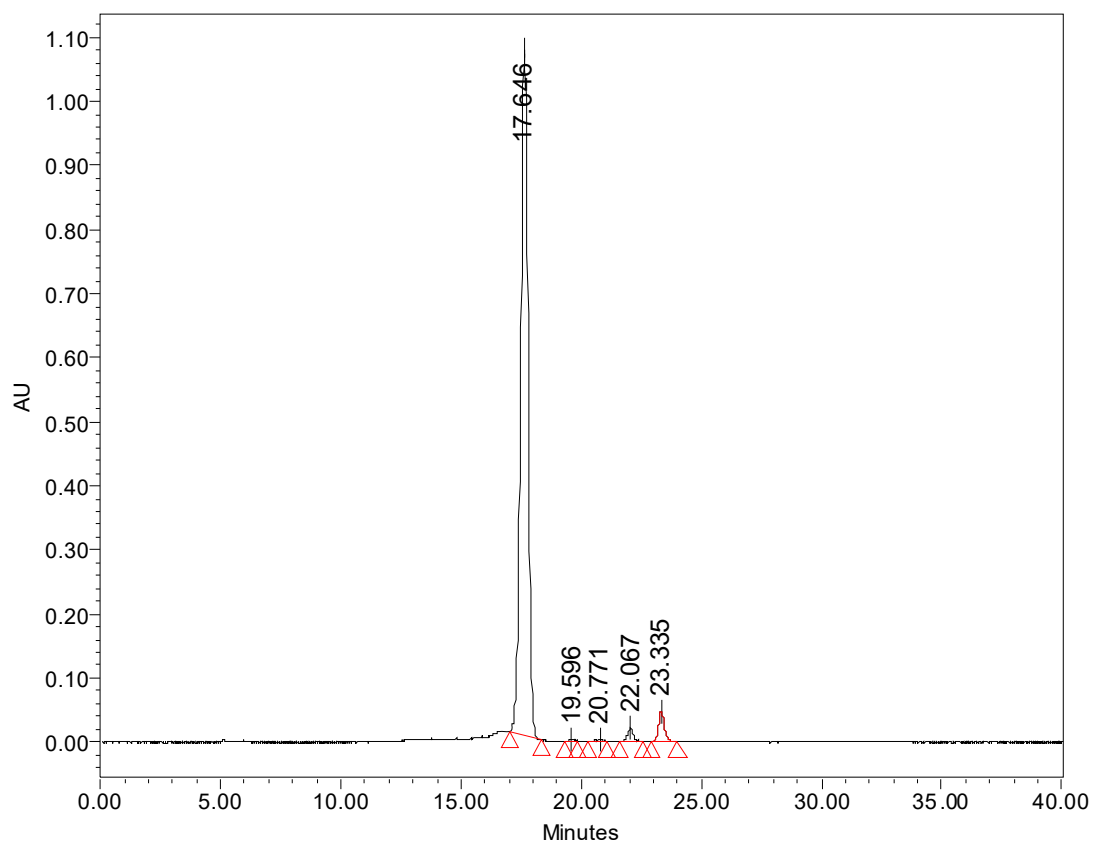
**Figure 4.3 Chromatogram of anthocyanin pigments of variety Nehru Centenary**



**Table 4.9 Chromatographic and spectral characteristics of anthocyanins in variety Nehru Centenary**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	17.623	91.42	Cyanidin 3,5-di-O-glucoside	452.12
2.	19.626	0.55	Pelargonidin 3,5-di-O-glucoside	5.43
3.	21.971	4.85	Unknown	23.10
4.	23.232	3.18	Unknown	15.74
Total				497.28

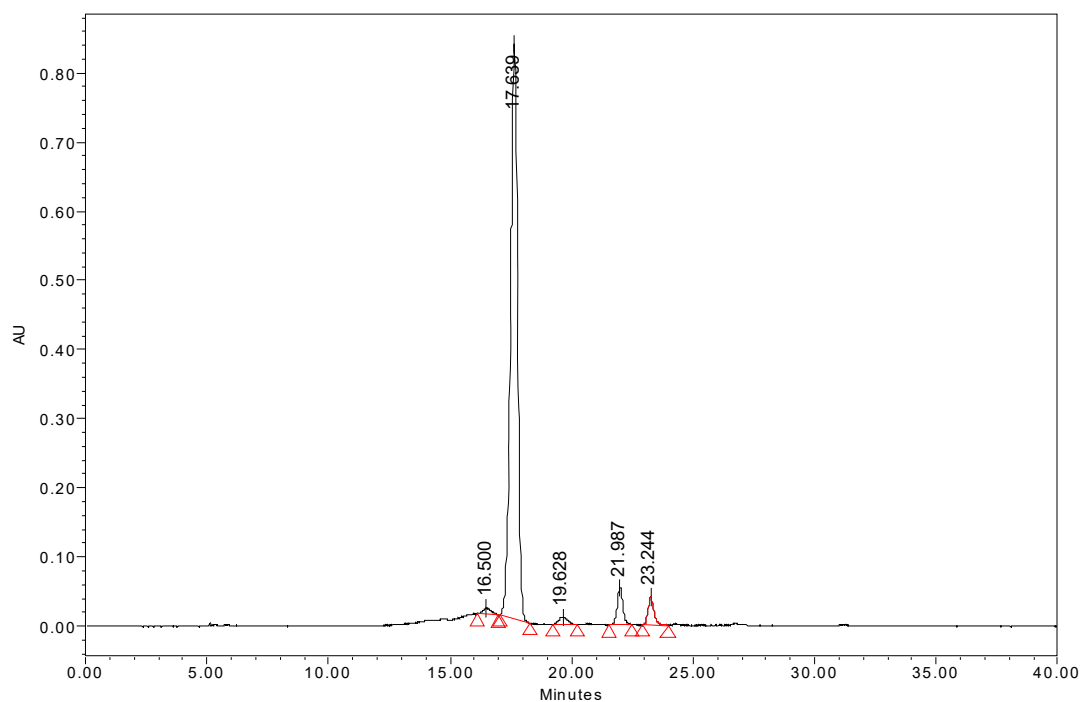
**Figure 4.4 Chromatogram of anthocyanin pigments of variety Raktagandha**



**Table 4.10 Chromatographic and spectral characteristics of anthocyanins in variety Raktagandha**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	17.646	94.65	Cyanidin 3,5-di-O-glucoside	457.66
2.	19.596	0.14	Pelargonidin 3,5-di-O-glucoside	1.31
3.	20.771	0.27	Unknown	1.31
4.	22.067	1.55	Unknown	7.48
5.	23.335	3.40	Unknown	16.42
Total				484.18

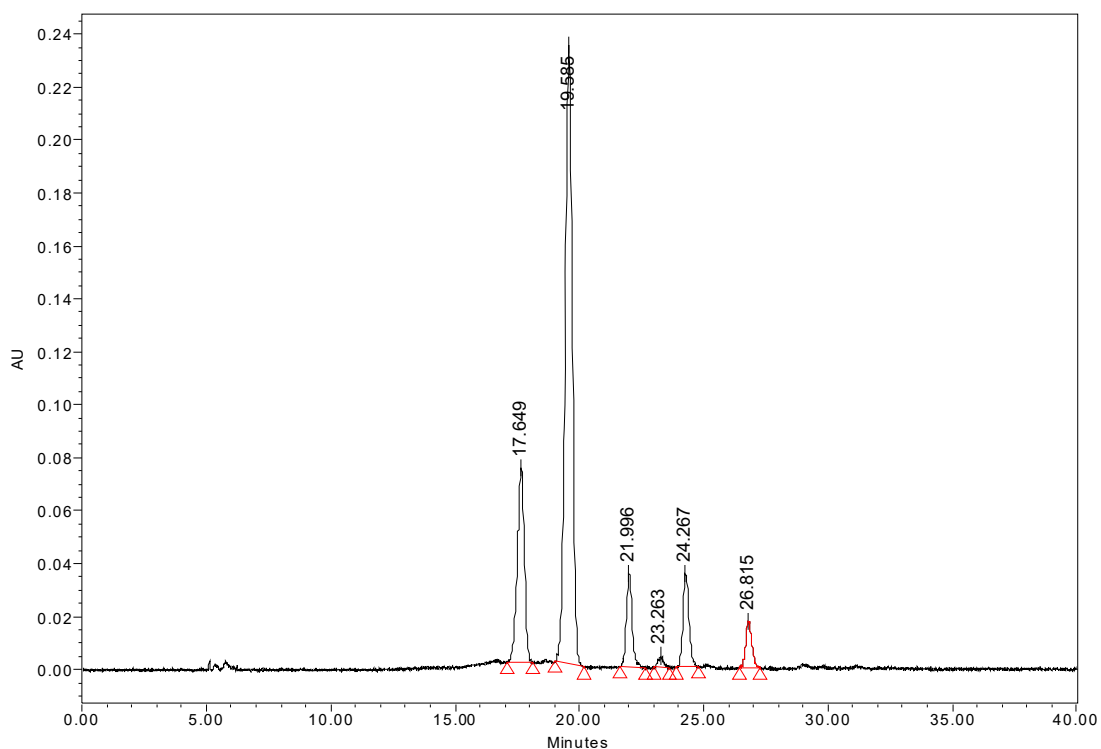
**Figure 4.5 Chromatogram of anthocyanin pigments of variety Raktima**



**Table 4.11 Chromatographic and spectral characteristics of anthocyanins in variety Raktima**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	17.639	89.20	Cyanidin 3,5-di-O-glucoside	338.91
2.	19.628	1.40	Pelargonidin 3,5-di-O-glucoside	10.66
3.	21.987	4.56	unknown	17.33
4.	23.244	3.62	unknown	13.77
Total				380.68

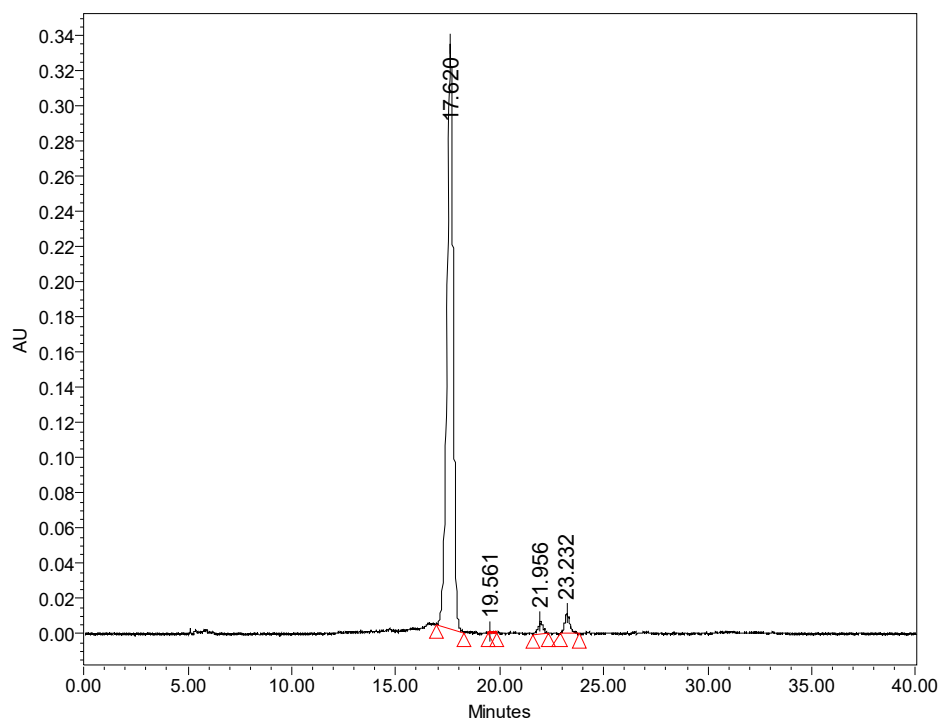
**Figure 4.6 Chromatogram of anthocyanin pigments of variety Suryakiran**



**Table 4.12 Chromatographic and spectral characteristics of anthocyanins in variety Suryakiran**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	17.649	19.21	Cyanidin 3,5-di-O-glucoside	29.28
2.	19.585	60.83	Pelargonidin 3,5-di-O-glucoside	185.43
3.	21.996	7.61	Unknown	11.60
4.	23.263	0.80	Unknown	1.22
5.	24.267	7.59	Unknown	11.56
6.	26.815	3.95	Unknown	6.02
Total				245.13

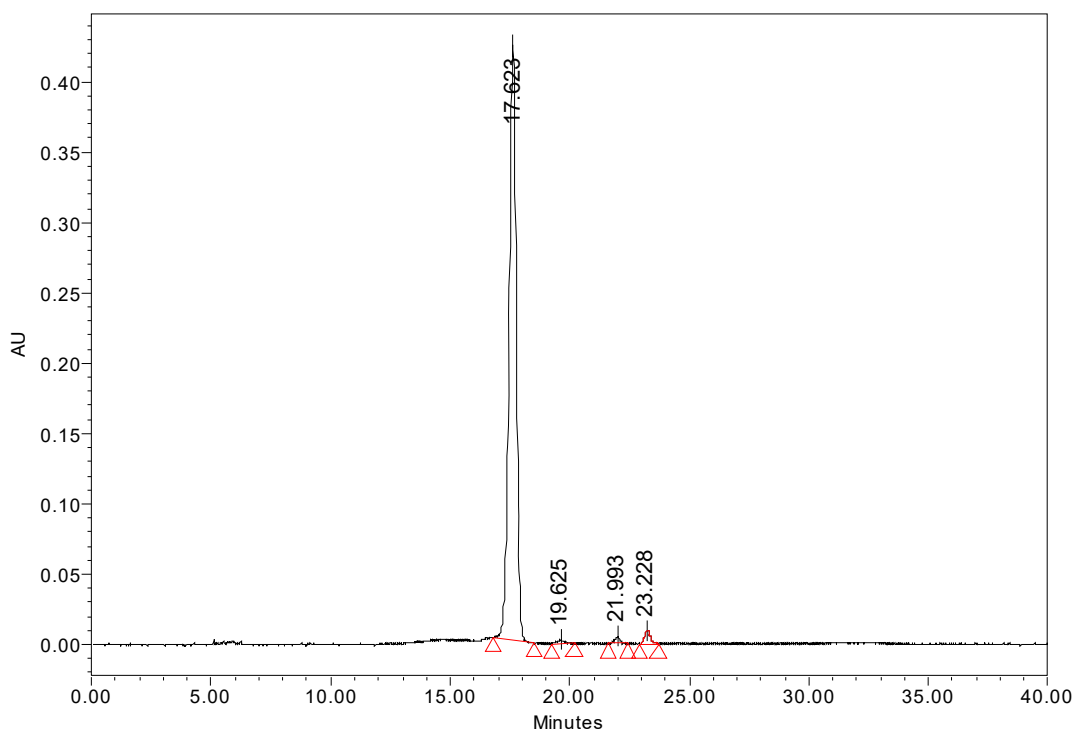
**Figure 4.7 Chromatogram of anthocyanin pigments of variety Pusa Bahadur**



**Table 4.13 Chromatographic and spectral characteristics of anthocyanins in variety Pusa Bahadur**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	17.620	95.62	Cyanidin 3,5-di-O-glucoside	137.59
2.	19.561	0.13	Pelargonidin 3,5-di-O-glucoside	0.39
3.	21.956	1.48	Unknown	2.14
4.	23.232	2.76	Unknown	3.97
Total				144.09

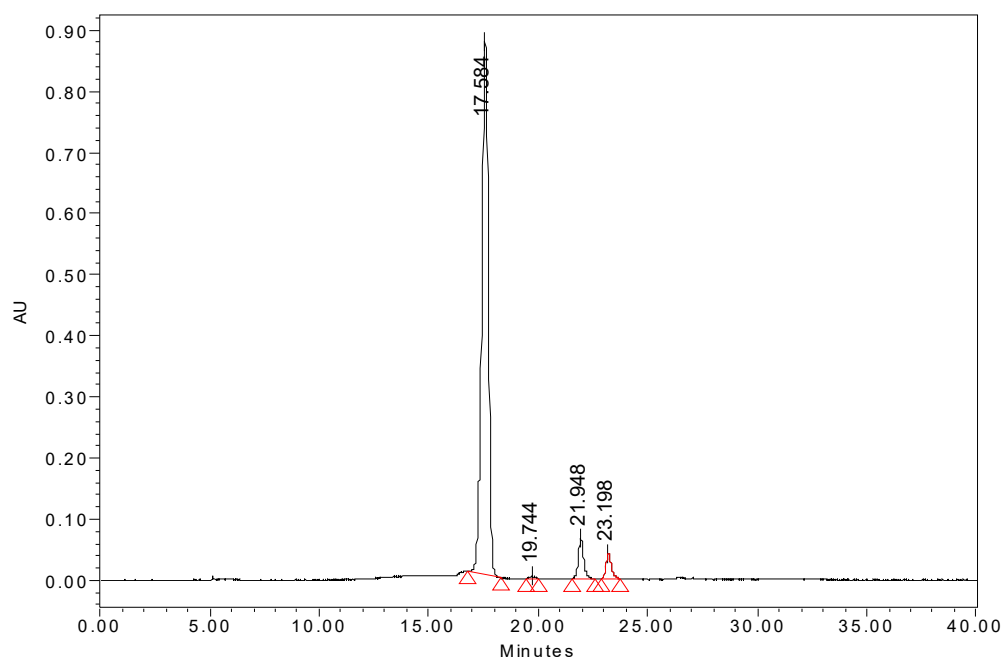
**Figure 4.8 Chromatogram of anthocyanin pigments of variety Bhim**



**Table 4.14 Chromatographic and spectral characteristics of anthocyanins in variety Bhim**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	17.623	96.72	Cyanidin 3,5-di-O-glucoside	175.80
2.	19.625	0.63	Pelargonidin 3,5-di-O-glucoside	2.31
3.	21.993	0.82	Unknown	1.50
4.	23.228	1.82	Unknown	3.32
Total				182.92

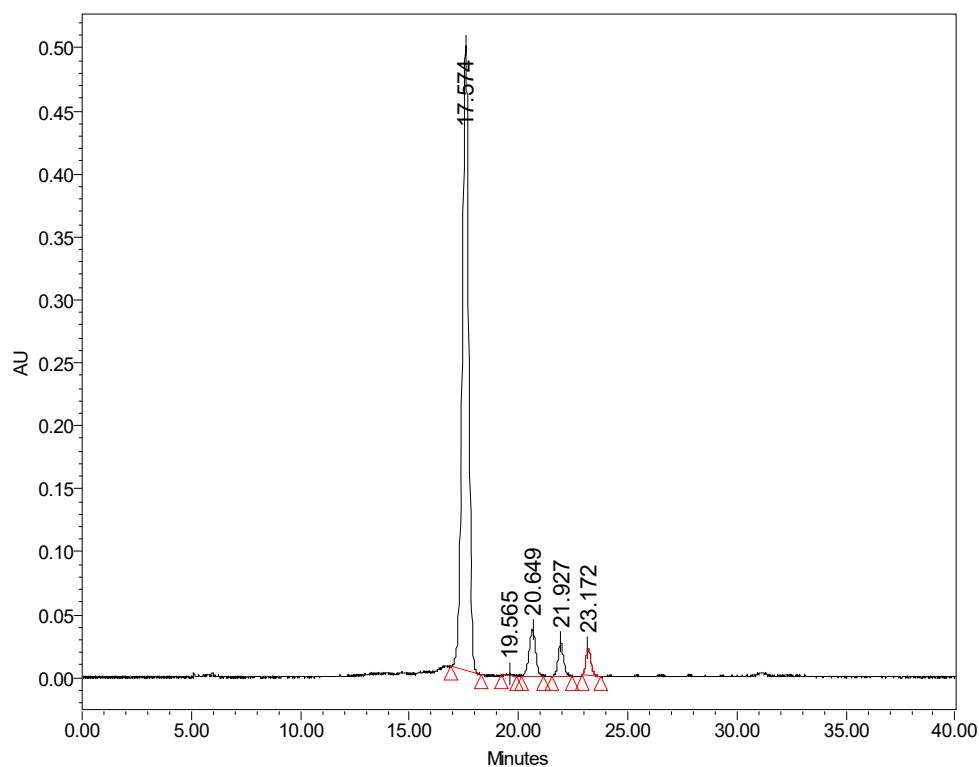
**Figure 4.9 Chromatogram of anthocyanin pigments of variety Dr. S.S. Bhatnagar**



**Table 4.15 Chromatographic and spectral characteristics of anthocyanins in variety Dr. S. S. Bhatnagar**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	17.584	90.78	Cyanidin 3,5-di-O-glucoside	362.40
2.	19.744	0.25	Pelargonidin 3,5-di-O-glucoside	1.98
3.	21.948	5.49	Unknown	21.91
4.	23.198	3.48	Unknown	13.90
Total				400.20

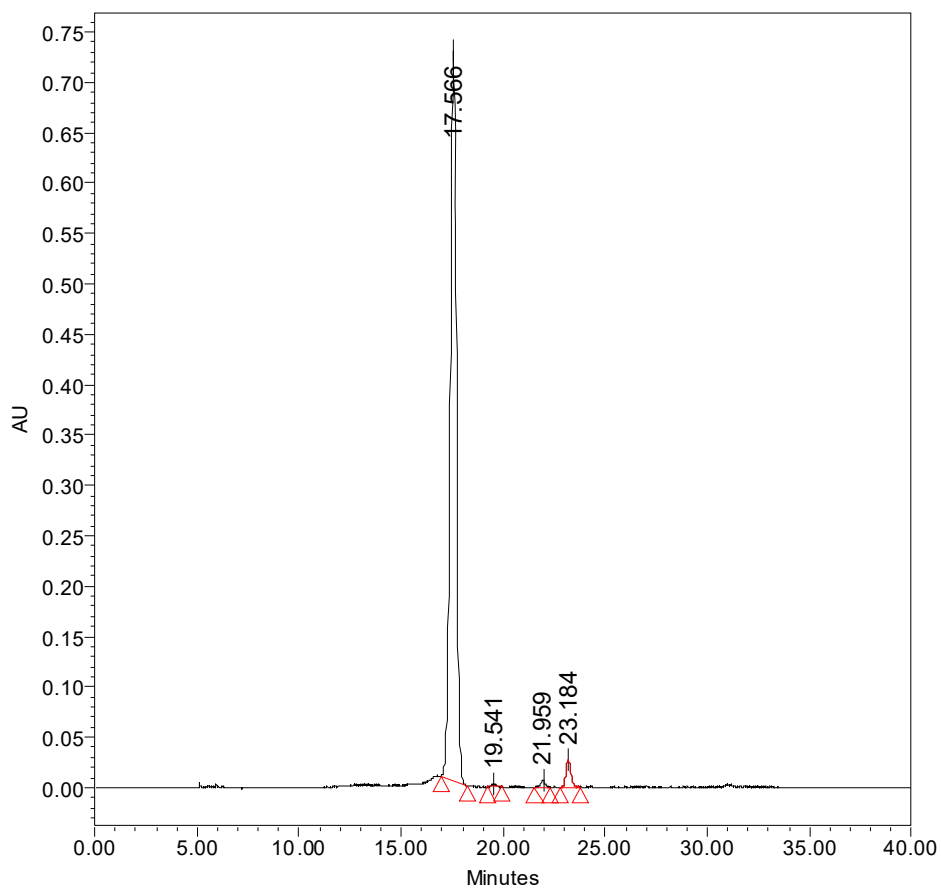
**Figure 4.10 Chromatogram of anthocyanin pigments of variety Rose Sherbet**



**Table 4.16 Chromatographic and spectral characteristics of anthocyanins in variety Rose Sherbet**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	17.574	86.35	Cyanidin 3,5-di-O-glucoside	206.57
2.	19.565	0.19	Pelargonidin 3,5-di-O-glucoside	0.93
3.	20.649	6.58	Unknown	15.75
4.	21.927	3.80	Unknown	9.08
5.	23.172	3.08	Unknown	7.36
Total				239.69

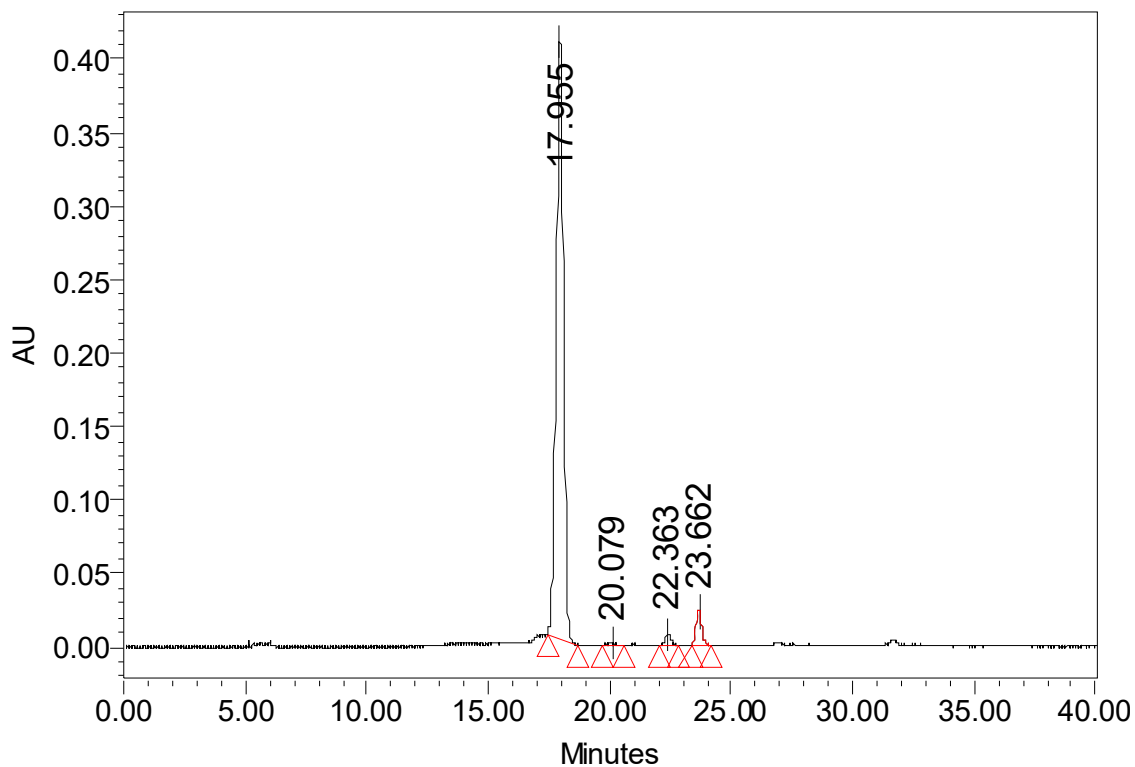
**Figure 4.11 Chromatogram of anthocyanin pigments of variety Pusa Virangana**



**Table 4.17 Chromatographic and spectral characteristics of anthocyanins in variety Pusa Virangana**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	17.566	96.04	Cyanidin 3,5-di-O-glucoside	301.66
2.	19.541	0.21	Pelargonidin 3,5-di-O-glucoside	1.30
3.	21.959	0.74	Unknown	2.34
4.	23.184	3.01	Unknown	9.45
Total				314.74

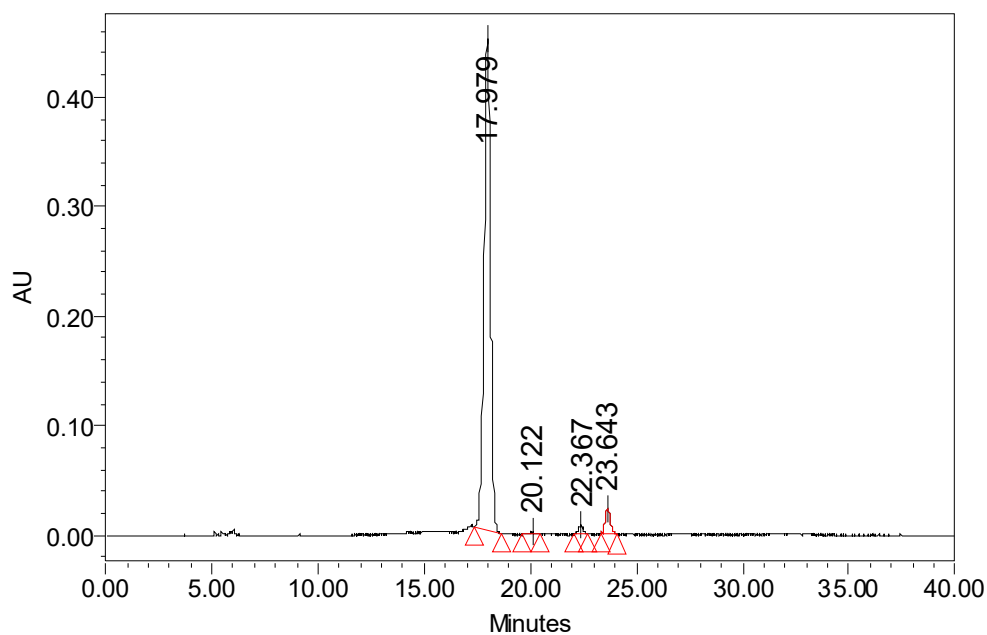
**Figure 4.12 Chromatogram of anthocyanin pigments of variety Surkhab**



**Table 4.18 Chromatographic and spectral characteristics of anthocyanins in variety Surkhab**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	17.955	93.63	Cyanidin 3,5-di-O-glucoside	173.60
2.	20.079	0.43	Pelargonidin 3,5-di-O-glucoside	1.58
3.	22.363	1.61	Unknown	2.99
4.	23.662	4.33	Unknown	8.03
Total				186.20

**Figure 4.13 Chromatogram of anthocyanin pigments of variety Pusa Ajay**



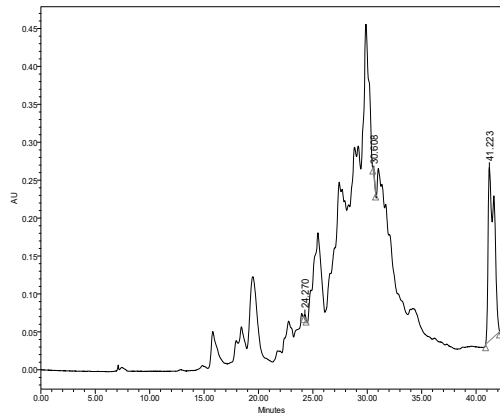
**Table 4.19 Chromatographic and spectral characteristics of anthocyanins in variety Pusa Ajay**

Peak number	RT (min) at 520 nm	Peak area (%)	Identified compound	Content (mg/100g)
1.	17.979	94.08	Cyanidin 3,5-di-O-glucoside	190.05
2.	20.122	0.53	Pelargonidin 3,5-di-O-glucoside	2.13
3.	22.367	1.27	Unknown	2.58
4.	23.643	4.12	Unknown	8.31
Total				203.06

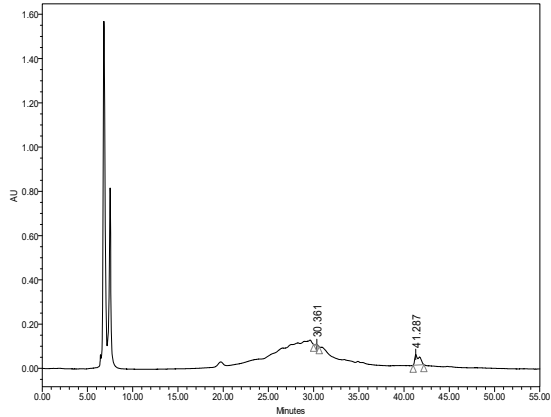
**Table 4.20 Anthocyanin content of rose varieties estimated by pH differential method and HPLC**

Varieties	Anthocyanin content (mg /100g)	
	pH differential method	HPLC
Ashwini	578.10	560.76
Dr. S. S. Bhatnagar	369.86	400.20
Nehru Centenary	288.15	497.28
Pusa Arun	282.60	527.91
Bhim	236.20	182.92
Raktima	202.63	380.68
Raktagandha	182.58	484.18
Pusa Virangana	180.50	314.74
Pusa Bahadur	124.87	144.09
Rose Sherbat	121.98	239.69
Suryakiran	96.69	245.13

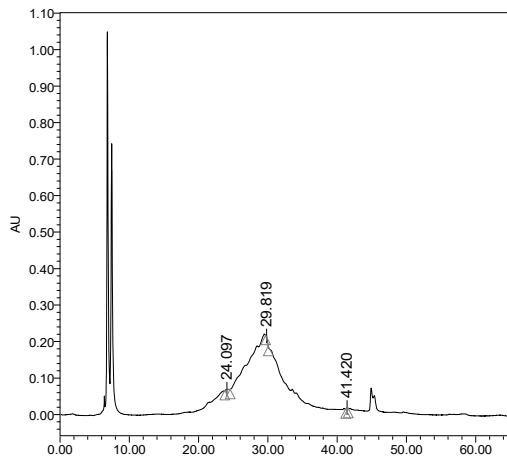
**Figure 4.14 Chromatograms of phenolic compounds of rose varieties**



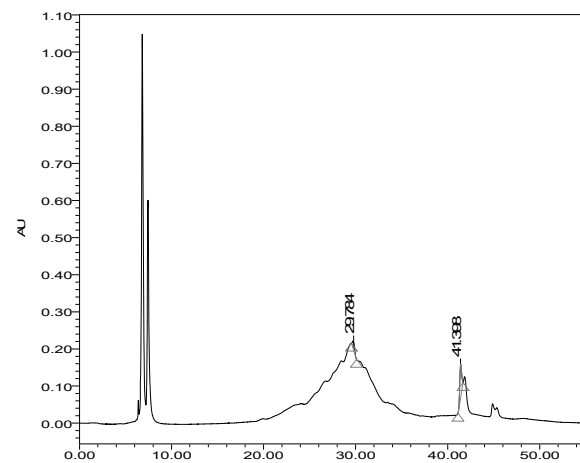
**Nehru Centenary**



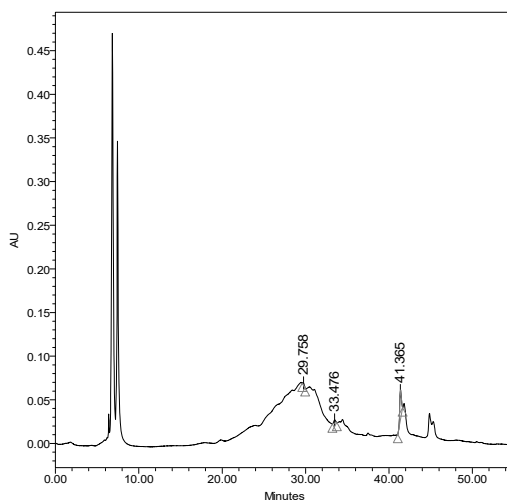
**Dr. S. S. Bhatnagar**



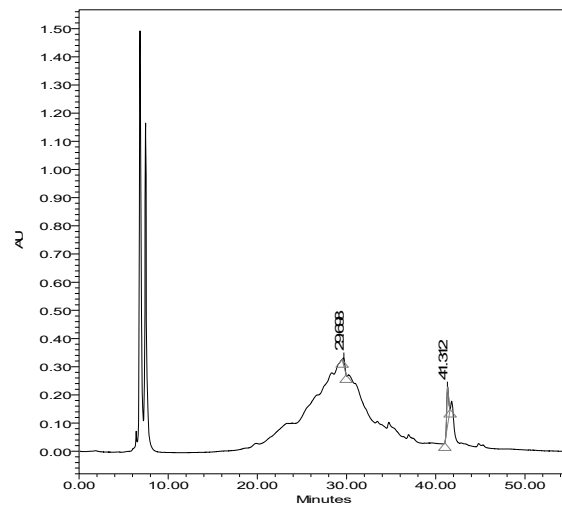
**Suryakiran**



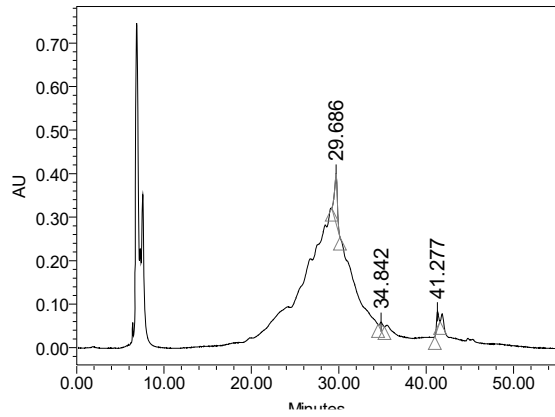
**Pusa Bahadur**



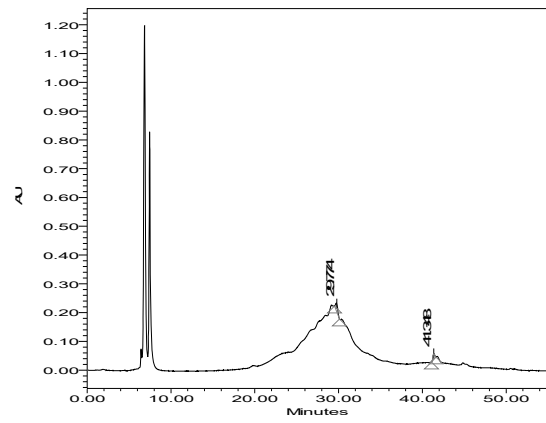
**Bhim**



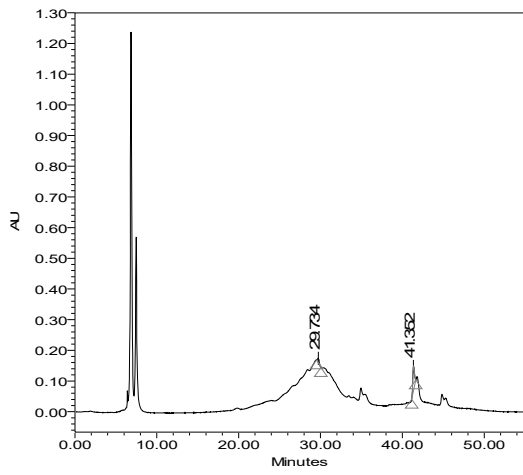
**Surkhab**



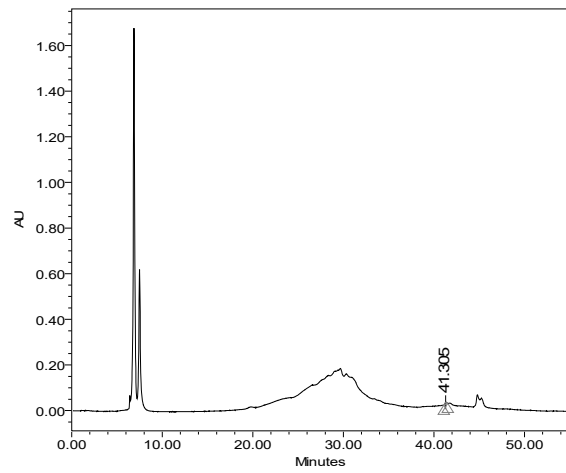
**Pusa Ajay**



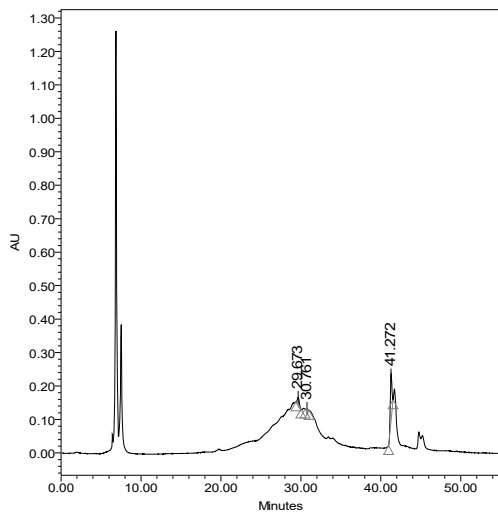
**Pusa Virangana**



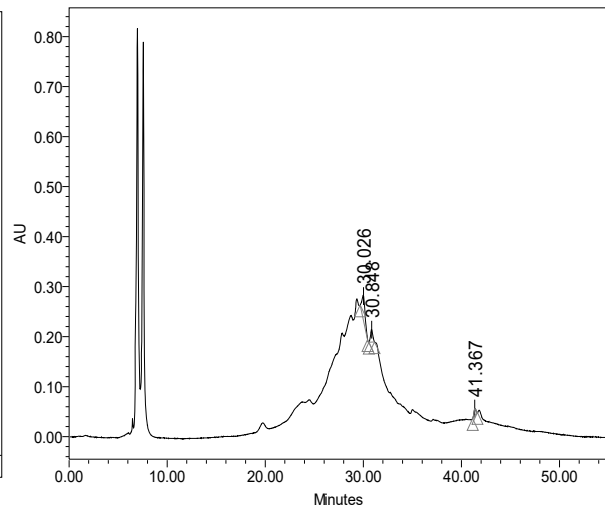
**Rose Sherbet**



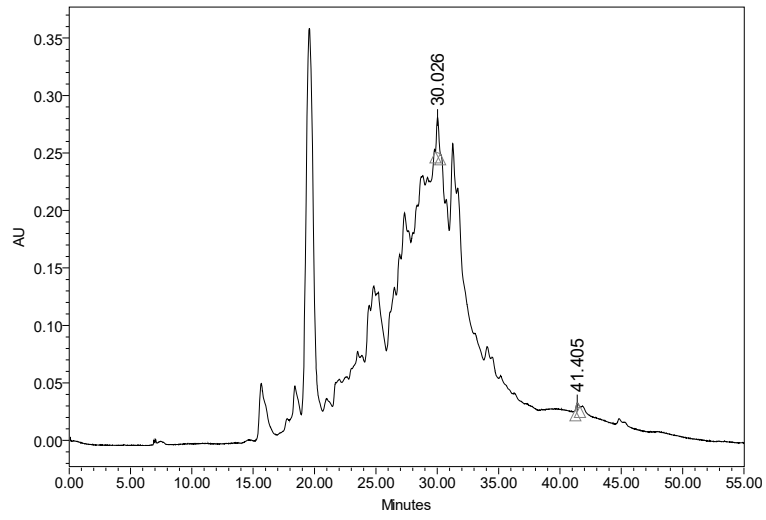
**Pusa Arun**



**Raktagandha**



**Raktima**



**Ashwini**

mg/100g). Minimum quercetin content was recorded in variety Suryakiran (2.38 mg/100g).

Catechin was found in Nehru Centenary (2.75 mg/100g) and Suryakiran (3.87 mg/100g). Epicatechin was found in all the varieties except variety Nehru Centenary and Pusa Arun. Epicatechin content ranged from 2.04 to 198.07 mg/100g. Maximum epicatechin content was recorded in variety Pusa Ajay (198.07 mg/100g) followed by Raktima (79.13mg/100g), Pusa Virangana (37.09 mg/100g) and Raktagandha (29.88 mg/100g). Minimum epicatechin content was recorded in Bhim (2.04 mg/100g).

Rutin was observed in Raktagandha, Bhim, Raktima and Nehru Centenary. Rutin content ranged from 1.17 mg/100g to 9.44 mg/100g. Maximum rutin content was observed in Raktima (9.44 mg/100g) followed by Nehru Centenary (2.03 mg/100g) and Bhim (1.37 mg/100g). The minimum rutin content was found in Raktagandha (1.17 mg/100g). 3-Hydroxy cinnamic acid was only observed in Pusa Ajay (0.98 mg/100g).

#### **4.5 *In vitro* induction of anthocyanin pigments in promising rose variety**

##### **4.5.1 Callus induction and multiplication from explants of *Rosa hybrida* var. Ashwini**

###### **4.5.1.1 Selection of explants for *in vitro* culture of rose**

Leaf and petal explants of rose were cultured *in vitro* and showed different response to culture initiation and callusing (Table 4.22). Microbial contamination was found maximum in leaf explants (30.15±0.28%) whereas, petal explants recorded minimum microbial contamination (14.12±1.17%). Percentage survival of explants was maximum in petal explants (83.62±0.38%) as compared to leaf explants (66.33±0.60%). Both the explants differed significantly. Earliest callus induction was noticed in leaf explants (9.00±0.32 days). Petal explants took 11.80±0.37 days for callus induction. Petal explants exhibited highest callusing frequency (69.49±0.28%) whereas leaf explants showed less callusing frequency (51.76±0.41%).

###### **4.5.1.2 Standardization of pre-treatments in rose**

As evident from the data presented in Table 4.23, pre-treatment of explants with different fungicidal and bactericidal treatments had significant effect on explants survival and microbial contamination. The culture infected with fungus and bacteria

did not survive for long period and turned black and died. On the other hand, the cultures which were free from infections remained green and responded well. It is evident from the Table 4.23 that maximum microbial contamination per cent ( $95.94 \pm 1.32\%$ ) in petal explants was recorded in control and minimum microbial contamination per cent ( $15.66 \pm 0.59\%$ ) was observed when carbendazim (0.2%) + mancozeb-45 (0.2%) + 8-HQC (200 mg/l) was used for 15 minutes. It was significantly followed by pre-treatment comprising of carbendazim (0.1%) + mancozeb-45 (0.1%) + 8-HQC (200 mg/l) dip for 1.5 h ( $35.28 \pm 0.74\%$ ). Pre-treatment shake of rose petal explants in solution comprising of carbendazim (0.2%) + mancozeb-45 (0.2%) + 8-HQC (200 mg/l) for 15 minutes gave highest survival ( $83.28 \pm 0.51\%$ ), which was significantly followed by carbendazim (0.1%) + mancozeb-45 (0.1%) + 8-HQC (200 mg/l) for 1.5 h ( $62.03 \pm 0.63\%$ ). Minimum survival of petal explant was recorded in control ( $1.67 \pm 1.41\%$ ).

#### **4.5.1.3 Standardization of culture medium for callus induction and multiplication from petal explants of *Rosa hybrida* var. Ashwini**

In rose increasing level of 2,4-D resulted in improvement of callusing (Table 4.24). Highest induction coefficient per cent was recorded ( $94.67 \pm 1.11\%$ ) when 2,4-D was added at 6.0 mg/l significantly followed by 2,4-D 5.0 mg/l ( $85.67 \pm 0.71\%$ ) and 2,4-D 4.0 mg/l ( $63.33 \pm 0.53\%$ ). Medium devoid of 2,4-D did not give rise to any callus. Earliest callus induction ( $8.67 \pm 0.67$  days) was observed when MS medium was supplemented with 2, 4-D (6.0 mg/l) which was significantly differed from other treatments. Days taken for callus induction was statistically at par when MS medium supplemented with 2,4-D 4.0 mg/l ( $13.33 \pm 0.33$  days) and 2,4-D 5.0 mg/l ( $11.33 \pm 1.20$  days). As evident from Table 4.33 and Plate 4.1 best induction and vigorous growth of callus was observed when petal explants were cultured on 2, 4-D (6.0 mg/l). Visual observations suggested that compact and creamish white callus was arisen from cut and punctured surface of petal explants (Plate 4.1a).

#### **4.5.1.4 Multiplication and maintenance of callus from petal explants of *Rosa hybrida* var. Ashwini**

The treatment combination which performed better for callus induction was further used to get sufficient quantity of callus during long-term culture for callus multiplication. In this regard, MS medium supplemented with 2, 4-D was altered to

**Table 4.22 Culture establishment and callus induction of different explants**

Explant	Contamination (%)	Survival (%)	Days to callus induction	Callusing (%)
Leaf	30.15±0.28 (33.29)	66.33±0.60 (54.52)	9.00±0.32	51.76±0.41 (45.99)
Petal	14.12±1.17 (21.96)	83.62±0.38 (66.11)	11.80±0.37	69.49± 0.28 (56.45)
SEm±	0.85	0.50	0.35	0.35
CD (P≤ 0.05)	2.82	1.66	1.15	1.17

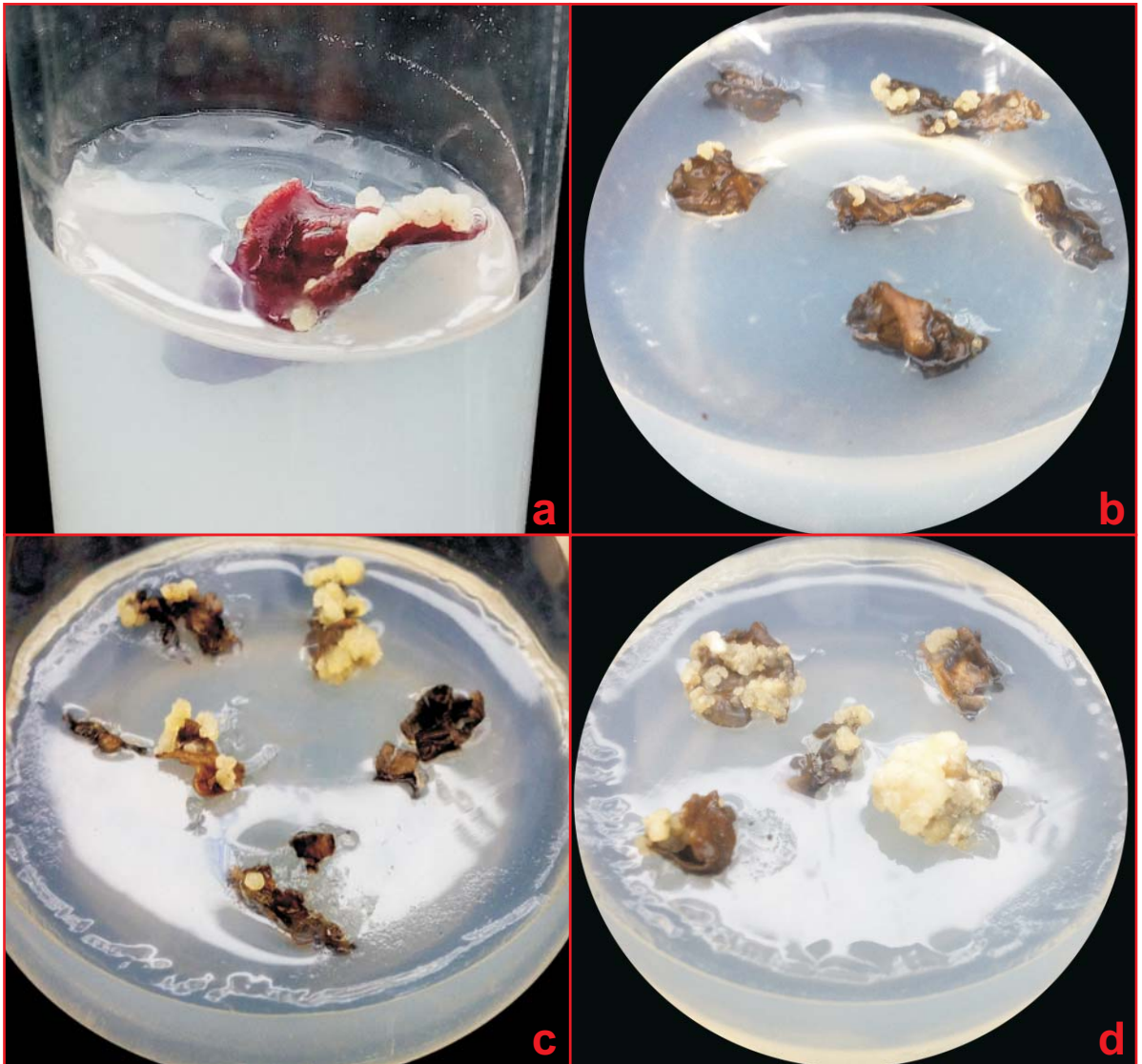
\*Values in parenthesis are arc sin transformed values

**Table 4.23 Effect of pre-treatment on *in vitro* culture initiation of rose (petals)**

Treatment	Contamination (%)	Survival (%)
T0 (control distilled water shake)	95.94±1.32 <sup>a</sup> (78.489)	1.67±1.41 <sup>d</sup> (7.15)
T1 (Carbendazim 0.1%+Mancozeb-45 0.1%+ 8 HQC (200 mg/l) 3 h)	42.11±0.54 <sup>b</sup> (40.44)	55.37±0.31 <sup>c</sup> (48.07)
T2 (Carbendazim 0.1%+Mancozeb-45 0.1%+ 8 HQC (200 mg/l) 1.5 h)	35.28±0.74 <sup>c</sup> (36.42)	62.03±0.63 <sup>b</sup> (51.95)
T3 (Carbendazim 0.2%+Mancozeb-45 0.2%+ 8 HQC (200 mg/l) 15 minutes)	15.66±0.59 <sup>d</sup> (23.26)	83.28±0.51 <sup>a</sup> (65.85)
SEm±	0.86	0.83
CD (P≤ 0.05)	2.84	2.75

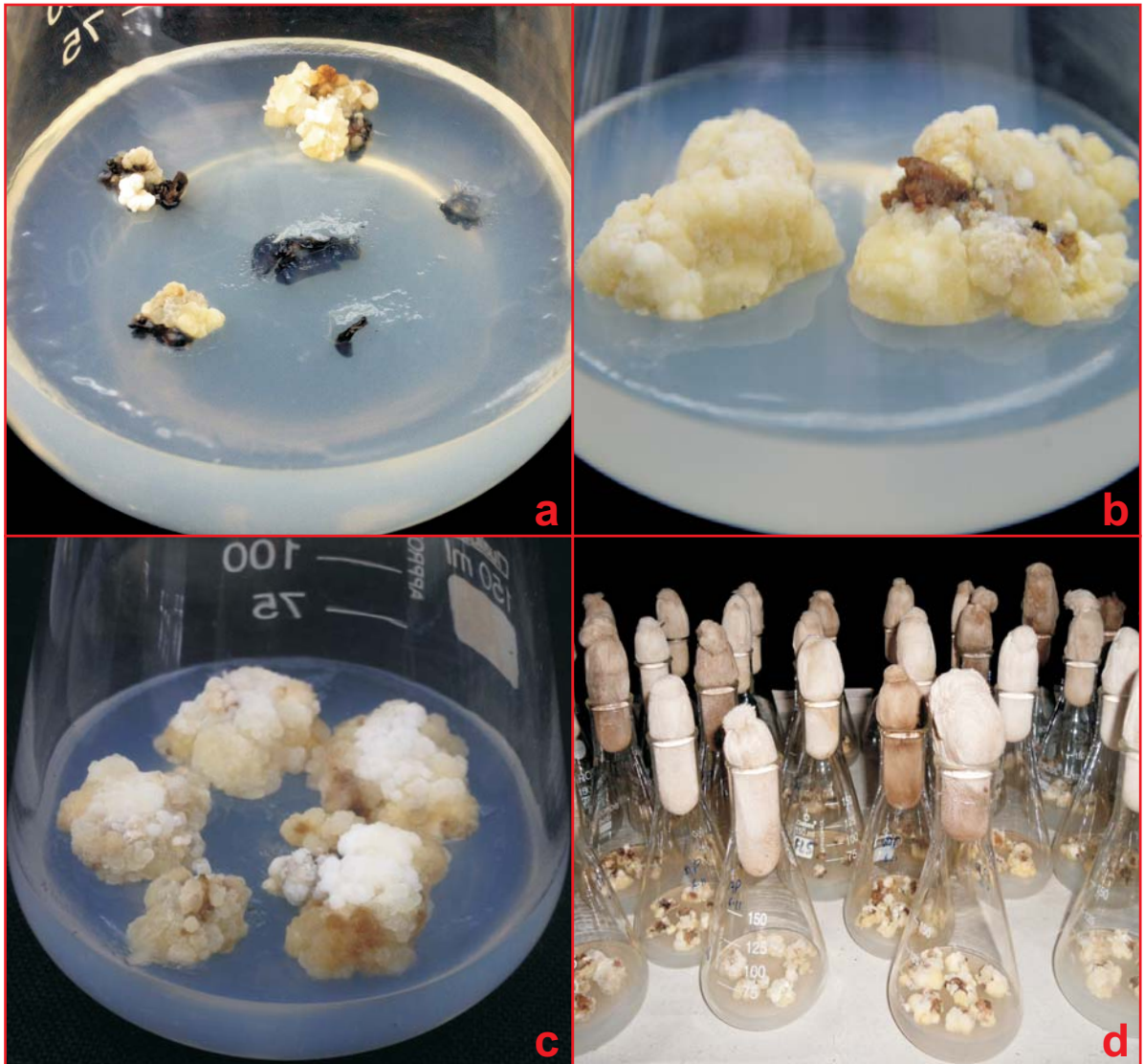
\*Values in parenthesis are arc sin transformed values

**Plate 4.1: Callus induction from petal explant of *Rosa hybrida* var. Ashwini**



- a. Callus initiation from petal explant
- b. Callus induction (MS+ 4 mg/l 2,4-D)
- c. Callus induction (MS+ 5 mg/l 2,4-D)
- d. Callus induction (MS+ 6 mg/l 2,4-D)

**Plate 4.2: Multiplication of petal derived callus of *Rosa hybrida* var. Ashwini in MS medium supplemented with 6 mg/l 2,4-D**



- a. 27 days old callus
- b. 50 days old callus
- c. 65 days old callus
- d. Callus maintenance in complete darkness at 24±1°C

**Table 4.24 Effect of 2,4-D levels on callus induction in rose (petals)**

Treatment	Induction coefficient per cent (%)	Days to callus induction	Callus growth status
T0 (MS + No hormone )	0.00	0.000	-
T1 (MS+2,4-D 4 mg/l)	63.33±0.53 <sup>c</sup> (52.71)	13.33±0.33 <sup>a</sup>	II
T2 (MS+2,4-D 5 mg/l)	85.67±0.71 <sup>b</sup> (67.74)	11.33±1.20 <sup>a</sup>	III
T3 (MS+2,4-D 6 mg/l)	94.67±1.11 <sup>a</sup> (76.70)	8.67±0.67 <sup>b</sup>	IV
SEm±	0.71	0.76	
CD ( P≤0.05)	2.36	2.31	

\*Values in parenthesis are arc sin transformed values

I = Weak callus initiation and poor growth

II = Good induction of callus but poor growth

III = Good initiation and moderate growth

IV = Best induction and vigorous growth of callus

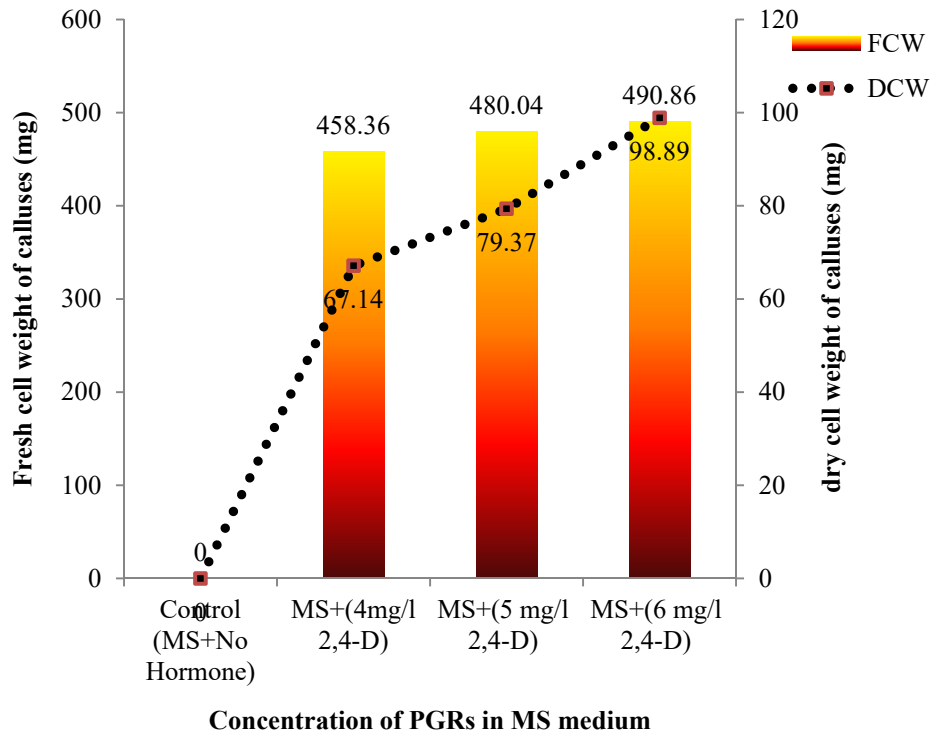
**Table 4.25 Pearson's correlation coefficient between different parameters recorded for callus induction and multiplication of petal explant of *Rosa hybrida* var. Ashwini**

Parameter	DCI	ICP	FCW	DCW	FCW:DCW R
DCI	1	0.804	0.972*	0.993**	0.829
ICP		1	0.918	0.771	0.999**
FCW			1	0.960*	0.935
DCW				1	0.799
FCW:DCW R					1

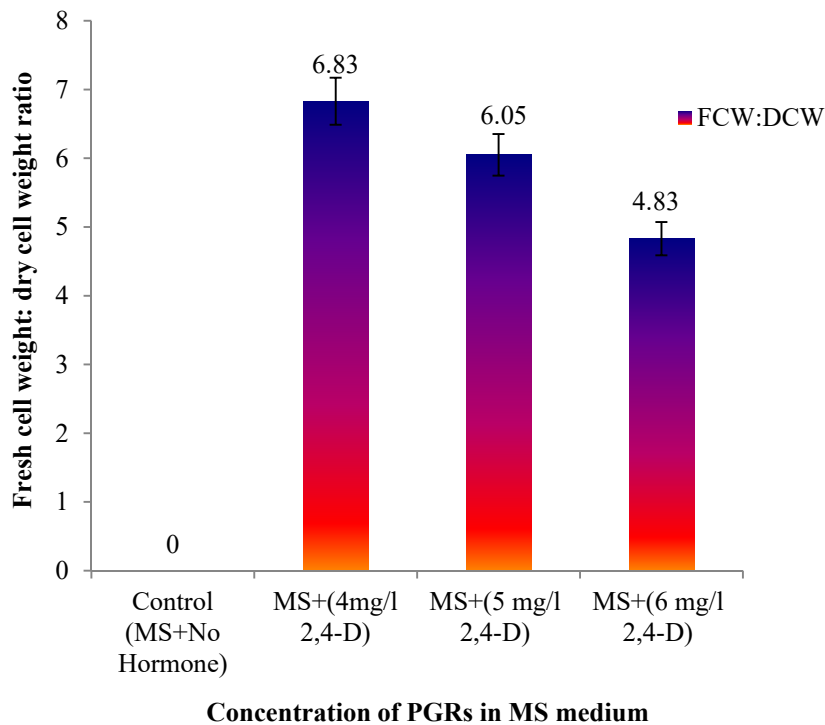
DCI= Days to callus initiation, ICP=Induction coefficient per cent, FCW=Fresh cell weight, DCW=Dry cell weight, FCW: DCW R = Fresh cell weight: dry cell weight ratio

\*, \*\*Correlation is significantly different at P≤ 0.05 and 0.01 respectively

**Figure 4.15 Effect of 2,4-D levels on callus fresh weight and dry weight of *Rosa hybrida* var. Ashwini**



**Figure 4.16 Effect of 2,4-D levels on fresh cell weight : dry weight ratio**



some extent and optimized for maintenance of callus growth. After 27 days of incubation, the calluses were transferred from induction to maintenance media and kept in complete dark for multiplication. After 7-9 days of culture, the calluses were divided and proliferated rapidly (Plate 4.2). The disintegrated mother petal explant was removed from proliferating callus to avoid browning due to release of phenolics. As the disintegrated mother petal explant was removed from the callus their growth was slow down. To neutralize this effect, quantity of vitamins of the MS basal medium was doubled.

#### **4.5.1.5 Effect of different 2,4-D levels on callus biomass accumulation of *Rosa hybrida* var. Ashwini**

To determine the callus growth in terms of biomass accumulation, fresh cell weight (FCW) at harvest, dry cell weight (DCW) after drying and weight ratio (FCW: DCW) were measured, after 42-day of culture. The fresh and dry cell weights were significantly affected by the various concentrations of 2,4-D in the culture medium (Figure 4.15). The petal derived calluses showed a steady growth. Maximum fresh cell weight ( $490.86 \pm 0.66$  mg) was recorded in when MS medium supplemented with 2, 4-D at 6.0 mg/l followed significantly by 2, 4-D at 5.0 mg/l ( $480.04 \pm 1.29$  mg) and 2, 4-D at 4.0 mg/l ( $458.36 \pm 0.92$  mg). Figure 4.15 showed that MS medium supplemented with 2, 4-D at 6.0 mg/l resulted in a high cell dry biomass production ( $98.89 \pm 0.99$  mg). This particular treatment had a significant effect on fresh as well as dry cell weight and differed significantly ( $P=0.05$ ) with the other tested treatments. Since there was no callus induction on the MS basal medium devoid of plant hormones (control), there was no fresh- and dry cell weight value for this.

To know the actual gain in callus biomass accumulation the ratio of fresh- to dry cell weight (FCW: DCW), an index of cell water content, was calculated. Callus contains approximately 96.5 per cent water content of the fresh weight. The callus with high relative dry weight has lower influx of water. Among the different treatments MS medium supplemented with 2,4-D at 6.0 mg/l resulted in the lowest FCW: DCW ratio ( $4.83 \pm 0.14$ ) indicating that the callus was not watery and gained good biomass (Figure 4.16). Explants cultured on MS medium without the plant growth regulator did not produce any callus; so there was no fresh- and dry cell weight value for this treatment.

#### 4.5.1.6 Correlation between callogenesis and growth parameters

Pearson's correlation coefficient was computed to identify the relationship between days to callus initiation, induction coefficient, fresh- and dry cell weights and weight ratio. Pearson's correlation coefficient identified a statistically significant positive correlation between days to callus induction, fresh and dry cell weight, as shown in Table 4.25. A strong statistically significant positive correlation ( $r = 0.999$ ) was observed between induction coefficient per cent and fresh cell weight: dry cell weight ratio. Positive correlation was also observed between induction coefficient per cent and days to callus initiation ( $r = 0.804$ ).

#### 4.5.2 Standardization of different concentrations of sucrose and ABA in the culture media for anthocyanin pigment induction from *in vitro* induced rose callus

Increasing the sucrose level in solid medium invariably led to higher anthocyanin accumulation in rose callus derived from petal explants (Table 4.26, Figure 4.17 and Plate 4.3). Maximum response coefficient percentage was registered with EM medium supplemented with 80 g/l ( $83.67 \pm 5.07\%$ ) followed non-significantly by EM + sucrose 70 g/l ( $81.33 \pm 1.39\%$ ) and EM + sucrose 60 g/l ( $70.33 \pm 3.87\%$ ). Minimum response coefficient percentage was established in control ( $29.33 \pm 0.55\%$ ). Earliest pigment induction ( $5.33 \pm 0.6$  days) was observed when 80 g/l sucrose was supplemented followed non-significantly by EM + sucrose (70 g/l) + ABA (30  $\mu$ M) ( $7.00 \pm 1.15$  days) and EM + sucrose (80 g/l) + ABA (20  $\mu$ M) (7.33 days). Anthocyanin induction was slow when EM medium supplemented with sucrose 50 g/l ( $17.33 \pm 1.20$  days). Anthocyanin intensification was earliest ( $8.67 \pm 1.20$  days) when EM medium supplemented with sucrose 80 g/l followed non-significantly by EM + sucrose (80 g/l) + ABA (20  $\mu$ M) ( $9.33 \pm 0.88$  days) and EM + sucrose (70 g/l) + ABA (30  $\mu$ M) ( $9.67 \pm 0.67$  days). Pigmentation was delayed maximum ( $19.67 \pm 0.67$  days) when sucrose was used at 50 g/l. Increasing sucrose concentration and combination of sucrose and ABA led to higher pigment intensification. Total anthocyanin content in calli ranged from  $5.23 \pm 0.66$  to  $53.42 \pm 2.38$  mg/kg fresh weight). Quantification of pigment content in calli was found highest ( $53.42 \pm 2.38$  mg/kg fresh weight) when EM medium supplemented with 80 g/l sucrose followed non-significantly by 70 g/l sucrose ( $51.55 \pm 1.33$  mg/kg FW). Lowest anthocyanin content ( $5.23 \pm 0.66$  mg/kg fresh weight) was recorded in control (EM+0). Pigment

content was  $46.67 \pm 1.53$  mg/kg fresh weight when EM medium supplemented with EM + ABA (30  $\mu$ M). It is evident that as ABA concentration increases in the EM medium beyond (30  $\mu$ M); pigment content decreases (Figure 4.17). The stereo microscopic study revealed that the callus tissues proliferated on 80 g/l sucrose level exhibited the presence of more red pigmentation (Plates 4.4c) than others (Plates 4.4a, b, d, e and f), thus clearly showing higher anthocyanin biosynthesis. From figure 4.18 it is evident that maximum fresh cell weight (FCW) of callus was observed in control  $396.71 \pm 1.25$  mg followed by EM + 50  $\mu$ M ABA ( $324.03 \pm 5.42$  mg). Minimum fresh cell weight ( $273.64 \pm 0.68$  mg) was recorded in treatment (EM + 80g/l sucrose).

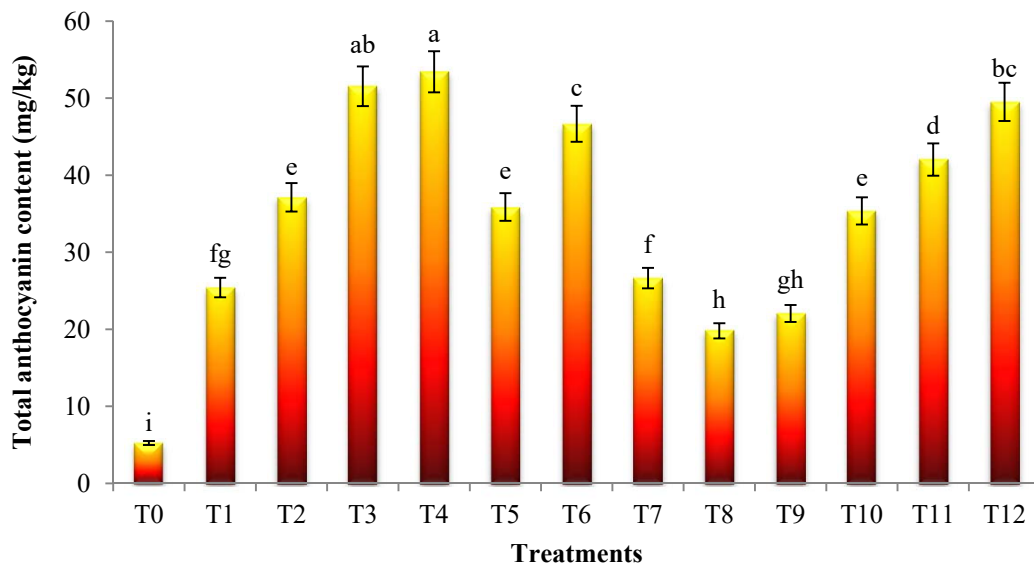
#### **4.5.2.1 Correlation between different parameters recorded for anthocyanin induction by sucrose and ABA in petal derived calli of *Rosa hybrida* var. Ashwini**

A statistically significant positive correlation between response coefficient and anthocyanin content ( $r = 0.960$ ) was noted (Table 4.27). A negative correlation was observed between response coefficient percentage, days required for pigment initiation and intensification. Days required for pigment initiation and intensification were negatively correlated with pigment content. Furthermore, significant positive correlations between days required for pigment initiation, pigment intensification and fresh cell weight of callus was found. However, a statistically significant relationship was detected between days required for pigment initiation, pigment intensification and fresh cell weight. A non-significant negative correlation was found between anthocyanin content and fresh cell weight ( $r = -0.891$ ).

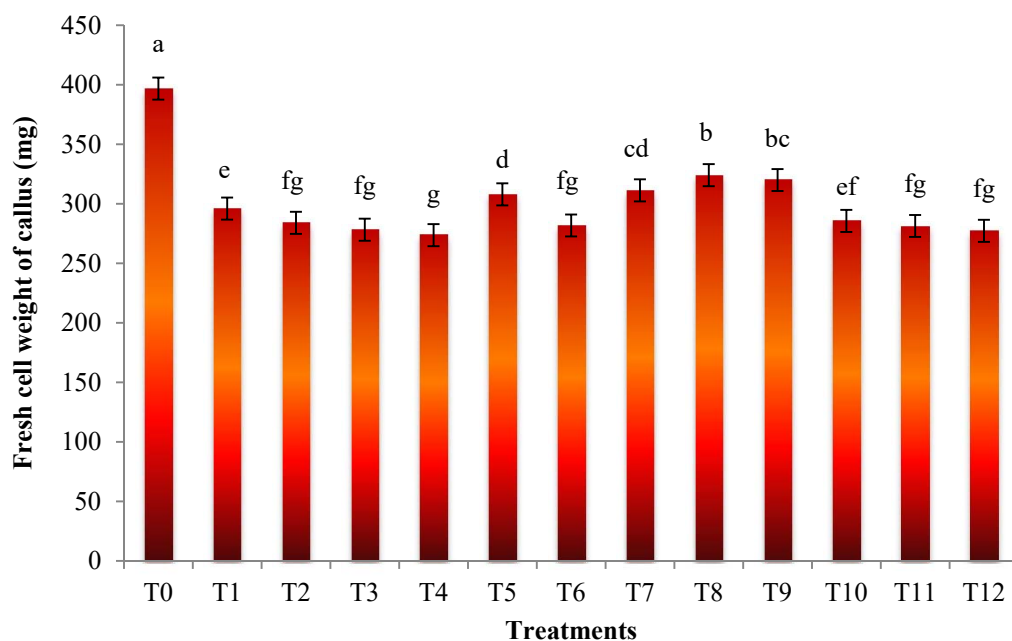
**Table 4.26 Effect of sucrose and ABA on anthocyanin pigment production in callus culture of *Rosa hybrida* var. Ashwini**

Treatment	Response coefficient (%)	Days to pigment initiation	Days to pigment intensification
T0=EM + 0 ( control)	29.33±0.55 <sup>h</sup> (32.78)	15.67±0.88 <sup>a</sup>	17.67±0.33 <sup>a</sup>
T1=EM + sucrose (50 g/l)	51.00±2.84 <sup>fg</sup> (45.56)	17.33±1.20 <sup>a</sup>	19.67±0.67 <sup>a</sup>
T2=EM + sucrose (60 g/l)	70.33±3.87 <sup>cd</sup> (57.22)	10.67±0.67 <sup>bcd</sup>	13.67±2.40 <sup>b</sup>
T3=EM + sucrose (70 g/l)	81.33±1.39 <sup>ab</sup> (64.43)	7.33±0.88 <sup>de</sup>	10.67±1.45 <sup>bcd</sup>
T4=EM + sucrose (80 g/l)	83.67±5.07 <sup>a</sup> (67.03)	5.33±0.66 <sup>c</sup>	8.67±1.20 <sup>d</sup>
T5=EM + ABA (20 µM)	53.67±0.83 <sup>fg</sup> (47.08)	8.49±1.81 <sup>cde</sup>	12.33±0.88 <sup>bcd</sup>
T6=EM + ABA (30 µM)	71.66±1.13 <sup>cd</sup> (57.84)	8.17±2.18 <sup>cde</sup>	11.67±1.764 <sup>bcd</sup>
T7=EM + ABA (40 µM)	44.33±1.26 <sup>fg</sup> (41.72)	10.82±0.62 <sup>bcd</sup>	12.33±0.66 <sup>bcd</sup>
T8=EM + ABA (50 µM)	43.33±0.97 <sup>g</sup> (41.15)	10.67±1.20 <sup>bcd</sup>	13.67±0.67 <sup>b</sup>
T9=EM + sucrose (50 g/l) + ABA (50 µM)	50.33±1.01 <sup>fg</sup> (45.17)	11.33±1.20 <sup>bc</sup>	13.66±1.86 <sup>b</sup>
T10=EM + sucrose (60 g/l) + ABA (40µM)	54.67±1.12 <sup>ef</sup> (47.66)	12.00±1.00 <sup>b</sup>	13.33±1.20 <sup>bc</sup>
T11=EM + sucrose (70 g/l) + ABA (30 µM)	64.33±0.72 <sup>de</sup> (53.31)	7.00±1.15 <sup>e</sup>	9.67±0.67 <sup>cd</sup>
T12=EM + sucrose (80 g/l) + ABA (20 µM)	75.000±0.76 <sup>bc</sup> (59.99)	7.33±1.20 <sup>de</sup>	9.33±0.88 <sup>d</sup>
SEm±	2.12	1.21	1.26
CD ( P≤0.05)	6.21	3.53	3.69

**Figure 4.17 Effect of sucrose and ABA on anthocyanin content on callus culture of *Rosa hybrida* var. Ashwini**



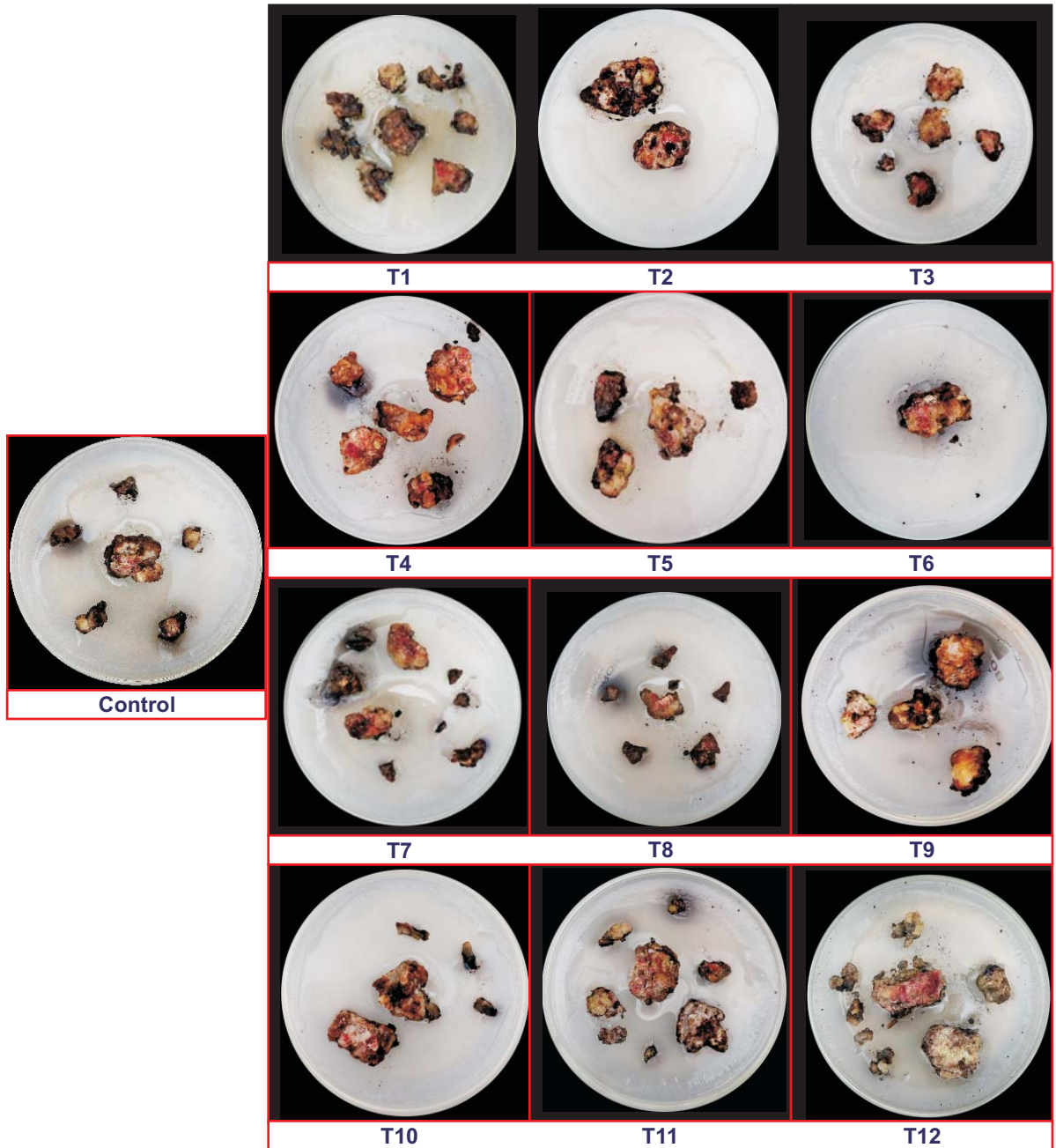
**Figure 4.18 Effect of sucrose and ABA on callus biomass accumulation *Rosa hybrida* var. Ashwini**



T0=EM + 0 (control) , T1=EM + sucrose (50 g/l), T2=EM + sucrose (60 g/l)  
 T3=EM + sucrose (70 g/l) , T4=EM + sucrose (80 g/l) , T5=EM + ABA (20 μM)  
 T6=EM + ABA (30 μM) , T7=EM + ABA (40 μM) , T8=EM + ABA (50 μM)  
 T9=EM + sucrose (50 g/l) + ABA (50 μM) , T10=EM + sucrose (60 g/l) + ABA (40 μM)  
 T11=EM + sucrose (70 g/l) + ABA (30 μM) , T12=EM + sucrose (80 g/l) + ABA (20 μM)

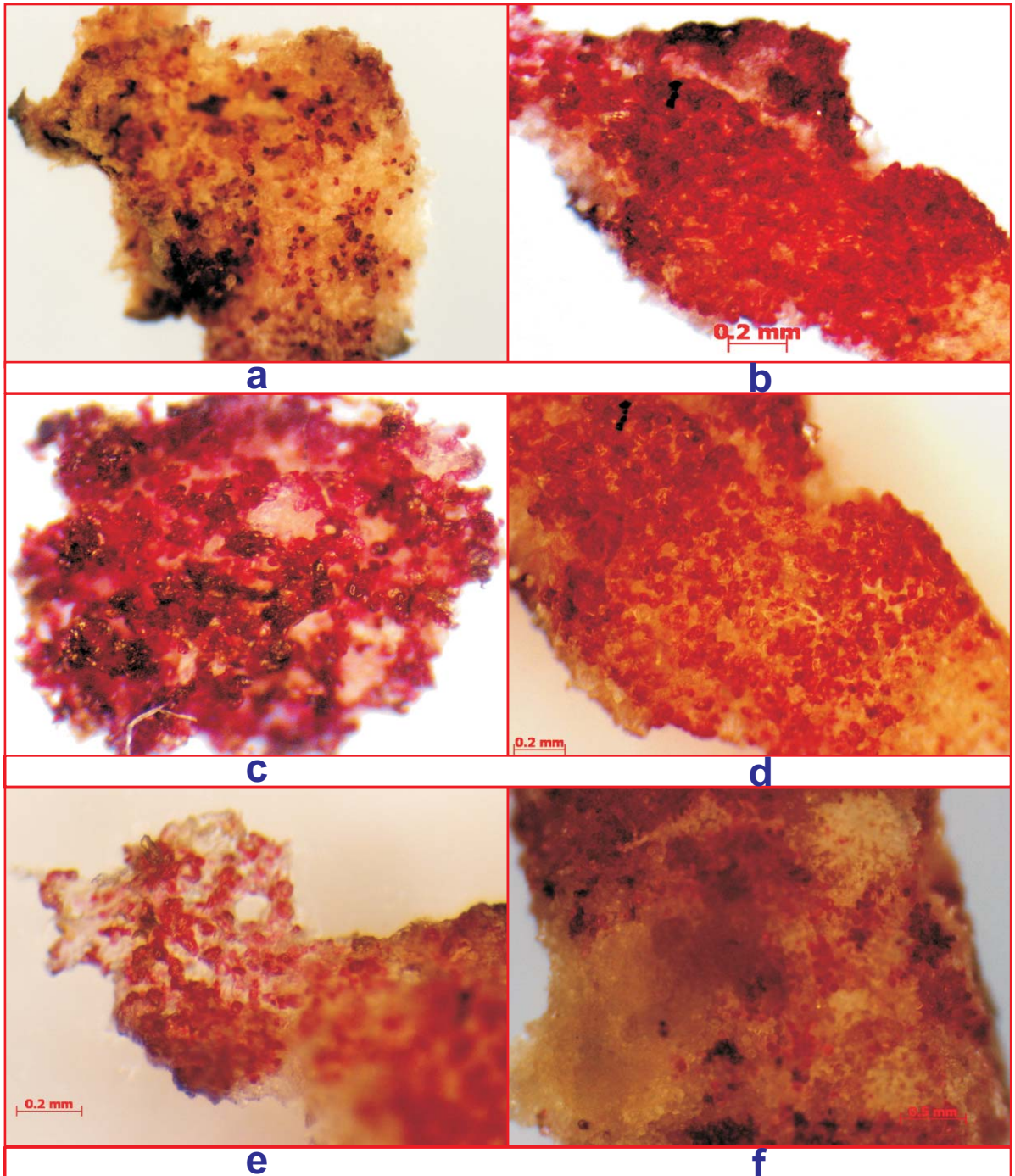
The same letter on the bar graph did not differ significantly at 5% level of significance when compared by Tukey's HSD test.

**Plate 4.3: Effect of sucrose and ABA on anthocyanin accumulation in petal derived callus of *Rosa hybrida* var. Ashwini**



T0 = EM + 0 (control), T1 = EM + sucrose (50 g/l), T2 = EM + sucrose (60 g/l)  
 T3 = EM + sucrose (70 g/l), T4 = EM + sucrose (80 g/l), T5 = EM + ABA (20 μM)  
 T6 = EM + ABA (30 μM), T7 = EM + ABA (40 μM), T8 = EM + ABA (50 μM)  
 T9 = EM + sucrose (50 g/l) + ABA (50 μM), T10 = EM + sucrose (60 g/l) + ABA (40 μM)  
 T11 = EM + sucrose (70 g/l) + ABA (30 μM), T12 = EM + sucrose (80 g/l) + ABA (20 μM)

**Plate 4.4: Stereomicroscopic (40 x) view of anthocyanin pigments in petal derived callus of *Rosa hybrida* var. Ashwini**



- a. EM + 0 (control)
- b. EM + sucrose (70 g/l)
- c. EM + sucrose (80 g/l)
- d. EM + ABA (30  $\mu$ M)
- e. EM + sucrose (70 g/l) + ABA (30  $\mu$ M)
- f. EM + sucrose (80 g/l) + ABA (20  $\mu$ M)

**Table 4.27 Correlation between different parameters recorded for anthocyanin induction by sucrose and ABA in petal derived calli of *Rosa hybrida* var. Ashwini**

<b>Parameters</b>	<b>RCP</b>	<b>NDTP Initi.</b>	<b>NDTP Inten.</b>	<b>AC</b>	<b>FCW</b>
<b>RCP</b>	1	-0.754**	-0.713**	0.960**	-0.849**
<b>NDTP Initi.</b>		1	0.976**	-0.813**	0.612*
<b>NDTP Inten.</b>			1	-0.790**	0.608*
<b>AC</b>				1	-0.891**
<b>FCW</b>					1

RCP= Response coefficient percentage, NDTP Initi.=Number of days taken for pigment initiation, NDTP Inten.= Number of days taken for pigment intensification, FCW=Fresh cell weight, AC=Anthocyanin content

\*,\*\*Correlation is significantly different at  $P \leq 0.05$  and  $0.01$  respectively

## 5. DISCUSSION

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Pigments responsible for the appearance of colours in higher plants are classified as chlorophylls, carotenoids, flavonoids and betalains. Pigments not only endow nature with attractive colours but also contribute towards improving health and lower the risk of diseases. The antioxidant properties of pigments have been suggested as being the main mechanism by which they impart their beneficial effects. Anthocyanin compounds have high free radical scavenging capacity and play an essential role in the prevention of cardiovascular disease, obesity, cancer, diabetes and other diseases.

Separation and characterization of these anthocyanin pigments are also utmost important for further research and its utilization for the use as a source of nutraceuticals. In spite of the health benefits the natural pigments accrue, the use of synthetic pigments comprising of heavy metals is rampant in the food and beverage industry. In this era of going back to nature it is imperative to use the natural pigments in pharmaceutical and food industry. Nowadays research is oriented more towards identification and isolation of pigments from natural sources, due to increased awareness about ill effects of manmade colourants on human health and environment. Pigment profiling involves the isolation, separation and characterization of pigments. Trends in pigment analysis reflect not only advances in analytical methodology and instrumentation, but also on current knowledge of their functions or actions. The biology and health benefits of plant pigments have lead researchers to discover modify and utilize techniques for the extraction, separation and quantification of these compounds from natural sources. Production of anthocyanin pigments by plant cell cultures has been suggested as feasible technology that has attracted industrial and academic interests in the past three decades. The present focus is on the strategies for enhancement of anthocyanin biosynthesis to achieve an economically viable technology for commercial applications. Through genotypes improvement, optimization of media and culture conditions and intelligent process strategies such as elicitation and two stage system, significant enhancement in productivity has been achieved in number of cultures. However, the yield of anthocyanins obtained so far is still far away from the full potential of anthocyanin synthesis by plant cell cultures. Thus continuous efforts are required to further improve the regulation of anthocyanin

biosynthesis pathway, accumulation, storage and breakdown that will eventually lead to genetic manipulation of anthocyanin biosynthesis. In recent years, research on plant pigments attracted more and more attention, especially their isolation and purification in some of the pigment rich fruit and vegetable crops. In contrast, there have been few studies on flower crops which are otherwise well known for the presence of a variety of pigments with myriad colours. So keeping this in view, there is a need to isolate, characterize and *in vitro* production of these nutraceutical pigments in rose varieties. In this chapter an attempt has been made to discuss the salient findings in the light of available literature and scientific wisdom. The salient findings are discussed hereunder;

5.1 Evaluation of Indian rose varieties for anthocyanin content, total phenolic content and antioxidant activity

5.2 Estimation of total anthocyanin content, phenolic content and chlorophyll content of rose leaves

5.3 Characterization of anthocyanin pigments of promising rose varieties using HPLC

5.4 Characterization of phenolic compounds of promising rose varieties using HPLC

5.5 *In vitro* induction of anthocyanin pigments in a promising rose variety

## **5.1 Evaluation of Indian rose varieties for anthocyanin content, total phenolic content and antioxidant activity**

### **5.1.1 Evaluation of rose varieties for total anthocyanin content**

Variability studies in anthocyanin content in rose varieties were carried out. The results indicated that there was a significant difference among the rose varieties for total anthocyanin content.

The total anthocyanin content of rose varieties in two consecutive growing seasons (December 2014 and March 2015) was investigated. Significant variation was observed for anthocyanin content of all the varieties in both the seasons. Higher anthocyanin content was observed for all the varieties in December 2014 as compared to March 2015. These results suggest that the anthocyanin contents of rose petals was strongly influenced by environmental factors (temperature and light) and action of osmotic adjusters during periods of drought and low temperatures (Stintzing and Carle, 2004). Howard *et al.* (2003) evaluated the total anthocyanin content of

blueberry in two growing seasons and they found that total anthocyanin content was affected more by differences in environmental growth conditions. Temperature is one of the major environmental factors that affect anthocyanin accumulation; anthocyanin content was less under high temperatures in petunia (Shvarts *et al.* 1997), rose (Dela *et al.* 2003), chrysanthemum (Nozaki *et al.* 2006) and grape (Mori *et al.* 2005). These results are also in confirmation with the findings of Ginova *et al.* (2013) who reported that anthocyanin content varied in response to environmental conditions such as growth temperature and light intensity. Anthocyanin concentration increases under low temperatures in the flowers of *Plantago lanceolata* (Stiles *et al.* 2007).

The total anthocyanins ranged from  $0.24 \pm 0.02$  to  $578.10 \pm 6.76$  mg/100g on fresh weight basis. Lin *et al.* (2014) reported that total content of anthocyanin rose petals varied from 0.025 mg/g to 18.69 mg/g. In the present study, rose variety Ashwini, a dark red colour variety recorded highest anthocyanin content ( $578.10 \pm 6.76$  mg/100g fresh weight) followed by Dr. S.S. Bhatnagar ( $369.86 \pm 0.16$  mg/100g fresh weight) and Nehru Centenary ( $288.15 \pm 1.29$  mg/100g fresh weight). The minimum anthocyanin content was recorded in white colour varieties *i.e.*, Iceberg, Mridula and Shabnam ( $0.24 \pm 0.02$  mg/100g,  $0.53 \pm 0.03$  mg/100g and  $1.38 \pm 0.13$  mg/100g fresh weight). High anthocyanin content was observed in dark red flowers followed by deep pink, orange, pink, yellow and white. The level of anthocyanin content significantly differed between dark red and other colours of the flowers. Similar results were reported by Park *et al.* (2015); they found that the red and purple coloured chrysanthemum varieties showed high amounts of anthocyanins in their petals compared to other colours. Our study is also supported by Lee *et al.* (2011) who reported anthocyanin content of 375 mg/100g in red petals of Korean edible rose (*Rosa hybrida* cv. Noble Red). Qin and Xiaojun (2013) reported the anthocyanins content in petals of Yunnan edible rose was (353.56 $\pm$ 2.50 mg cyanidin 3,5-di-O-glucoside per 100g fresh weight. Gonzalez *et al.* (2012) investigated the anthocyanin content of 209 mg/100g of fresh weight in hibiscus. The anthocyanin content in the petals ranged from 13 mg/g to 18 mg/g in cultivar Magic and Relance respectively. The red and purple coloured varieties showed high amounts of anthocyanins in their petals. Influence from all the study indicated that red, purple, magenta colours have high anthocyanin content compared to other colours. Compared with the content of anthocyanins in vegetables, Takeoka *et al.* (2012) reported that the concentration of

anthocyanins in rhubarb ranged from  $19.8 \pm 1.5$  (Crimson Red) to  $341.1 \pm 41.6$  mg/100 g dry weight (Valentine).

### 5.1.2 Evaluation of rose varieties for total phenolic content

Among 50 rose varieties taken into the study the total phenolic content varied from  $5.21 \pm 0.39$  mg GAE/100g fresh weight to  $427.59 \pm 3.47$  mg GAE/100g fresh weight of petals. It was evident from the results that light colour variety showed lesser phenolic content whereas the bright red colour variety showed higher phenolic content in the petals. Our studies are supported by Garzon and Wrolstad (2009). They reported that the total phenolic content as 406 mg GAE/100g fresh weight in nasturtium flowers (*Trapaeolum majus*). Qin and Xiaojun (2013) reported a total phenolic content of  $2087.43 \pm 17.37$  mg gallic acid equivalents (GAE) per 100g fresh weight (FW) in rose petals. Our results are in confirmation with the findings of Vinokur *et al.* (2006). Roman *et al.* (2013) also reported that the total phenolic content in *Rosa canina*, varied from 326 mg/100g frozen pulp to 575 mg/100 g frozen pulp. Lim *et al.* (2014) screened different varieties of *Portulaca grandiflora* for total phenolic content. Their total phenolic content were ranging from 46.39-82.19 mg GAE/100g, 36.72-56.45 mg GAE/100g and 41.46-85.70 mg GAE/100g for methanolic, acetone and ethanolic extract, respectively.

### 5.1.3 Evaluation of rose varieties for antioxidant activity

Antioxidants are capable of deactivating free radical before the latter attack cells and biological targets (Atoui *et al.* 2005). Therefore, their activity will be critical for maintaining optimal protection. The antioxidant activities of fifty varieties of rose were investigated by three different assays. There was a significant difference between the antioxidant activities of the diverse genotypes. Our studies revealed that the CUPRAC values of rose varieties ranged from  $13.93 \pm 0.55$   $\mu$ M trolox/g for variety Iceberg to  $512.71 \pm 2.01$   $\mu$ M trolox/g for Ashwini.

FRAP values among rose varieties ranged from  $8.92 \pm 0.36$   $\mu$ mol trolox/g fresh weight to  $397.15 \pm 0.82$   $\mu$ mol trolox/g fresh weight. The DPPH value among rose varieties ranged from  $4.45 \pm 0.30\%$  (Iceberg) to  $93.47 \pm 0.19\%$  (Ashwini). As evident from results, high antioxidant activity was observed in dark red flowers followed by deep pink, orange, pink, yellow and white. Similar results were reported by Sadighara *et al.* (2012); they studied antioxidant activity of *Althaea officinalis* by CUPRAC and

found that reddish pink flowers of *Althaea officinalis* have more antioxidant activity than pink and white flowers. Result of the present investigation showed that the flower colour plays a role not only on the content of anthocyanins, but also on antioxidant power. Specifically, red colour is generally associated to high values of antioxidant power and white colour to the lowest ones. The possible reason for this variation in antioxidant content of rose varieties may be due to genotypic effect. Suzan and Sezai (2011) concluded that antioxidant activity was influenced by genotype in *Rosa* taxa. Red flowered cultivars reported higher antioxidant activity as compared to others. The results of present investigation are in agreement with the findings of Benvenuti *et al.* (2016) and Sadighara *et al.* (2012). Soare *et al.* (2015) reported that antioxidant activity of *Rosa canina* varied from 99.3 to 363.44  $\mu\text{mol TE/g fw}$ . Sayed *et al.* (2012) investigated the antiradical activity of flowers of fresh taif rose by 1,1-diphenyl picrylhydrazyl (DPPH.) and reported high antiradical activity with  $\text{SC}_{50}=49.44 \mu\text{g/ml}$ . Qin and Xiaojun (2013) also investigated antioxidant activities of rose by DPPH assay and found DPPH radical scavenging activity value was 2089 mg GAE/100g fresh weight. Our results are also in confirmation with the findings of Zeng *et al.* (2014). They evaluated the extracts from 19 Chinese edible flowers for antioxidant extracts. The results showed the extracts of *Paeonia suffruticosa*, *Paeonia lactiflora* and *Rosa rugosa* possessed stronger DPPH radical scavenging activity ( $94.221\pm 0.102$ ;  $93.739\pm 0.424$  and  $94.244\pm 0.163\%$ , respectively). The antioxidant activity of aqueous and methanol extracts of *Rosa damascena* flower petals was determined by Tatke *et al.* (2015). The  $\text{IC}_{50}$  values of aqueous and methanol extract were  $8.30\mu\text{g/ml}$  and  $4.85 \mu\text{g/ml}$  respectively by DPPH method. Petrova *et al.* (2016) investigated the antioxidant activity of five edible flowers (*Tagetes erecta* L., *Calendula officinalis* L., *Geranium macrorrhizum* L., *Bougainvillea spectabilis* Willd. and *Helianthus tuberosus* L.) and found that geranium showed highest antioxidant capacities using two methods, DPPH and FRAP assays were obtained for geranium (*Geranium macrorrhizum* L.) extracts as 242.9 and 106 mM TE/g fw, respectively.

#### **5.1.4 Correlation between total anthocyanin content, antioxidant activities and phenolic content**

A strong statistically significant positive correlation was found between total anthocyanin content, antioxidant activity and total phenolic content of rose petals. In

the present study, the results suggested that a strong positive correlation was found between antioxidant activities and the total anthocyanin content of rose petals. The strong correlation between these parameters indicates that varieties with high anthocyanin content constitute a good index for antioxidant activities. Our result is in agreement with Nowak and Gawlik-Dziki (2007). They reported strong positive correlation coefficient ( $r = 0.8485$ ) between the DPPH radical scavenging activity and phenolic content of rose. Ozgen *et al.* (2010) reported that the total anthocyanin content of *Sambucus canadensis* is highly correlated to antioxidant capacity values ( $r = 0.70-0.85$ ). Adina *et al.* (2010) also found that antioxidant activity of berries is directly proportional to the anthocyanin content. A strong and positive correlation was found between the total antioxidants (between the CUPRAC, FRAP and DPPH) value and total phenolic content. This result was in agreement with previous reports which revealed that the phenolic compounds contribute significantly to the antioxidant activity. Our results are well supported by Ginova *et al.* (2013). They reported that antioxidant capacity correlated well,  $r = 0.78-0.88$  with the contents of total polyphenols in rose petals. Burin *et al.* (2010) observed strong positive correlation ( $R = 0.956$ ) between the antioxidant activity and total phenolic content for the commercial grape juice. Kalt *et al.* (1999) found a strong correlation between antioxidant capacity and total phenols ( $r = 0.83$ ) in four species of berries. Katalinić *et al.* (2006) reported high linear correlation (0.9825) between the total phenolic content and FRAP values of selected medical plant extracts.

## **5.2 Estimation of total anthocyanin content, total phenol and chlorophyll content of rose leaves**

### **5.2.1 Evaluation of rose leaves for total anthocyanin content**

Among the 50 rose genotypes studied, the total anthocyanin content ranged from  $20.94 \pm 1.04$  to  $242.53 \pm 4.61$  mg/100g fresh weight of leaves. Variety Pusa Priya showed highest total anthocyanin content ( $242.53 \pm 4.61$  mg/100g fresh weight of leaves). The lowest total anthocyanin content was obtained by Shabnam, ( $20.94$ mg/100g fresh weight of leaves). Notably high amount of anthocyanin pigment was found in rose leaves. The exact role of anthocyanins is unclear: both photo protective (Gould *et al.*1995) and defensive (Coley and Aide, 1989) functions have been attributed to these pigments. It is possible that anthocyanins, which are often located in epidermal vacuoles, serve a similar photo protective function by absorbing

visible light when leaves have not fully developed photosynthetic competence. Beside these roles anthocyanin pigment also act as a source of antioxidants. Stone (1979) noted that anthocyanins in young developing leaves of *Iguanura geonomaeformis* and *Pinganga* species appear brown due to the masking effect of chlorophyll.

### 5.2.2 Evaluation of rose leaves for total phenolic content (TPC)

Recently roses have gained growing attention due to their antioxidant and other beneficial properties (Daels-Rakotoarison *et al.* 2002; Gao *et al.* 2000). The antioxidant properties of plant extracts were well connected with the content of polyphenols (Sroka, 2005). The total phenolic content varied from  $4.96 \pm 0.46$  mg GAE/100g fresh weight to  $134.32 \pm 1.20$  mg GAE/100g fresh weight of leaves among various varieties of *Rose* sp. taken into the study. Variety Pusa Priya ( $134.32 \pm 1.20$  mg GAE/100g fresh weight) showed highest phenolic content followed by Pusa Ajay ( $73.38 \pm 0.52$  mg GAE/100g fresh weight). Shabnam exhibited lowest phenolic content ( $4.96 \pm 0.46$  mg GAE/100g fresh weight). Total phenolic content of rose leaves was in the same range that has been previously reported for 17 rose species (Nowak and Gawlik-Dzik *et al.* 2007). Our results are in confirmation with the finding of Bitis *et al.* (2017) who reported total phenolic (203.8 mg gallic acid equivalents/g) content of *Rosa sempervirens* leaves.

### 5.2.3 Total chlorophyll content

In the present study it was found that the chlorophyll content depends upon genotypes. Among the rose genotypes studied, the total chlorophyll content ranged from  $0.25 \pm 0.01$  mg/g to  $1.45 \pm 0.01$  mg/g fresh weight of leaves. Variety Shabnam showed highest total chlorophyll ( $1.45 \pm 0.01$  mg/g). However, lowest total chlorophyll was obtained in variety Pusa Priya ( $0.25 \pm 0.01$  mg/g). Results obtained are in agreement with the finding of Dolatkahi *et al.* (2013) on *Rosa hybrida* cv. Avalanche, who indicated that content of total chlorophyll, chlorophyll a and chlorophyll b varied from 0.93 to 1.84 mg/g, 0.67 to 1.29 mg/g and 0.25 to 0.55 mg/g respectively.

### 5.2.4. Correlation between pigments and phenolic content of rose leaves

A strong statistically significant positive correlation ( $r = 0.971$ ) between total anthocyanin content and phenolic content of rose leaves was observed. Chlorophyll a, chlorophyll b and total chlorophyll content in leaves showed a statistically significant

negative correlation with total anthocyanin content. In accordance with this studies, the same relationship was detected between black and violet pepper groups by Stommel *et al.* (2014), indicating that decreasing of chlorophyll content was due to the high concentration of anthocyanin. Total phenol content also showed a statistically significant negative correlation with Chlorophyll a, chlorophyll b and total chlorophyll content in leaves.

### **5.3 Characterization of anthocyanin pigments of promising rose varieties using HPLC**

Chromatographic separation of anthocyanins is prerequisite for their identification by co-chromatography or by mass spectrometric analysis using tandem techniques. In the present study, anthocyanins were characterized from 13 selected rose varieties (Ashwini, Pusa Arun, Nehru Centenary, Dr. S.S. Bhatnagar, Raktima, Raktagandha, Pusa Bahadur, Bhim, Suryakiran, Pusa Virangana, Rose Sherbet, Surkhab and Pusa Ajay) using HPLC. Petal anthocyanins of these varieties were systematically identified and characterized by high-performance liquid chromatography (HPLC) coupled with photodiode array detector among thirteen rose varieties. Individual anthocyanins were identified according to the HPLC retention time, elution order and by comparison with authentic standards and published data.

Several peaks were detected in all varieties corresponding to different kinds of anthocyanin fractions. Among all the rose varieties, cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-O-glucoside were major anthocyanins identified according retention time, elution order and by comparison with external standards and published data. As standards of all anthocyanins were not available, the cyanidin-3-glucoside (C3G) was taken as standard to quantify the unknown peaks, which was expressed in terms of C3G equivalents. The total anthocyanin content estimated by HPLC ranged from 144.08 mg/100 g to 558.81 mg/100g. Total anthocyanin content was highest in Ashwini (558.81 mg/100g). Lowest anthocyanin content was found in Pusa Bahadur (144.08 mg/100 g). Maximum cyanidin 3,5-di-O-glucoside content was recorded in Ashwini (497.79 mg/100g), whereas maximum pelargonidin 3,5-di-O-glucoside content was recorded in Suryakiran (185.43 mg/100g). According to Eugster *et al.* (1991) anthocyanin types of roses flowers petals depend upon plant variety, being mostly cyanidin 3,5-di-O-glucoside, pelargonidin 3,5-di-O-glucoside and peonidin 3,5-di-O-glucoside. In our study, cyanidin 3,5-di-O-glucoside content was maximum

in red and pink coloured varieties, whereas in orange colour variety pelargonidin 3,5-di-O-glucoside content was maximum. Our results are well supported by the findings of Ludmila *et al.* (2015). They evaluated red, pink and orange coloured varieties of rose and reported cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-O-glucoside anthocyanin. They concluded that pink and red coloured varieties have more fraction of cyanidin 3,5-di-O-glucoside, whereas in orange coloured variety peak area of pelargonidin 3,5-di-O-glucoside exceeded that for cyanidin 3,5-di-O-glucoside. Anthocyanin types and contents may be affected by factors, including environmental stresses, variety, genetics and agronomic conditions (Jing *et al.* 2007; Lopes-da-Silva *et al.* 2007).

Our findings suggest that cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-O-glucoside were most predominant anthocyanin found in Indian roses using reversed-phase C18 column chromatography. These results are similar to the findings of Lee *et al.* (2011) who characterized anthocyanins from rose as cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-O-glucoside. The most predominant anthocyanin was cyanidin 3,5-di-O-glucoside in all the varieties. This result is in accordance with a previous study reporting that cyanidin 3,5-di-O-glucoside was detected as the absolutely dominant component in most rose varieties (Raimond *et al.* 1995). Qin and Xiaojun (2013) also strongly supporting our study, they detected 3 kinds of anthocyanins in rose and found that cyanidin 3,5-di-O-glucoside was the predominant constituent which accounted for approximately 94.9% of total anthocyanins according to the analysis results of high performance liquid chromatography-photodiode array detection (HPLC-PAD). Biolley *et al.* (1994) found cyanidin 3,5-diglucoside and pelargonidin 3,5-diglucoside in 100 cyanic cultivars of *Rosa x hybrida* by HPLC technique. Mikanagi *et al.* (2000) reported Eleven anthocyanins: 3-glucosides and 3,5-diglucosides of cyanidin (Cy), pelargonidin (Pg) and peonidin (Pn), 3-rutinosides and 3-o-coumaroylglucoside-5-glucosides of Cy and Pn, and Cy 3-sophoroside from 44 taxa of three sections (*Cinnamomeae* (*Rosa*) 26, *Chinenses* 8 and *Gallicanae* 10) and eight modern garden roses.

#### **5.4 Characterization of phenolic compounds of promising rose varieties using HPLC**

Indian rose varieties were not extensively studied; the present study revealed that they are a rich source of anthocyanins, flavonol glycosides and phenolic acids.

The identification of phenolic compounds present in rose petal crude extract was performed by comparing their UV-Vis spectra with published data and available standards. Several peaks were detected in all varieties corresponding to different kinds of phenolic fractions. A total of five different types of phenolic compounds were identified in crude extract. Quercetin was present in all the varieties. Quercetin content ranged from 2.38 to 603.88 mg/100g. Catechin was found in Nehru Centenary (2.75 mg/100g) and Suryakiran (3.87 mg/100g). The presence of catechin in rose hip has been previously reported (Hvattum, 2002; Guimarães *et al.* 2013). Epicatechin was found among all the varieties except Nehru Centenary and Pusa Arun. Epicatechin content ranged from 2.04 to 198.07 mg/100g. Rutin was observed in Raktagandha, Bhim, Raktima and Nehru Centenary. Rutin content ranged from 1.17 mg/100g to 9.44 mg/100g. 3-Hydroxy cinnamic acid was only observed in Pusa Ajay (0.98 mg/100g), which is in agreement with literature (Hvattum, 2002; Guimarães *et al.* 2013). The identified compounds in this study are in agreement with published data related to the phenolic compounds in rose hip (Sumere *et al.* 1993; Hvattum, 2002; Widen *et al.* 2012; Barros *et al.* (2013); Guimarães *et al.* 2013; Stănilă *et al.* 2015). The present results are in agreement with the findings of Kumar *et al.* (2008), who reported gallic acid, catechin, epicatechin, rutin, m-coumaric acid, quercitrin, myricetin, quercetin, apigenin and kaempferol in fresh flowers of *Rosa bourboniana* and *Rosa brunonii*.

## **5.5 *In vitro* induction of anthocyanin pigments in promising rose variety**

### **5.5.1 Callus induction and multiplication from explants of *Rosa hybrida* var. Ashwini**

#### **5.5.1.1 Selection of explants for *in vitro* culture of *Rosa hybrida* var. Ashwini**

Both Leaf and petal explants varied considerably with regard to culture initiation, callus induction and contamination percentage. *In vitro* cultures are usually not microorganism free, as microorganisms can survive the surface decontamination within a plant body. Bacteria may appear soon after the initiation of cultures but often only after several passages (Cassels, 2011). There was marked difference in microbial contamination in both explants. This difference may be due to endogenous level of microbes present in explants tissue. It is evident from the experimental results that minimum microbial contamination was observed when petal was used as explants as

compared to leaf explants. Earliest callus induction was noticed in leaf explants as compared to petal explants. Similar results were reported by Ram (2012).

#### **5.5.1.2 Standardization of pre-treatments in rose**

Pre-treatments in *in vitro* cultures are generally applied to minimize the microbial load. Surface contaminants are comparatively easy to control as both fungicidal and bactericidal compounds can effectively be used as pre-treatments to eliminate such contaminants (Enjalric *et al.* 1988). Pre-treatment improved establishment of *in vitro* culture of different plant species (George and Sherrington, 1984). As evident from the results, minimum microbial contamination was observed when carbendazim (0.2%) + mancozeb-45 (0.2%) + 8-HQC (200mg/l) was used for 15 minutes. This treatment is more efficient depending upon the nature of fungi or bacteria infecting the explant excised. Carbendazim and mancozeb are common fungicides, and 8-HQC has bactericidal action and these effectively control different fungal and bacterial contaminations in the explant. Results obtained signified that pre-treatments not only reduced the microbial contaminations (15.66±0.59%) but also increased the explant survival (83.28±0.51%) rate when applied with optimum time duration. This finding is in agreement with previous results on rose (Choudhary, 1991; Prasad, 1995; Ram, 2012) and chrysanthemum (Ravindra, 2003). Our results are also supported with the findings of the da Camara Machado *et al.* (1991) and Krishnamurthy *et al.* (2001), who noticed improved explant survival and less microbial contaminations in apple and tuberose, respectively. On the other hand, treatment without the use of any fungicide, bactericide failed due to high microbial contamination of all cultured explants.

#### **5.5.2 Standardization of culture medium for callus induction and multiplication from petal explants of *Rosa hybrida* var. Ashwini**

A callus culture system offers many advantages as a model system for several biological investigations. Callus cultures have been widely used in rose for various physiological studies (Weinstein *et al.* 1962). Growth and morphogenesis of plant tissue in *in vitro* is mostly governed by culture media composition. Murashige and Skoog's (1962) medium (MS) was found to be the most commonly used for rose organogenesis and regeneration from the cultured explants (Pati *et al.* 2006). As evident from the results, increasing level of 2,4-D resulted in improvement of

callusing. Highest induction coefficient per cent was recorded ( $94.67 \pm 1.11\%$ ) when 2,4-D was added at 6.0 mg/l. Usually auxins promote callus induction (Yang *et al.* 2009, Mousavi *et al.* 2012); however others found that auxin-cytokinin combination were much important for callus induction in some plant species (Dhar and Joshi 2005, Lim *et al.* 2009). Such variations may be due to the type and species of plant, endogenous hormonal balance and explant type. The suitable PGRs for callus induction vary among plant species. In the present investigation, healthy and vigorous callus with high induction response was recorded with auxin only. MS medium supplemented with 6 mg/l 2,4-D was rated best in rose petal explants for induction coefficient per cent, earliest callus induction and callus quality.

The results of present investigation are in accordance with findings of Anand *et al.* (2017), who reported that when MS medium supplemented with 6 mg/l 2,4-D recorded maximum induction coefficient (98.75%) and minimum days (8.50) was required for callus initiation in callus culture of bougainvillea. There are a number of studies which describes the effect of 2,4-D on callus initiation and callus growth in various cultured explants. Ram (2009) reported that 4 mg/l 2,4-D in culture medium increased the callusing frequency and recorded earliest callus induction in leaf explants of rose. Yang *et al.* (2009) reported the highest frequency of callus induction in *Euphorbia helioscopia* cultures when cultured on MS medium supplemented with 3.0 mg/l 2,4-D. Jala (2014) cultured rose stem node with axillary bud on MS medium supplemented with different concentrations of 2,4-D. It was found that MS medium supplemented with 0.5 mg/l 2,4-D gave the highest number of callus. El-Nabarawy *et al.* (2015) reported that 2,4-D was the most effective in inducing and maintaining callus cultures of ginger derived from shoot tip segments of auxiliary buds. Visual observations suggested that compact and creamish white vigorous callus was obtained from cut and punctured surface of petal explants. High callusing on outer and inner punctured places was observed. Better morphogenic responses in wounded explants were also described in other plant species (D' Onofrio and Morini, 2003, Pacheco *et al.* 2008). This may be a reflection of different endogenous hormonal levels and cell differentiation levels in tissue segments (Korocho *et al.* 2002). It is assumed that the cell proliferation that started at the injured part of petal segment may have been due to the accumulation of auxins at the point of injury, which stimulated cell proliferation in the presence of PGRs and their derivatives. The results are consistent with a

hypothesis which postulates that the accumulation of auxins at the point of injury stimulated cell proliferation (Ahmad *et al.* 2010).

#### **5.5.2.1 Effect of 2, 4-D levels on callus biomass accumulation *Rosa hybrida* var. Ashwini**

As indicated in the results, petal derived calli showed a steady growth. Maximum fresh cell weight ( $490.86 \pm 0.66$  mg) and dry cell weight ( $98.89 \pm 0.99$  mg) of callus was recorded in when MS medium supplemented with 2,4-D at 6.0 mg/l. Duangporn and Siripong (2009) found that 2,4-D had more effect on callus growth individually when compared to NAA. These results are in consistence with the finding of Ram (2009) ; Anand *et al.* (2017), who recorded high callus biomass accumulation with high levels of 2, 4-D in rose and bougainvillea respectively. El-Nabarawy *et al.* (2015), reported that increasing level of 2,4-D (0.5 and 1.0 mg/l) combined with the increasing level of BA enhanced the callus fresh and dry weight gradually. Verma *et al.* (2012) found that, MS media supplemented with 1.0:0.5 and 0.5:1.0 mg/l of 2, 4-D: BA were found to be the most promising media in relation to callus biomass.

To know the actual gain in callus biomass accumulation the ratio of fresh- to dry cell weight (FCW: DCW), an index of cell water content, was calculated. Callus contains approximately 96.5 per cent water content of the fresh weight. The callus with high relative dry weight has lower influx of water. Among the different treatments, MS medium supplemented with 2, 4-D at 6.0 mg/l resulted in the lowest FCW: DCW ratio ( $4.83 \pm 0.14$ ) which indicated that the callus produced was not watery and gained better biomass accumulation. Similar results were obtained by Ram (2012); Anand *et al.* (2017). Explants cultured on MS medium without the plant growth regulator did not produce any callus; so there was no fresh- and dry cell weight value for this treatment.

#### **5.5.2.2 Correlation between callogenesis and growth parameters**

As evident from the results, strong statistically significant positive correlation existed between days to callus induction, fresh- and dry cell weight. The direct correlation between different parameters such as explant type, callus induction, callus biomass accumulation and regeneration capacity were reported earlier in some plant

species by researchers (Bírsin *et al.* 2001; Gandonou *et al.* 2005; Mohebodini *et al.* 2011; Ram *et al.* 2015).

### **5.5.3 Standardization of different concentrations of sucrose and ABA in the culture media for anthocyanin pigment induction from *in vitro* induced rose callus**

On the basis of the knowledge of the biosynthetic pathways, several organic compounds have been added to the culture medium in order to enhance the biosynthesis of secondary metabolites. The exogenous factors such as biosynthetic precursor to culture medium may increase the yield of the desired product (Namdeo, 2007). The stimulation of anthocyanin biosynthesis in plant cell cultures has been shown to depend on numerous factors, such as growth regulators, light, temperature, phosphate, nitrate, sugar and initial inoculum density (Hall and Yeoman, 1986; Zhang *et al.* 1997; Sato *et al.* 1996; Takeda *et al.* 2003). Plant cell and tissue-based production of anthocyanins and other natural colorants is now being viewed with renewed interest (Lila, 2004). To enhance the productivity, optimization of medium composition or addition of enhancer such as elicitor has been investigated (Cormier *et al.* 1990). Elicitors interact with plant membrane receptors, generating signal compounds, which subsequently activate specific genes for enzymes involved in secondary metabolite biosynthesis (Brooks and Watson, 1991). The augmentative effects of sugars and ABA on anthocyanin accumulation have been reported mainly in the cultured callus of various species (Simões *et al.* 2009; Chan *et al.* 2010; Ram *et al.* 2011; Usha *et al.* 2014). Therefore, EM medium supplemented with varied sucrose and ABA levels were tested in order to enhance the biosynthesis of anthocyanins in rose callus culture. As indicated in results, all the cultures initiated on the tested sucrose and ABA levels were able to synthesize anthocyanins, but they varied in response coefficient, number of days required for pigment initiation and intensification, fresh weight and anthocyanin content. As evident from the results, the stereozoom microscopic study showed that the callus tissues proliferated on optimal sucrose level (80g/l) and exhibited the presence of more red pigmentation compared to others, thus clearly showing higher anthocyanin biosynthesis. This reduction may be due the fact that elevated sucrose levels caused high osmotic stress in the culture medium and in response to which the uptake of nutrients might have decreased. Further, these results showed that anthocyanin biosynthesis in rose callus were

associated with the supplementation of sucrose concentration in the EM medium. Results of the present investigation, suggest that the addition of sucrose up to an optimum level in the culture medium showed an up-regulated anthocyanin biosynthesis in the callus cultures of *Rosa hybrida* var. Ashwini. Although sucrose is an indispensable nutrient, it also acts as an osmotic agent when used at high concentrations (Nagira and Ozeki, 2004). The present experiment showed earliest and higher accumulation of anthocyanins in response to optimum sucrose level (80 g/l), which may be attributed to the high osmotic potential of culture and in response to that the cultures biosynthesized the secondary metabolite, *i.e.* anthocyanins. The present findings are supported by number of researchers. Higher (6-7%) level of sucrose in culture medium resulted more accumulation of anthocyanin in *Vitis vinifera* (Cormier *et al.* 1990) and *Rosa hybrida* cv. 'Charleston' (Hennayake *et al.* 2006). Sucrose increases the osmotic potential of culture medium which imposes the osmotic stress in the cultures, thereby inducing the anthocyanin biosynthesis (Cállebaut *et al.* 1990; Konczak-Islam *et al.* 2003; Hennayake *et al.* 2006; Mathur *et al.* 2010; Ram *et al.* 2011). Anthocyanin accumulation was promoted in cell suspension culture of *Melastoma malabathricum* using sucrose as osmoticum (See *et al.* 2011). Our results are in accordance with the findings of Ram *et al.* (2011), they found that the medium containing 7 per cent sucrose led to the higher anthocyanin induction in callus cultures of *Rosa hybrida*. They suggested that osmotic-stress imposed by high sucrose levels favoured production of anthocyanins. In anthocyanin biosynthesis, sugars could act as precursors of metabolic processes and as a signalling molecule for promoting gene expression (Solfanelli *et al.* 2006; Weiss, 2000). Genes coding for dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS) were up-regulated and the accumulation of anthocyanins was strongly increased by sucrose in grape cells (Gollop *et al.* 2001, 2002). Dai *et al.* (2014) suggested that the sugar-induced enhancement of anthocyanin accumulation results from altered expression of regulatory and structural genes (especially UDP-glucose:anthocyanidin 3-O-glucosyltransferase), together with massive reprogramming in signalling transduction pathways.

In the present investigation, anthocyanin accumulation was enhanced in petal derived rose calli in response to ABA at certain levels (30  $\mu$ M). Abscisic acid was reported to show enhancing effects on the synthesis of anthocyanin in many plant

species like tomato hypocotyl (Carvalho *et al.* 2010), grapevine leaves (Pirie and Mullins, 1976) and torenia shoots (Nagira *et al.* 2006) and our results are in accordance with that. This may be due to stress induced by ABA in culture medium. This is supported by an increase in the expression of the UFGT (UDP- glucose: flavonoid-3-O-glycosyl tranferase coding for a specific to the anthocyanin pathway) and VvMYBA1 genes (coding for a transcriptional regulator controlling anthocyanin biosynthesis), as well as other genes coding upstream located enzymes (PAL- phenylalanine ammonia-lyase, CHI-chalcone isomerase, CHS- chalcone synthase etc.). Moreover, it was also postulated that ABA induces phenylalanine ammonia-lyase (PAL), a key enzyme for the biosynthesis of anthocyanin and other phenolic compounds.

When sucrose (80 g/l) and ABA (20  $\mu$ M) were applied in combination, promotive effect was pronounced synergistically on anthocyanin production. This implies that the combination of the best level of sucrose and ABA in the culture medium had a positive effect on anthocyanin production in comparison with the use of 20  $\mu$ M ABA alone. Our results are in agreement with the findings of Hiratsuka *et al.* (2001). It is evident from the results that as the level of sucrose in the culture medium increased, the growth of callus tissues was significantly decreased. The maximum growth of calluses in terms of biomass accumulation (FW) was recorded in control, *i.e.* EM medium only (396.71 $\pm$ 1.25 mg). Minimum fresh cell weight (273.64 $\pm$ 0.68 mg) was recorded in treatment (EM+80 g/l sucrose). Thus, the high sucrose level adversely affected the callus biomass accumulation which may be linked to the high osmotic stress brought about by high sucrose level in the culture medium which may have resulted in lesser uptake of nutrients and thereby, reduced callus biomass accumulation. This result is in line with the findings of Sato *et al.* (1996). They observed decreased cell growth in media with high sucrose concentration. This decrease in cell growth was attributed to inhibition of nutrient uptake in strawberry suspension culture due to an increase in the osmotic potential or high viscosity of the medium.

#### **5.5.3.1 Correlation between different parameters recorded for anthocyanin induction by sucrose and ABA in petal derived calli of *Rosa hybrida* var. Ashwini**

The strong correlation between response coefficient and anthocyanin content indicates that callus cultures with high response coefficient constitute a good index for ability to produce more anthocyanins. Furthermore, significant positive correlations between days required for pigment initiation and fresh cell weight of callus was found. Strong correlation was detected between days required for pigment initiation, pigment intensification and fresh cell weight. However, a statistically significant relationship was detected between days required for pigment intensification and fresh cell weight and pigment content, indicating the dependence of these parameters on each other and in increasing the recovery of the final product. Negative correlation was detected between anthocyanin content and fresh cell weight. These results are in agreement with the findings of (Ram *et al.* 2011, 2013).

## 6. SUMMARY AND CONCLUSION

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The present investigation entitled “**Isolation and Characterization of Anthocyanin Pigments from Indian Rose Varieties as a Potential Source of Nutraceuticals**” was carried out at the Division of Floriculture and Landscaping, Division of Agricultural Chemicals and Central Tissue Culture Laboratory, ICAR-Indian Agricultural Research Institute, New Delhi-110012, during 2014-2016. To achieve the objectives, 50 varieties of rose were used as plant material and experiments were conducted to find out the best rose varieties for high anthocyanin content with higher antioxidant activities and to decipher the anthocyanin profiles of Indian bred roses, as well as *in vitro* induction of these nutraceutical pigments with the following objectives.

1. Isolation and quantification of total anthocyanin pigments from Indian rose varieties.
2. Identification and characterization of anthocyanin pigments from selected rose varieties.
3. *In vitro* induction of nutraceutical pigments in promising rose variety.

The salient findings of the investigation are summarized below

### 6.1 Estimation of total anthocyanin content, total phenolic content and antioxidant activities in rose petals

- The total anthocyanin content of rose varieties varied significantly among all the varieties in two consecutive growing seasons (December 2014 and March 2015). Higher anthocyanin content was observed for all the varieties in December 2014 as compared to March 2015.
- The total anthocyanin content ranged from  $0.24 \pm 0.02$  to  $578.10 \pm 6.76$  mg/100g on fresh weight basis.
- Ashwini, a dark red coloured variety recorded highest anthocyanin content ( $578.10 \pm 6.76$  mg/100g fresh weight) followed by Dr. S.S. Bhatnagar ( $369.86 \pm 0.16$  mg/100g fresh weight), Nehru Centenary ( $288.15 \pm 1.29$  mg/100g fresh weight) and Pusa Arun ( $282.60 \pm 2.74$  mg/100g fresh weight).
- Higher anthocyanin content was observed in dark red flowers followed by deep pink, orange, pink, yellow and white.

- Among all the varieties tested, Ashwini ( $427.59 \pm 3.47$  mg GAE/100g fresh weight) recorded highest phenolic content and Iceberg showed lowest phenolic content ( $5.21 \pm 0.39$  mg GAE/100g fresh weight).
- The highest antioxidant activity estimated by CUPRAC, FRAP, DPPH were observed in variety Ashwini. Ashwini had CUPRAC value of  $512.71 \pm 2.01$   $\mu$ mol trolox/g fresh weight; FRAP value of  $397.15 \pm 0.82$   $\mu$ mol trolox/g fresh weight and DPPH value of  $93.47 \pm 0.19\%$  respectively.
- Ashwini, Dr. S.S. Bhatnagar, Nehru Centenary, Pusa Arun and Bhim were rated as best varieties for high phenolic content and high antioxidant activities.
- Varieties with higher anthocyanin content recorded high phenolic content as well as high antioxidant activities.
- Statistically significant positive correlation was observed between total anthocyanin content, antioxidant activity and total phenolic content of rose petals.

## **6.2 Estimation of total anthocyanin content, total phenol and chlorophyll content of rose leaves**

- Pusa Priya was rated as the best variety for total anthocyanin content and total phenolic content whereas, lowest anthocyanin content and phenolic content was observed in variety Shabnam.
- The total chlorophyll content was found maximum in Shabnam ( $1.45 \pm 0.01$  mg/g). However, lowest total chlorophyll was obtained in variety Pusa Priya ( $0.25 \pm 0.01$  mg/g).
- The rose varieties with higher anthocyanin content in their leaves recorded lowest chlorophyll content.

## **6.3 Characterization of anthocyanin pigments of promising rose varieties using HPLC (High Performance Liquid Chromatography)**

- Two major anthocyanins were identified in all the varieties *i.e.* cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-O-glucoside.
- The total anthocyanin content estimated by HPLC ranged from 144.08 mg/100g to 558.81 mg/100g. Total anthocyanin content was highest in Ashwini (558.81 mg/100g).

- In all the red and pink coloured varieties, cyanidin 3,5-di-O-glucoside was predominant anthocyanin, whereas in orange colour variety pelargonidin 3,5-di-O-glucoside was predominant anthocyanin.
- Ashwini recorded highest cyaniding 3,5-di-O-glucoside content (497.79 mg/100g), whereas minimum cyanidin 3,5-di-O-glucoside content was recorded in Suryakiran (29.28 mg/100g).
- Highest pelargonidin 3,5-di-O-glucoside content was recorded in Suryakiran (185.43 mg/100g), whereas minimum pelargonidin 3,5-di-O-glucoside content was recorded in Pusa Bahadur (0.39 mg/100g).

#### **6.4 Characterization of phenolic compounds of promising rose varieties using HPLC**

- A total of 5 different types of phenolic compounds (quercetin, catechin, epicatechin, rutin and 3-Hydroxy cinnamic acid) were identified in rose petal extract.
- Quercetin content ranged from 2.38 to 603.88 mg/100g among all the selected varieties.
- Catechin was found in the varieties, Nehru Centenary (2.75 mg/100g) and Suryakiran (3.87 mg/100g).
- Epicatechin was found in all the varieties except Nehru Centenary and Pusa Arun.
- Rutin was observed in the varieties, Raktagandha, Bhim, Raktima and Nehru Centenary.
- 3-Hydroxy cinnamic acid was only observed in Pusa Ajay (0.98 mg/100g).

#### **6.5 *In vitro* induction of anthocyanin pigments in promising rose variety**

##### **6.5.1 Callus induction and multiplication from explants of *Rosa hybrida* var. Ashwini**

- In rose, petal was found to be the most suitable explant which exhibited highest callusing frequency (69.49±0.28%) and maximum survival percentage (83.62±0.38%).

- Pre-treatment with carbendazim (0.2%) + mancozeb-45 (0.2%) + 8-HQC (200 mg/l) was used for 15 minutes resulted in minimum culture contamination ( $15.66\pm 0.59\%$ ) and maximum survival ( $83.28\pm 0.51\%$ ) of explants.

#### **6.5.2 Standardization of culture medium for callus induction and multiplication from petal explants of *Rosa hybrida* var. Ashwini**

- In rose, highest induction coefficient per cent ( $94.67\pm 1.11\%$ ) and earliest callus induction ( $8.67\pm 0.67$  days) was observed when MS medium was supplemented with 2, 4-D (6.0 mg/l).
- Maximum fresh cell weight ( $490.86\pm 0.66$  mg) and high cell dry biomass production ( $98.89\pm 0.99$  mg) was recorded when MS medium supplemented with 2, 4-D at 6.0 mg/l.

#### **6.5.3 Standardization of different concentrations of sucrose and ABA in the culture media for anthocyanin pigment induction from *in vitro* induced rose callus**

- In rose petal calli, earliest pigment induction, intensification and higher anthocyanin accumulation was observed when EM medium was supplemented with 80g/l sucrose.
- Anthocyanin accumulation was enhanced in petal derived rose calli in response to ABA at certain levels (30  $\mu$ M). Higher ABA concentration in the EM medium reduced the pigment accumulation.
- Sucrose was found to have more profound effect on anthocyanin induction.
- A significant positive correlation was found between response coefficient and anthocyanin content ( $r = 0.960$ ).

Results obtained in the present investigation provided useful information on anthocyanin content, total phenolic content and antioxidant activities of rose, their isolation and characterization by using HPLC, as well as *in vitro* induction of these nutraceutical pigments, for efficient utilization of roses in food, nutraceutical and pharmaceutical industry, *etc.* The recommendations from this study will provide opportunities for the rose growers to diversify into novel niche areas.

## **Isolation and Characterization of Anthocyanin Pigments from Indian Rose Varieties as a Potential Source of Nutraceuticals**

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### **ABSTRACT**

Rose (*Rosa hybrida* L.) is one of the most important commercial flower crop with wide range of colour spectre, mainly attributed to the presence of anthocyanin pigments. The potential of anthocyanin pigments from Indian rose varieties, however, is not completely understood. A study was therefore, conducted to find out the best rose varieties for high anthocyanin content with higher antioxidant activities and to decipher the anthocyanin profiles of Indian bred roses, as well as *in vitro* induction of these nutraceutical pigments. Among 50 rose varieties, Ashwini recorded highest total anthocyanin content, total phenolic content and highest antioxidant activities (CUPRAC- Cupric reducing antioxidant capacity, FRAP- Ferric Reducing Antioxidant Potential and DPPH free radical scavenging activity) on fresh weight basis followed by Dr. S.S. Bhatnagar, Nehru Centenary, Pusa Arun and Bhim. Significant positive correlation was observed between total anthocyanin content, antioxidant activity and total phenolic content of rose petals. Rose varieties which recorded highest anthocyanin content were analysed for their anthocyanin composition by High Performance Liquid Chromatography (HPLC) coupled with a photodiode array (PDA) detection. Two anthocyanins, cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-O-glucoside were characterized as major components in all selected rose varieties. In all the red and pink coloured varieties, cyanidin 3,5-di-O-glucoside was predominant anthocyanin, whereas in orange colour variety pelargonidin 3,5-di-O-glucoside was predominant anthocyanin. Five different types of phenolic compounds (quercetin, catechin, epicatechin, rutin and 3-Hydroxy cinnamic acid) were identified in rose petal extract by High Performance Liquid Chromatography. Anthocyanin pigments were also induced *in vitro* in promising rose variety, Ashwini. Best callusing in rose petal explants was obtained on MS medium supplemented with 2, 4-D (6.0 mg/l). In rose petal calli, earliest pigment induction, intensification and higher anthocyanin accumulation was observed when EM medium was supplemented with 80g/l sucrose. Sucrose was found to have more profound effect on anthocyanin induction. A significant positive correlation was found between response coefficient and anthocyanin content. It is concluded that the rose can be

exploited as a potential source of nutraceuticals for its constitutive anthocyanins and its biological activity. Its nutraceutical potential will definitely provide momentum towards further research for its application in food systems especially in functional foods.

## भारतीय गुलाब की किस्मों से पौष्टिक-औषधीय पदार्थों के संभावित स्रोत के रूप में एन्थोसयानिन्स वर्णको (पिगमेंट्स) का पृथक्करण और उनका निरूपण

### सार

गुलाब (रोजा हाइब्रिडा एल.) सबसे महत्वपूर्ण वाणिज्यिक फूलों की फसलों में से एक है, जोकि अपनी विस्तृत वर्णकों की श्रृंखला के लिए प्रसिद्ध है, इस श्रृंखला में एन्थोसयानिन पिगमेंट्स मुख्य वर्णक है। फिर भी भारतीय गुलाब की किस्मों से एन्थोसयानिन्स वर्णको (पिगमेंट) की क्षमता को पूरी तरह से जांचा नहीं गया है। इसलिए, उच्च ऑक्सीकरण प्रतिरोधी (एंटीऑक्सीडेंट) गतिविधियों के साथ उच्च एन्थोसयानिन्स मात्रा वाली सबसे अच्छी गुलाब की किस्मों का पता लगाने के लिए तथा भारतीय नस्ल के गुलाब की एन्थोसयानिन्स प्रोफाइल को समझने के साथ-साथ इन पौष्टिक-औषधीय वर्णको (पिगमेंट्स) के इन-विट्रो प्रवर्तन के बारे में भी पता लगाने हेतु यह अध्ययन किया गया। गुलाब की 50 किस्मों में से, एन्थोसयानिन्स और फिनोलिक की कुल मात्रा और सबसे अधिक एंटीऑक्सीडेंट गतिविधियों (CUPRAC, FRAP and DPPH) के साथ ताज़ा फूलों के वजन (ग्राम) के आधार पर "अश्विनी" को उच्चतम तथा इसके बाद डॉ एसएस भटनागर, नेहरू सेंटेनरी, पूसा अरुण और भीम में अधिक मात्रा में पाए गए। गुलाब की पंखुड़ियों में मौजूद एन्थोसयानिन की मात्रा, एंटीऑक्सीडेंट गतिविधि और कुल फिनोलिक की मात्रा के बीच महत्वपूर्ण सकारात्मक सहसंबंध देखा गया है। गुलाब की वे किस्में, जिनमें एन्थोसयानिन्स की उच्चतम मात्रा दर्ज की गई थी, उनका फोटोडायोड एर्र (पीडीए) के साथ उच्च निष्पादन तरल क्रोमैटोग्राफी (एचपीएलसी) द्वारा उनके एन्थोसयानिन्स संरचना के लिए विश्लेषण किया गया था। दो एन्थोसयानिन्स, साइनाइडिन 3,5-डी-ओ-ग्लूकोसाइड और पेलेर्गोनिडिन 3,5-डी-ओ-ग्लूकोसाइड, गुलाब की सभी चयनित किस्मों में प्रमुख घटकों के रूप में वर्णित थे। सभी लाल और गुलाबी रंग की किस्मों में, साइनाइडिन 3,5-डी-ओ-ग्लूकोसाइड प्रमुख एन्थोसयानिन्स था, जबकि नारंगी रंग की किस्मों में पेलेर्गोनिडिन 3,5-डी-ओ-ग्लूकोसाइड प्रमुख एन्थोसयानिन्स था। उच्च निष्पादन तरल क्रोमैटोग्राफी द्वारा गुलाब की पंखुड़ी के निष्कर्षण में पांच अलग-अलग प्रकार के फेलोनिक यौगिक कैटेर्सिटिन, कैटचिन, एपिकैतचिन, रुटिन और 3-हाइड्रोक्सी सिनामिक एसिड की पहचान की गई। गुलाब की सर्वोत्तम किस्म 'अश्विनी' में एन्थोसयानिन्स रंगद्रव्यों का भी इन-विट्रो उत्प्रेरित किया गया। गुलाब की पंखुड़ी में, 2, 4-डी (6.0 मिलीग्राम प्रति लीटर) के एमएस मीडियम पर सर्वश्रेष्ठ कॉलसिंग प्राप्त किया गया। ईएम मीडियम 80 ग्राम/लीटर सूक्रोज रखने पर, गुलाब की पंखुड़ी में कॉली, प्रारंभिक वर्णक (पिगमेंट) प्रवर्तन, एन्थोसयानिन्स का तीव्रीकरण और उच्च संचयन देखा गया। एन्थोसयानिन्स के प्रवेश करवाने पर सूक्रोज का अधिक गहरा प्रभाव देखा गया। प्रतिक्रिया गुणांक और एन्थोसयानिन्स की मात्रा के बीच एक महत्वपूर्ण सकारात्मक सहसंबंध पाया गया था। यह निष्कर्ष निकाला गया है कि गुलाब को अपने गठनक एन्थोसयानिन्स और इसकी जैविक गतिविधि के लिए पौष्टिक-औषधीय पदार्थों के संभावित स्रोत के रूप में प्रयुक्त किया जा सकता है। निश्चित रूप से इसकी पौष्टिक क्षमता, खाद्यान्न प्रणालियों विशेषकर क्रियाशील खाद्य में इसके इस्तेमाल पर अनुसंधान को बल प्रदान करेगी।

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## APPENDIX

### Monthly record of weather parameters from January 2014 to December 2015 at IARI

Month	Temperature (°C)		Mean Relative Humidity (%)	Rainfall (mm)
	Maximum	Minimum		
<b>2014</b>				
January	18.6	6.8	81.6	18.6
February	21.4	7.51	79.5	63.5
March	27.2	12.7	69.0	63.5
April	34.8	17.9	56.8	16.4
May	38.7	22.6	57.9	79.6
June	41.8	26.4	53.0	59.6
July	35.9	26.3	75.6	227.8
August	35.8	25.8	72.8	98.9
September	34.0	24.0	70.9	124.3
October	33.2	18.8	61.6	0
November	28.3	10.6	61.0	0
December	20.6	6.73	76.40	26.4
<b>2015</b>				
January	16.9	6.8	83	35.8
February	24.6	10.6	70.2	0
March	27.2	13.1	71.1	201.8
April	33.9	19.3	60.4	51.8
May	41.1	24.0	45.4	0.8
June	38.5	25.6	64.0	124.4
July	33.8	25.6	76.3	381.0
August	33.5	25.5	78.2	261.3
September	35.2	23.6	62.6	67.2
October	33.8	17.5	64.4	0.0
November	28.1	11.9	69.1	2.2
December	22.6	6.11	72.0	0.0
Average	(30.8)	(17.5)	(68)	1123