SEPARATION OF ANTHOCYANINS FROM HIMALAYAN YELLOW RASPBERRY RUBUS ELLIPTICUS USING HIGH-RESOLUTION LIQUID CHROMATOGRAPHY−ELECTROSPRAY IONIZATION−TANDEM MASS SPECTROMETRY

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Abstract
Five anthocyanin derivatives were identified in the fruit of *Rubus ellipticus (Hisalu)* by using advanced analytical techniques, HRLC-ESI-MS/MS. Five major anthocyanin derivatives of cyanidin, petunidin, and delphinidin were extracted from the fruit of *Rubus ellipticus* (RE) and relative concentrations of the identified anthocyanin derivatives of petunidin, delphinidin, cyanidin were found to be: 0.18 ± 0.02 mg/ g, 0.26 ± 0.02 mg/ g and 0.058 ± 0.02 mg/g fruit weight respectively, and for cyanidin-3-galactoside/glucoside and for petunidin-3-*O*-glucose the relative concentration was found to be 0.078 ± 0.02 mg/g fruit weight. Total anthocyanin content (TAC) was found to be: 0.58 ± 0.02 mg/g fruit weight. The content of anthocyanin was found to be higher in the fruit of *Rubus ellipticus* compared with *Prunus carmeniaca, Pyracantha crenulata*, and *Celtis australis* fruits.

Keywords: *Rubus ellipticus;* Anthocyanins; Cyanidin; Petunidin; Delphinidin, TAC

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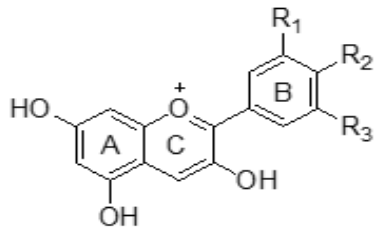
1. Introduction

Anthocyanins have applications in food, pharmaceutical and cosmetic industries on account of their inherent colors and biological activities[1].Anthocyanins are poly-hydroxylated/methoxylated glycosides or acylglycosides of anthocyanidins which are oxygenated derivatives of 2-phenyl benzopyrylium or flavylium salts[32] **(Figure 1)**.



Anthocynidin	R_1	R_2	R_3	MW
pelargonidin (Pg)	H	OH	H	271
cyanidin (Cy)	OH	OH	H	287
delphinidin (Dp)	OH	OH	OH	303
peonidin (Pn)	OMe	OH	H	301
petunidin (Pt)	OMe	OH	OH	317
malvidin (Mv)	OMe	OH	OMe	331

Figure.1 Chemical structures structure of anthocyanins

Anthocyanins are responsible for blue purple and red color in plants. They are mostly abundant in berries and other fruits.[2] Anthocyanins are natural colorants. Due to this property anthocyanins are highly demanded in commercial markets such as food, beverage and in pharmaceutical industries because synthetic colors are showing adverse effects on human health. Generally six common anthocyanins are found in plants as described in figure 1.[3, 4]

Several kinds of research on anthocyanidins have been discontinued on account of the difficulty of unraveling the particular distribution and chemical structure of anthocyanins in several fruits, flowers and other sources. Nevertheless, approximately 600 anthocyanins have been studied by using various experimental techniques.[5, 6]However, the knowledge regarding their chemical structure remains incomplete. HRLC-ESI-MS/MS is a modern technique to identify, separate and quantify the compounds derived from plant residues. This is possible because of its high sensitivity in elucidating the structure of compounds and its accuracy in mass measurement up to 0.001 amu within the secondary metabolites.[7]This technique has been used in structure elucidation and distribution of anthocyanins in foods.

Uttarakhand is a Himalayan state which is rich in plants. These plants have been exploited by local communities for a variety of uses such as extraction of pharmaceuticals, medicine, drugs, and edible foods to name but a few of them. Within the Indian Himalayan Region, over 675 wild edibles are known[8]. The North Himalayan Region, usually known as Kumaon hills. Kumaon hills fruits have great potential to contribute towards economic development[9]. The species *Rubus ellipticus* is classified as belonging to the Rosaceae family and is locally known as Hisalu or Hissar and is present in the forest between 800 m and 2400 m above the sea level[10]. *Rubus ellipticus* is shrub species of the Kumaon Himalayan region, Uttarakhand, India. The stem is brownish with prickles having leaves. *Rubus ellipticus* fruiting are available from April to May. *Rubus ellipticus* fruits are generally yellow in color and shown to exhibit many biological activities.[11-13]*Rubus ellipticus* has great potential to generate income because of the presence of anthocyanins as natural colorants.[14]

This study will create job opportunities for the people of Uttarakhand and it will help to stop the migration of people from Uttarakhand (India) due to un-employability. Therefore, this study is aimed at detecting and isolating anthocyanins in edible fruits of *Rubus ellipticus* followed by using advanced analytical techniques for structure elucidation, as described earlier.

2. Materials and Methods

2.1. Sample Collection and Reagents

Rubus ellipticus fruits were gathered from deep forest of Niglaate, District Nainital, India and transported to the laboratory immediately. Water, methanol, ethanol, acetonitrile, formic acid, KCl, sodium acetate, and cyanidin-3-glucoside, HPLC Grade reagents were used.

2.2. Sample Preparation

Rubus ellipticus fruits were washed to remove dust dregs and then the samples were air-dried under darkness at room temperature. The dried sample became converged into powder with the help of mortar and pestle. 50 g powder samples was extracted with 80 % v/v methanol-water solvent at RT, 25°C in dark by using modified method.[15]The aqueous methanol water fruits sample extract was filtered and centrifuged at

8000 rpm for 10 min under very cool condition (at below 30°C). The liquid part was separated from solid by decantation. The volume of the liquid was reduced to 1/3rdof the starting volume under vacuum at 32°C, using a rotary evaporator. The concentrated fruit of *Rubus ellipticus* (RE) extract was stored below -20°C for analytical investigation and characterization using HRLC-ESI-MS/MS.

2.3. Analysis of Anthocyanins

The detailed analysis of RE sample was executed by high - resolution liquid chromatography-electrospray ionization – tandem mass spectrometry integrated with ESI-QTOF-MS. The aqueous methanol extract of RE was further analyzed by GC, using C-18 column. A 10% mixture of 0.1% aqueous formic acid and 90% acetonitrile was used to elude the RE extract from the column at a flow rate of 3 ml/min. In addition, the mass of the analyte was obtained using a mass spectrometer (130 m/z- 1000 m/z). From the library of spectra and available data the compounds present in the RE extract were elucidated on the basis of their retention times.

2.4. Estimation of TAC

The TAC present in RE aqueous methanol was obtained with he help of pH-differential method. The absorbance values of the mixture were recorded at 520 nm and 700 nm using a UV spectrophotometer. The molar extinction coefficient (ε) for anthocyanin was found to be 26900 L m^{-1} mol^{-1}. In this investigation cyanidin-3-glucoside (standard) and distilled water (Blank) was utilized. Absorbance (A) was determined equation below:

A = [(A_{520} − A_{700}) at pH=1.0] − [(A_{520} − A_{700}) at pH=4.5]

$$\text{TAC (mg/g)} = \frac{\text{Abs} \times \text{MW} \times \text{DF} \times 1000}{\varepsilon \times \text{l}}$$

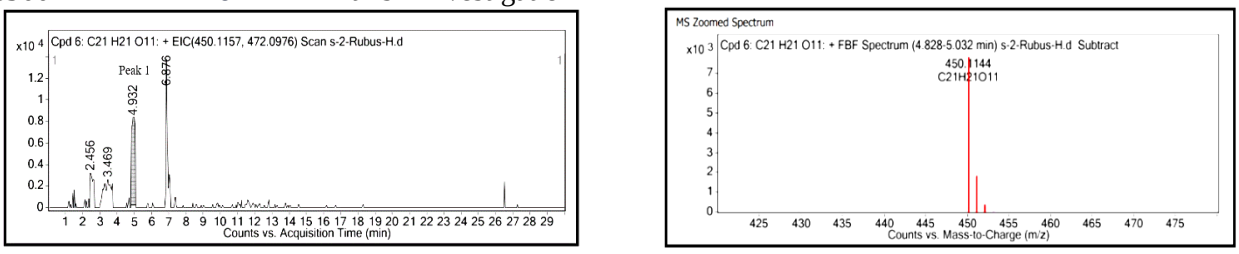
Where Abs = absorbance, MW = standard molecular weight (449.2 g/mol), DF = dilution factor, l = path length (1 cm). TAC total Monomeric Ananthocyanin content calculated in mg/g fruit weight.

3. Results and Discussion

3.1. Peak recognition

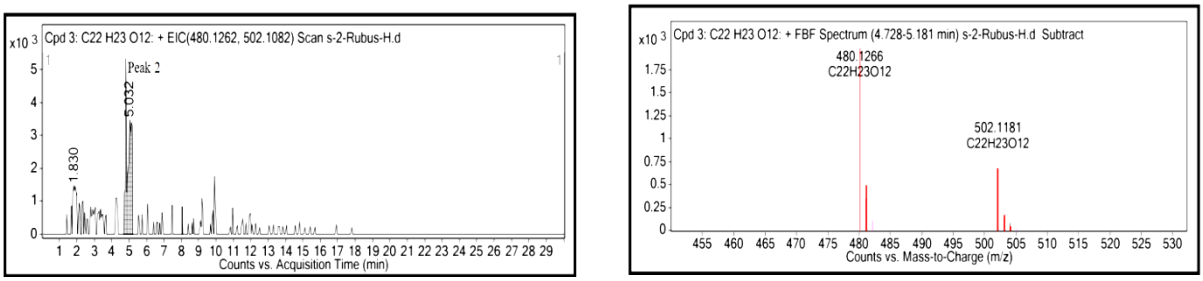
Rubus ellipticus extract showed more peaks than that expected for a single anthocyanin. This suggested that a mixture of different anthocyanin compounds was present. Since the polarity and chemical behavior of these compounds happen to be similar, they exhibit the same retention time. However, if the peaks are resolved, they may reflect more than one compound.[16]

Anthocyanins were identified by comparison of retention time (RT), molecular formula (MF) and m/z data with already available mass spectral literature.[17]Five anthocyanins, isolated from RE and being reported for the first time, are cyanidin-3-galactoside/glucoside, petunidin-3-*O*-glucoside, petunidin 3-(6"-acetylglucoside), delphinidin-3-*O*-arabinoside and cyanidin3.5-di-*O*- β-D-glucoside as shown in Figures 2 - 6.



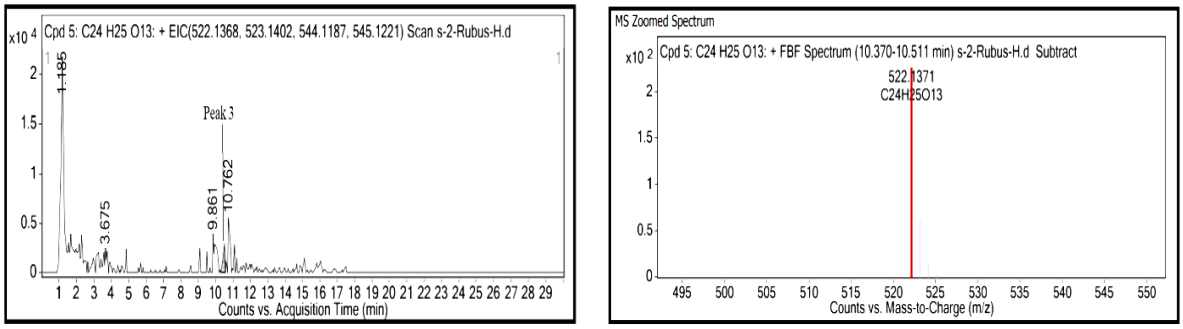
(a) **(b)**

Figure 2 (a) Zoom Chromatogram of, (b) **HRLC (ESI^+):** m/z calculated for (Peak 1, RT: 4.932) $C_{21}H_{21}O_{11}^+$ **$[M+H]^+$:**450.1162, **found**:450.1144.



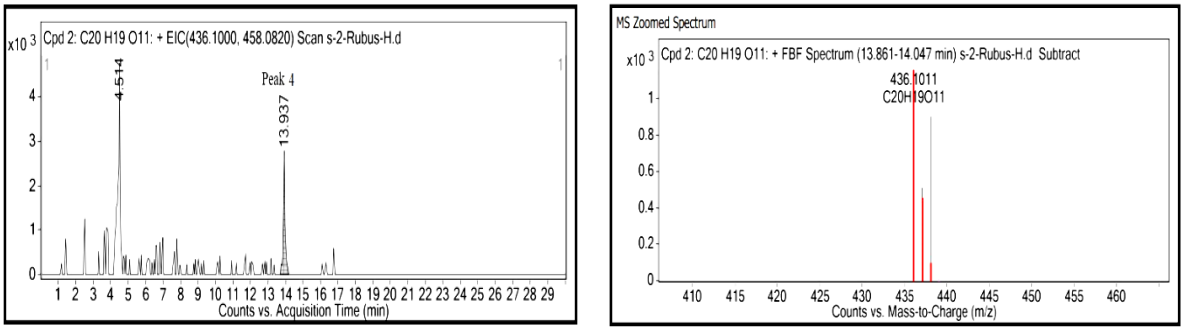
(a) **(b)**

Figure 3. (a) Zoom Chromatogram, (b) **HRLC (ESI^+):** m/z calculated for (Peak 2, RT:5.032) $C_{22}H_{23}O_{12}^+$ **$[M+H]^+$:** 480.1268**, found:** 480.1266.



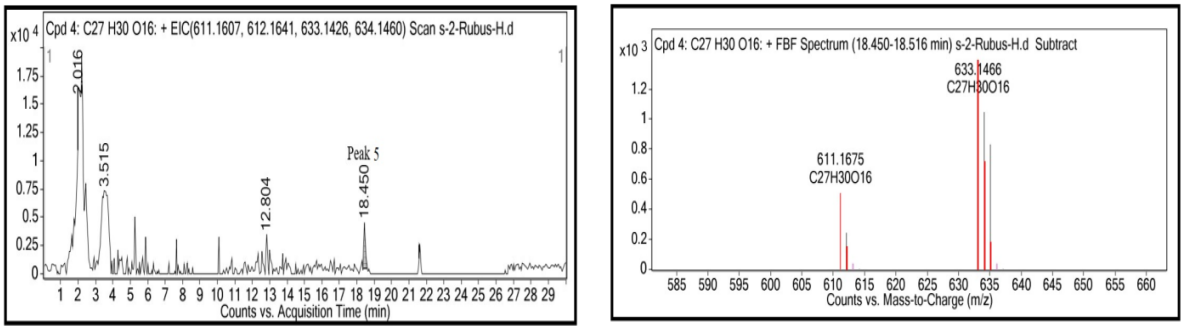
(a) **(b)**

Figure 4. (a) Zoom Chromatogram, (b) **HRLC (ESI⁺):** m/z calculated for (Peak 3, RT: 10.511) $C_{24}H_{25}O_{13}^{+}$ **[M+H]⁺:** 522.1373**, found:** 522.1371.



(a) **(b)**

Figure 5. (a) Zoom Chromatogram, (b) **HRLC (ESI⁺):** m/z calculated for (Peak 4, RT: 13.937) $C_{20}H_{19}O_{11}^{+}$ **[M+H]⁺:** 436.1006**, found:** 436.1011.



(a) **(b)**

Figure 6. (a) Zoom Chromatogram, (b) **HRLC (ESI⁺):** m/z calculated for (Peak 5, RT: 18.45) $C_{27}H_{30}O_{16}^{+}$ **[M+H]⁺ :** 611.1612**, found:** 611.1675.

In the Rubus ellipticus aqueous methanol extract, peak 1 with M^+(molecular ion) at m/z 449.107 and RT 4.93, peak 2 with M^+ at m/z 479.1216 and RT 5.03, peak 3 with M^+ at m/z 521.1298 and RT 10.51, peak 4 with M^+ at m/z 435.0932 and RT 13.93 and peak 5 had a molecular ion M^+ at m/z 610.1539 and RT 18.45, was identified as cyanidin-3-galactoside/glucoside, petunidin-3-*O*-glucoside, petunidin 3- (6"-acetyl glucoside), delphinidin-3-*O*-arabinoside and cyanidin3.5-di-*O*-β-D-glucoside respectively.[18-32] The relative concentration determine by :

$$= \frac{RelativeArea\,\%\ \times TAC}{\text{Total Area \% of Anthrocynin}} = \frac{RelativeArea\,\%\ \times 0.58\ \pm\ 0.02}{15.64}$$

Relative concentrations of Petunidin 3-(6"-acetyl glucoside), delphinidin-3-*O*-arabinoside, cyanidin 3.5-di-*O*-β-D-glucoside were found to be 0.18 ± 0.02 mg/ g FW, 0.26 ± 0.02 mg/ g FW and 0.058 ± 0.02 mg/g FW, respectively. Relative concentrations for both cyanidin -3- galactoside

/glucoside and petunidin-3-*O*-glucose were found to be 0.078 ± 0.02 mg/ g FW as a combination (Table 2) because the peak for both compounds is found at the same place in the HRLC spectra; therefore, it is not possible to find out relative concentration for both compounds separately. Total monomeric anthocyanin content (TAC) was found in the fruit of *Rubus ellipticus* is 0.58 ± 0.02 mg/g fruit weight.

Table 2. Relative Concentration of Identified Anthocyanins

Peak	Start	End	RT	Compounds	Peak Area	Area %	Relative Area %	Relative Conc. mg/ g FW
1	4.828	5.779	4.932	Cyanidin-3-alactoside/glucoside	24788259.06	12.9	2.12	0.078 ± 0.02
2			5.032	Petunidin-3-*o*-glucoside				
3	10.511	11.291	10.511	Petunidin 3-(6"-acetyl glucoside)	56752528.2	29.53	4.85	0.18 ± 0.02
4	13.344	14.194	13.937	Delphinidin-3-O-arabinoside	83076422.32	43.23	7.1	0.26 ± 0.02
5	18.378	19.483	18.45	Cyanidin-3.5-di-o-beta-D-glucoside	18431341.34	9.59	1.57	0.058 ± 0.02

Total Peak Area= 1168993822; Total Area % = 608.26; Area % of Anthocyanin Content = 95.25; Relative area % of Anthocyanin in w.r.t 100% = 15.64 (2.12+ 4.85 +7.1+1.57 = 15.64); the total monomeric Anthocyanin content was found 0.58 ± 0.02 mg/ g FW (fruit weight)

3.2. Comparison of Total Anthocyanin Content with other Plants

A literature review showed that the fruit of *Rubus ellipticus* is richer in anthocyanin content compared to other similar fruits. The highest TAC was found in *Rubus ellipticus* (0.58 ± 0.02 mg/g fw) followed by *Pyracantha crenulata* (0.44 ±0.05 mg/g fw), *Celtis australis* (0.54 ±0.05 mg/g fw), and *Prunus armeniaca* (0.27 ±0.01 mg/g fw) (Table SM3)[31]. The order of the species based on the total anthocyanin content is as follows: *Rubus ellipticus*> *Prunus armeniaca*> *Pyracantha crenulata*> *Celtis australis.*

Table 1. Comparison of total anthocyanin content with other plants

S. No	Wild fruit species	Anthocyanins (mg/g fw)
1	*Prunus armeniaca*	0.27 ±0.01
2	*Pyracantha crenulata*	0.44 ±0.05
3	*Celtis australis*	0.54 ±0.05
4	***Rubus ellipticus***	0.58 ± 0.02

4. Conclusions

This study confirm the presence of anthocyanins viz cyanidin-3-galactoside/glucoside, petunidin-3-*O*-glucoside, petunidin 3-(6"-acetylglucoside), delphinidin-3-*O*-arabinoside and cyanidin3.5-di-*O*-β-D-glucoside in *Rubus ellipticus* fruits. TAC (Total anthocyanin content) found in the fruit of *Rubus ellipticus*was 0.58 ± 0.02 mg/g fruits. The content of anthocyanin was found to be higher in the fruit of *Rubus ellipticus*as compared to *Prunus armeniaca*, *Pyracantha crenulata* and *Celtis australis* fruits. This is can help in the rural development of hilly region by increasing economic growth of people due to high demand of anthocyanins in different industries sectors.

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