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Research Article

HRLC-ESI-MS BASED SEPARATION AND IDENTIFICATION OF ANTHOCYANINS EXTRACTED FROM POPULAR WILD EDIBLE FRUIT OF HIMALAYA: *MYRICA ESCULENTA* (HIMALAYAN BAYBERRY)

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ABSTRACT

The four anthocyanins derivative in the fruit of *Myrica esculenta* were identified. The separation and identification of anthocyanins extracted from *Myrica esculenta* (Himalayan Bayberry) based on advanced analytical instrumentation technique High Resolution Liquid Chromatography Electrospray Ionization Mass Spectrometry (HRLC-ESI-MS). Anthocyanins were identified by correlating their retention time, mass, molecular formula, and mass per charge ratio (m/z) with published data. We extract and identify four major anthocyanins derivative of Malvidin, Cyanidin, and Delphinidin from *Myrica esculenta* fruit (MEF) and Relative concentrations of identified anthocyanin Malvidin 3-(6''-acetylglucoside), Cyanidin3-O-(6''-acetylglucoside), Delphinidin -3-O-arabinoside, Cyanidin-3.5-di-O-beta-D-glucoside were found to be 0.205 ± 0.4 mg/100 g FW, 0.342 ± 0.4 mg/100 g FW, 0.421 ± 0.4 mg/100 g FW and 0.610 ± 0.4 mg/100 g FW, respectively. The total monomeric anthocyanin content (TMAC) was found to be 1.58 ± 0.4 mg/100 g FW (fruit weight). These anthocyanin derivatives were identified first time in this *Myrica esculenta fruit*.

Keywords: *Myrica esculenta,* Anthocyanins, Cyanidin, Malvidin, Petunidin, Delphinidin.

1. INTRODUCTION

In recent times, phytochemical and pharmacological studies are conducted on various plants. *Myrica esculanta* (family: *Myricaceae*) is a medicinal plant. Traditionally, it has been used as an herbal medicine for the treatment of cough, asthma, fever, chronic bronchitis, diarrhea, rheumatism, and inflammation; roots have been used in bronchitis, asthma, cholera, and flowers claimed to treat earache, diarrhea and paralysis. The barks of the plant are used as an antiseptic, anti-asthmatic, and analgesic. Fruits are sedative and carminative, utilized in food industries in different forms like syrups, jam, squash, and refreshing drinks. It *is a potential shrub of* family *Myricaceae*. This family got only one genus *i.e. Myrica* and 45 species. In India, only one species occurs. *M. esculenta* fruits are wild edible species of the Indian Himalayan Region, locally known as Kafal or Kaphal [1]. It occurs in forests from 900 to 2100 meters above sea level. *It* is a large woody, evergreen, tree, having light brown bark, crowded lanceolate leaves, and is about 3 to 15 meters in height [2]. The fruits are succulent drupe with small ellipsoidal or ovoid to globose in shape, initially green, and become reddish during ripening. *M. esculenta* contains smaller fruits of around 4-5 mm, their shelf life is less than 2-3 days and repined fruits are available from April to June [3]. *M. esculenta* fruit was shown to exhibit a variety of pharmacological activities like antioxidant, Analgesic, Anticancer; Antipyretic effect, and antimicrobial against bacteria due to richness in phytochemical constituents like Reducing Sugars, Tammins, Vitamin C, Catechin, Gallic acid, Chlorogenic acid and Anthocyanins [4].

The term anthocyanin is coined from the Greek *anthos*, meaning flower, and *kyanos*, meaning blue. The anthocyanins have great importance in the field of pharmacological research because of their sparkling color, high water solubility and valuable biological properties. These are the biggest group of vacuolar natural colors that are water soluble [5]. Their color is due to their resonating structure of flavylium cation

which is also the basis of their instability. Being phytochemical, these molecules cannot be synthesized by humans and animals and they have to rely on plants for their requirements. Anthocyanins are found in many plant tissues predominantly in the epidermal tissues, palisade, spongy mesophyll of leaves, flesh of fruits /stem, and underground storage organs [6-7]. But most commonly accumulated in flowers and fruits [8]. Anthocyanins provide color to plant tissues (flowers, stems, leaves, roots) ranging from red, purple to blue according to the pH and their structural composition. Chemically, anthocyanins belong to the flavonoids group that is glycosylated polyhydroxy and polymer-thoxy derivatives (3,5,7,3,9-tetrahydroxy flavylium cation) of flavylium salts, possessing a characteristic C-6 (A ring)-C-3 (C ring)-C-6 (B ring) carbon structure. Anthocyanins occurring in nature contain several anthocyanidins or aglycones, but only six are common in foods e.g. Cyanidin (Cy), Pelargonidin (Pg), Delphinidin (Dp), Peonidin (Pn), Petunidin (Pt) and Malvidin (Mv) are the commonly found anthocyanidin in plant [9-10].

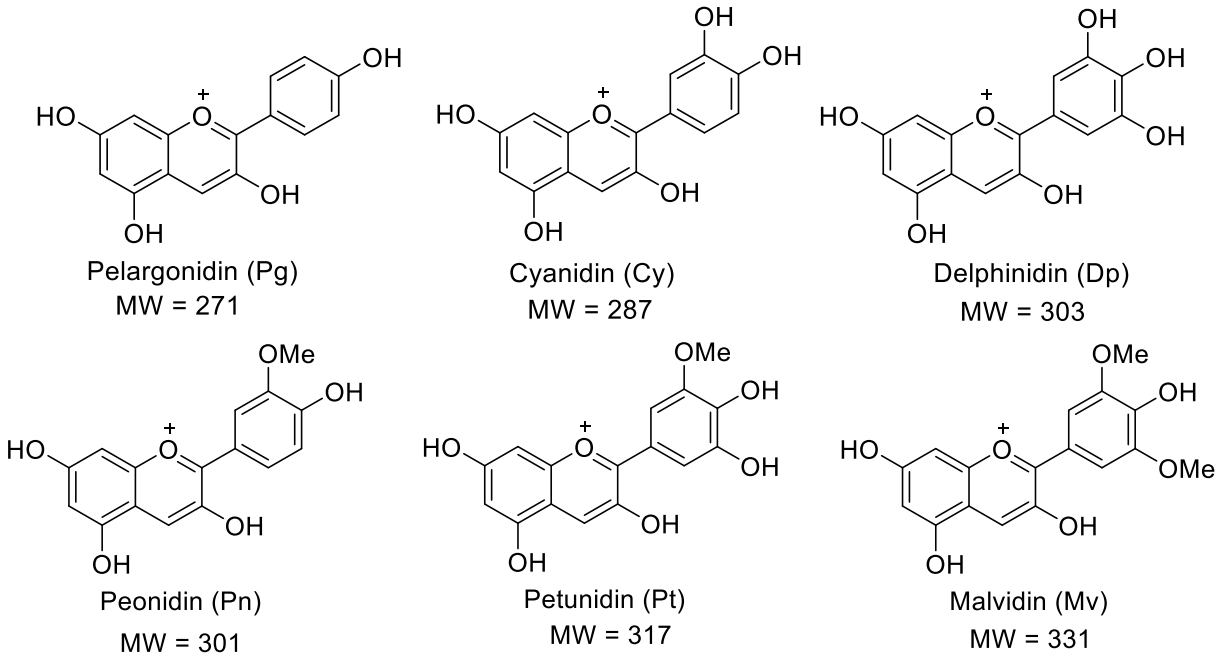


Fig. 1: Basic Chemical structures and molecular weights (MW) of six common anthocyanidins.

Approximately 600 anthocyanins are acknowledged in numerous studies by using different experimental techniques [11-12]. We have limited knowledge about the chemical structure of anthocyanins due to the limitation of analytical instrumentation techniques. High-Resolution Liquid Chromatography Electrospray Ionization Mass Spectrometry (HRLC-ESI-MS) is an advanced analytical technique to separate, identify and quantify the anthocyanins compound due to its high sensitivity to detect trace levels of compounds and accuracy in mass measurement within the secondary metabolites [13].

Indian Himalayan Region is rich in plant diversity, used by local communities in various forms as a medicament (medicine & drugs), fuel, wood, farm equipment, agro forestry, edible food, etc. [14]. The North-Western Himalayan Region is usually known Kumaon hills are one of the regions with a high diversity of edible fruits, which have great potential to contribute towards food security, nutrition, health, and income generation programmed [15]. *M. esculenta* is a popular wild edible fruit among the local people and tourists due to its medicinal importance. Therefore, it has high commercial importance for people of Kumaon Himalaya, local people earned approx 14 lakh/season from selling of the fruits [2]. Anthocyanins and phytochemical constituents have high demand in food, pharmaceutical, and cosmetic industries. Therefore, *M. esculenta* has great potential towards income generation due to the presence of anthocyanins natural colorant (poly-phenols), phytochemical constituents, and their medicinal properties (biological activities) [16].

This research will generate job opportunities and contribute to stop the migration of Sub-Himalayan

residents due to un-employability from the remote hilly area of Uttarakhand (India). Therefore, this study is design for the Separation and identification of anthocyanins in wild edible fruits of *Myrica esculenta* based on the analytical instrumentation technique High Resolution Liquid Chromatography Mass Spectrometry with Electrospray Ionization (HRLC-ESI-MS).

2. MATERIAL AND METHODS

2.1. Plant sample and material

The fresh fruit sample (*Myrica esculenta*) were collected from the nearby forest area of Shyamkhet (Bhowali), District-Nainital, Uttarakhand, India, which is situated at 1854 meter above sea level (*latitude and longitude* 29.384735° N, 79.460834° E. The sample collection was based on ethnophamocological and ethnobotanical literature, in consultation with Dr. Brij M Upreti, Associate Professor, Dept of Botany, Uttaranchal College, Dehradun, Uttarakhand. All used chemicals and reagents in the analysis were of analytical Grade like water, methanol, ethanol, acetonitrile, formic acid, potassium chloride, sodium acetate, cyanidin-3-glucoside, and obtained from Merck, Germany.

2.2. Sample Preparation

Fresh fruit sample (about 200 g) was accurately washed with running tap water then distilled water to eliminate dust residues. After proper washing, cleaning, the pulps of the fruits were removed carefully and put the sample for air drying under dark at room temperature. Dried sample was grinded into powder form by mortar and pestle, and kept at-20°C until being analyzed. The dried powder (50g) fruit sample was extracted by methanol-water (80:20, v/v, MeOH/H_2O) at room temperature under the dark environment with some modification as reported [17]. The extract solution was filtered and centrifuged at 8000 rpm for 10 min under the cooling condition below 30°C using a centrifugal machine to remove the residue. The fruit extract was concentrated under vacuum at 34°C using a rotavap till 1/3 of its initial volume. Concentrated *Myrica esculenta* fruit (MEF) extract was stored at -20°C for further analysis and supply the sample for HRLC-ESI-MS/MS analysis.

2.3. HRLC-ESI-MS/MS Analysis of Anthocyanins

Profiling of active compounds present in MEF extract was carried out by using the HR-LCMS (Agilent Technologies India Pvt. Ltd., India) coupled with ESIQTOF-MS (Agilent G6550A) system. The MEF methanolic extract was injected for the analysis. For the chromatographic separation, Hypersil Gold C-18 column 3 µm, 100 x 2.1 mm (Agilent G1316 C) with the mobile phases, A: 0.1% Formic Acid in water and B: 90% Acetonitrile (ACN)+10% Water+0.1% Formic Acid was used. The elution gradient optimized for the MEF extract sample was, 95% of solvent A for 0 to 2 min, followed by 0%, and 95% for 2 to 25 min and 25 to 30 min respectively. The constant flow rate of 0.3 ml/min was maintained throughout the run and gradient flow was controlled by the binary pump system (Agilent G4220B). Separated analyte fractions were introduced into Mass spectrometer (MS). Dual ESI ionization was used for this study and the acquisition was done for the m/z range of 130 to 1000. The compounds were predicted based on retention time and mass spectrum data values using the spectral library and published data.

2.4. Determination of Total Monomeric Anthocyanin Content (TMAC)

The total anthocyanin content from MEF methanolic extract was determined by the spectrophotometric pH-differential method. MEF extract was taken and potassium chloride buffer (pH 1.0) and sodium acetate buffer (pH 4.5) were added. The absorbance of the MEF extract mixture was measured at 520 nm and 700 nm using a UV-Visible spectrophotometer. Absorbance (A) and Total Monomeric Anthocyanin Content (TMAC) was calculated using following equations - $A = [(A_{520} - A_{700}) \text{ at pH } 1.0] - [(A_{520} - A_{700}) \text{ at pH } 4.5]$.

Total Monomeric Anthocyanin content (mg/g)= {(Abs x MW x DF x 1000)/ ε x l}

The distilled water was used as a blank and cyanidin-3-glucoside was used as the standard. Where Abs = absorbance, MW = the molecular weight of cyanidin-3-glucoside (449.2 g/mol), DF = dilution factor, ε (molar absorptivity) = 26,900 of cyanidin-3-glucoside, l = path length (1 cm), 10^3 = factor for conversion from g to mg, TMAC were reported on the basis mg/100g fruit weight (FW).

3. RESULTS AND DISCUSSION

3.1. Peak Identification and Assignment

High resolution mass spectrometry (HR-MS) instruments measure the precised mass of analytes without fragmentation. The main important feature of this system is that it's very selective since it measures the precised mass of a compound allowing even minor

changes in structure to be distinguished. However, analytes having the same molecular formula and exact mass are required for separation through LC. In high resolution, liquid chromatographic separation of MEF extract represented several numbers of peaks which indicates the presence of different anthocyanin compounds. Because of similarity in polarity and chemical properties several compounds may exhibit the same retention time. Therefore, a resolved peak may correspond to more than one compound [18].

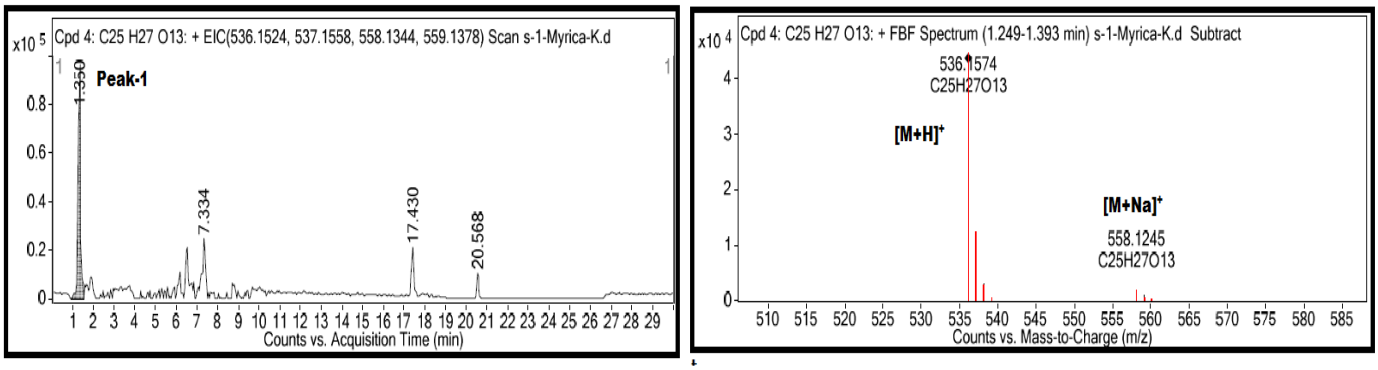
Table 1: List of Anthocyanins compounds detected from HRLC--MS analysis of MEF extract

Peak	RT (min)	Compound	Adduct	m/z calcd.	m/z found	Mol. Wt.[a]	Molecular Formula[a]
1	1.35	Malvidin3-(6"-acetylglucoside)	$[M+H]^+$	536.153	536.1574	535.1492	$C_{25}H_{27}O_{13}^+$
			$[M+Na]^+$	558.1349	558.1245		
2	2.23	Cyanidin3-O-(6"-acetylglucoside)	$[M+Na]^+$	514.1087	514.1108	491.1215	$C_{23}H_{23}O_{12}^+$
3	4.43	Delphinidin -3-O-arabinoside	$[M+Na]^+$	458.0825	458.0817	435.0936	$C_{20}H_{19}O_{11}^+$
4	4.99	Cyanidin-3.5-di-O-beta-D-glucoside	$[M+H]^+$	611.1612	611.1587	610.1522	$C_{27}H_{30}O_{16}^+$
			$[M+Na]^+$	633.1432	633.1404		

[a]*Anthocyanin*

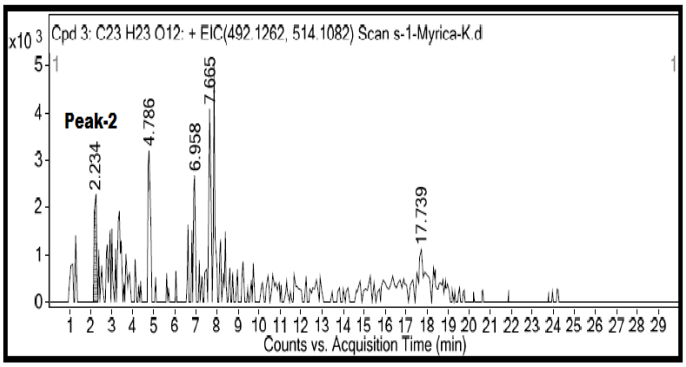
The four anthocyanins were detected from MEF extract in HRLC-ESI-MS analysis. These four common anthocyanins were identified by comparing their retention time, molecular formula, and mass spectral data of each anthocyanins molecule with published data [19-22]. *M. esculenta* fruit which is also known as Himalayan bayberry, found in the North-Western Himalaya region commonly known as Kumaon Hills. Anthocyanins identified in the fruit sample reported the first time, are given in Table 1.

M. esculenta bayberry was found to contain four anthocyanins by comparing elution order, molecular mass (m/z), and molecular formula. The major anthocyanin were tentatively characterized and identified as Malvidin3-(6"-acetylglucoside) Figure 2a, 2b (peak-1), Cyanidin 3-O-(6"-acetylglucoside) Figure 3a, 3b (peak-2), Delphinidin 3-O-arabinoside Figure 4a, 4b (peak-3) and Cyanidin-3.5-di-O-beta-D-glucoside Figure 5a, 5b (peak-4) In the MEF extract, Peak 1 with M^+(molecular ion) at m/z 535.1492 and retention time 1.35 was identified as Malvidin 3-(6"-acetylglucoside), Peak 2 with M^+ at m/z 491.1215 and retention time 2.23 was identified as Cyanidin 3-O-(6"-acetylglucoside), Peak 3 with M^+ at m/z 435.0936 and retention time 4.33 was identified as Delphinidin 3-O-arabinoside, Peak 4 with M^+ at m/z 610.1522 and retention time 4.99 was identified as Cyanidin-3.5-di-O-beta-D-glucoside tentatively. Other peaks left as without characterizations in zoom chromatogram (Figures 2a, 3a, 4a, 5a, and 6a) of HRLC-ESI-MS are nor-anthocyanins. Therefore, these are not discussed in the manuscript. The real chromatogram and its qualitative compound reports are given in supporting information.

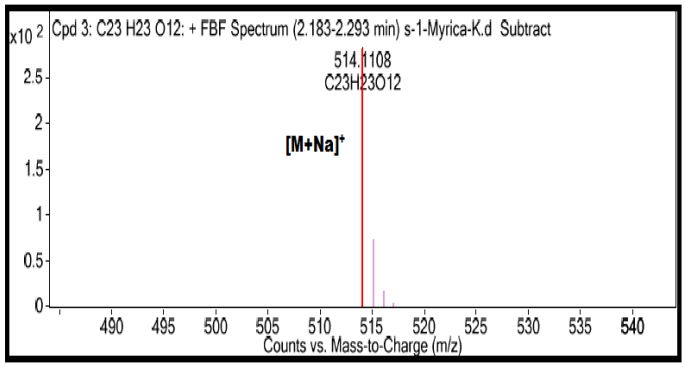


2a 2b

Fig. 2: (a) Zoom Chromatogram (b) HRLC (ESI^+): m/z calcd for (Peak 1, RT: 1.35) C_{25} H_{27} O_{13}^+ $[M+H]^+$:536.153, found:536.1574

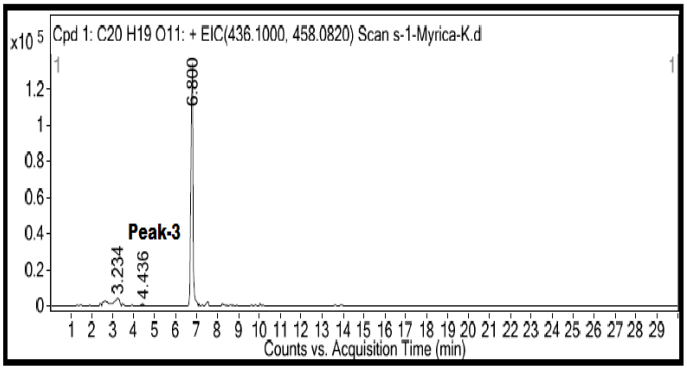


3a

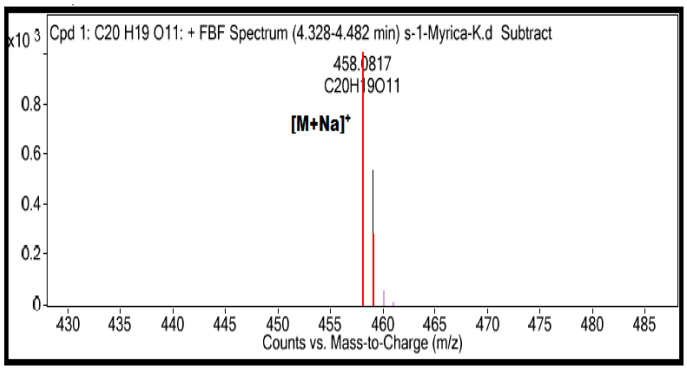


3b

Fig. 3: (a) Zoom Chromatogram (b) HRLC (ESI$^+$): m/z calcd for (Peak 2, RT: 2.23) $C_{23}H_{23}O_{12}^+$ [M+Na]$^+$ 514.1087, Found: 514.1108

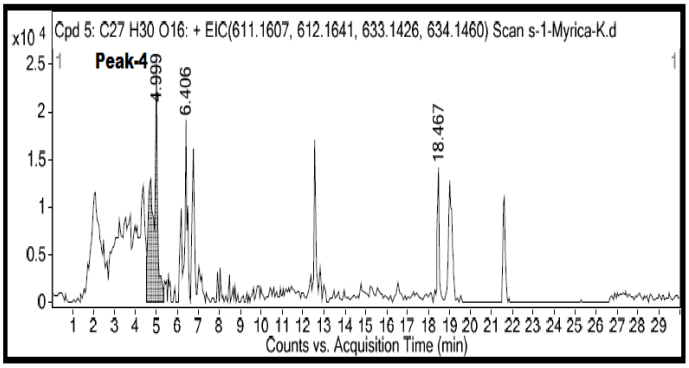


4a

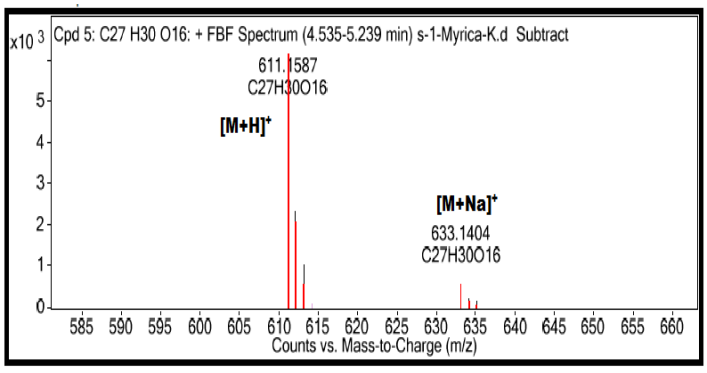


4b

Fig. 4: (a) Zoom Chromatogram, (b) HRLC (ESI$^+$): m/z calcd for (Peak 3, RT: 4.43) $C_{20}H_{19}O_{11}^+$ [M+Na]$^+$: 458.0825, found: 458.0817



5a



5b

Fig. 5: (a) Zoom Chromatogram, (b) HRLC (ESI$^+$): m/z calcd for (Peak 4, RT: 4.99) C_{27} H_{30} O_{16}^+ [M+H]$^+$:611.1612, found:611.1587

The relative Concentration was calculated by using following formula:

={(Relative Area % X total monomeric Anthocyanin content)/ Total area % of Anthocyanin}

={(Relative Area % X 1.58±0.4)/ 30.82}

The Relative concentrations of identified anthocyanin Malvidin 3-(6''-acetylglucoside), Cyanidin3-O-(6''-acetylglucoside), Delphinidin -3-O-arabinoside, Cyanidin-3.5-di-O-beta-D-glucoside were found to be 0.205 ± 0.4 mg/100 g FW, 0.342 ± 0.4 mg/100 g

FW, 0.421±0.4 mg/100 g FW and 0.610±0.4 mg/100 g FW, respectively (Table 2). Total monomeric anthocyanin content was found in *Myrica esculenta fruit* is 1.58 ± 0.4 mg/100 g FW (fruit weight)

Table 2: Relative Concentration of Identified Anthocyanins Compounds

Peak	Start	End	RT	Compounds	Peak Area	Area %	Relative Area %	Relative Conc. mg/100 g FW
1	1.199	1.63	1.35	Malvidin3-(6"-acetylglucoside)	45255015.95	31.38	4.01	0.205 ± 0.4
2	1.63	2.293	2.23	Cyanidin3-O-(6"-acetylglucoside)	75389234.68	52.27	6.69	0.342 ± 0.4
3	4.377	4.635	4.43	Delphinidin -3-O-arabinoside	92652376.74	64.24	8.22	0.421 ± 0.4
4	4.635	5.052	4.99	Cyanidin-3.5-di-O-beta-D-glucoside	134130145.5	93	11.9	0.610 ± 0.4

Total Peak Area = 1126787794; Total Area % = 781.25; Area % of Anthocyanin Content= 240.89; Relative area % in w.r.t.100% = 30.82; the total monomeric anthocyanin content was found to be 1.58 ± 0.4 mg/100 g FW (fruit weight).

4. CONCLUSION

The present study of MEF extract revealed the presence of four common anthocyanins most of which are described the first time in the sample species. The four major anthocyanins Malvidin 3-(6"-acetylglucoside), Cyanidin-3-O-(6"-acetylglucoside), Delphinidin-3-O-arabinoside and Cyanidin-3.5-di-O-beta-D-glucoside identified in the MEF extract tentatively. Total monomeric anthocyanin content was found in *Myrica esculenta* fruit is 1.58±0.4 mg/100 g FW (fruit weight). *Myrica esculenta fruit* also naturally contributing as an antioxidant, anti-diabetic and antimicrobial agent and this study provides a reliable scientific approach for the further application of anthocyanins in *Myrica esculenta* fruit. This research will play an important role for the economic growth of people in Uttarakhand.

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