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Analysis and characterization of anthocyanin from phalsa (*grewia asiatica*)

Abstract

Anthocyanins (ACNs) are water-soluble plant pigments having a vital role in plant physiology and human health. Grewia asiatica L., genus (Tiliaceae) is a fruit producing shrub, originating from south Asia (Pakistan, India), East (Cambodia), cultivated mainly for its comestible fruit and well reported diverse medicinal uses. The current study was the first attempt to characterize ACNs from G. asiatica, native to Pakistan. Results revealed that maximum anthocyanin extraction yield (1193.8µg/g) was obtained with acidified methanol as compared to other investigated solvents (water, methanol, ethanol and their binary mixture). Total seven ACNs were identified including non-acylated (delphinidin-3-O-glucoside, peonidin-3-O-glucoside, pelargonidin-3-O-malonyl glucoside), acylated (cyanidin-peonidin-and pelargonidin-3-O-6"-acetylglucoside) and pyranoanthocyanin (Malvidin-3-O-glucoside pyruvic acid). Among them cyanidin-3-O-(6"acetylglucoside) was the major ACN comprising 44-63% (695µg/g) of total ACNs composition followed by peonidin-3-O-glucoside consisting of 3-30% (163.6µg/g) and pelargonidin-3-O-(6"-acetyl glucoside) 8-14% (140.4µg/g). Moreover, the study of ACNs stability at the temperature 10-55°C, testified the better temperature tolerance by ACNs under dark at temperature ranging, 10-40°C. Hence, the results revealed that G. asiatica is a potent fruit rich in ACNs and can be used as food colorant and nutraceutical.

Keywords: grewia asiatica, cyanidin-3-o-6"acetyl glucoside, pH differential method, LC-MS/MS

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Abbreviations: ACN, anthocyanins; Cy, cyanidin; Dp, delphinidin; Pt, petunidin; Pn, peonidin; Pg, pelargonidin; Mv, malvidin

Introduction

Anthocyanins (Greek anthos, "flower" and Greek kyanose, "blue"), an extensive group of flavonoids, are water-soluble plant pigments.[1] They exist in vacuoles of plant cells and are responsible for vigorous blue, purple, and red colours of various botanic organs such as flowers, grains, and fruits.[2]

They occur primarily as glycosides or acylglycosides of their respective aglycone anthocyanidins. There are almost seventeen natural anthocyanidins; however, only 6 of them, cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin are universally distributed.[3] Over 600 naturally occurring ACNs have been reported differing in (a) the number and position of hydroxyl and methoxyl groups in the basic anthocyanidin skeleton, (b) identity, number and positions at which sugars are attached and (c) the extent of sugar acylation and identity of acylating agent.[3] They differ from other natural flavonoids on the basis of colour ranges that can be derived from them and their[4] capability to form resonance structures through pH variation.[5] Therefore, ACNs are regarded as potential candidates for natural colorants in the food industry.[6,7] Similarly, in the past years, questions were raised regarding the safety of synthetic colorants due to their toxicity, thus the interest in natural pigments has been increased significantly as a consequence of both the legislative action and consumer awareness.[8]

ACNs are considered as substantial dietary compounds also, owing to their potent free radical scavenging and antioxidant nature in vitro leading to their antihypertensive and anticarcinogenic[4] behaviour against oral, esophageal and colon cancer.[5] In addition, ACNs could promote sharp-sightedness, immune response, blood circulatory system and protection against cardiac diseases.[6] The research has shown that acylated ACNs may behave quite differently from their nonacylated counterparts, in terms of their stability and bioavailability; (Stoner et al., 2005), hence it is not reasonable to consider all ACNs equally. Therefore, knowledge regarding the concentrations and daily intake of specific ACNs is essential.

The main sources of ACNs are fruits and some vegetables such as grapes, blackberries, blueberries, egg-plants (aubergine) and avocado.[9] Recently, indigenous fruits such as Syzyguim cumini have been found as potential candidates for polyphenols and flavonoids, particularly. In this context *Grewia asiatica* L., (called Phalsa locally)[10,11] which is agronomically crucial indigenous plant, native to South Asia (Pakistan and India) and Cambodia, cultivated mainly for its comestible fruit and well-reputed diverse medicinal uses.[12,13] Also, it is used to manufacture soft drinks, jams, pies, squashes and chutneys. Incontrovertible evidence has been documented that G. asiatica fruit has an antioxidative and antiradical capacity,[14] particularly its peel, mush and seed. In this regard, assessed the antioxidant activity of G. asiatica leaves extract while Rymbai, H et al.[8] evaluated its greater antiradical potential up to 95.87% in Swiss albino mice.[8] Later on, they found a significant hepatoprotective efficacy of its fruit extract in the same organism.[8] In addition declared *G. asiatica* fruit extract as a rich source of ACNs (by evaluating total ACNs) and found its effective antimicrobial activity against four different species of gram-

positive and negative bacteria. Despite its diverse use, it has suffered remarkable disregard, as is evident from the lack of literature about the described plant.[11] Therefore, it was necessary to strengthen the research about the *G. asiatica* fruit pigment in order to promote the industrialization of its fruit products.

Keeping in mind the therapeutic importance and possible industrial utilization as a natural colorant, the present study is focused on the identification and quantification of individual ACNs from *G. asiatica* fruit. Also, their solubility in different solvents and stability at various temperatures under the influence of light and dark have been highlighted.

Materials and methods

Standards and solvents

Analytical grade methanol and ethanol (for extraction purpose) were procured from Sigma Aldrich (Darmstadt, Germany), while for HPLC analysis and cleanup procedure, HPLC grade chemicals such as Methanol, formic acid, ethyl acetate and hydrochloric acid (HCl) were purchased from Fischer scientific Ltd (Lough borough, UK). Standards of the 3-O-α-glucoside chloride of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin and cyanidin-3-O-rutinoside (Keracyanin Chloride, 99% pure) were obtained from Applicam (Darmstadt, Germany).

Samples collection and storage

Ripe fruit samples of *G. asiatica* (dark purplish and hemispherical single-seeded) were purchased from local markets of Hyderabad, Sindh province, Pakistan; placed into the cold insulated sampling box immediately followed by whole fruit homogenization and storage at -20°C. The homogenized samples were lyophilized for 48h using Trio science lyophilize Model TR-FD-BT-50 (Tokyo, Japan) and stored at -4°C until analysis.

Anthocyanin extraction and purification

ACNs were extracted from G. asiatica fruit samples following a reported method with slight modifications. Briefly, 10ml acidified methanol (HCl, 0.1% v/v) was added to 1g of lyophilized sample and incubated (30rpm) at 20°C for 24h in dark followed by separation of supernatant through centrifugation (5000rpm) at 4°C for 20min.

For purification of filtered supernatant, a standard procedure[15] was employed. In brief, ACN extract was loaded on C18 Sep-Pak cartridge (Waters Associates, Milford, USA), preconditioned with deionized water and methanol (HPLC grade), and washed with 5-fold volume of ultra-pure acidified water (HCl, 0.01% v/v) and ethyl acetate followed by elution with methanol containing 0.1% HCl. The ACN solution was collected and condensed at 40°C using a rotary evaporator under vacuum for the spectroscopic and chromatographic analysis. All analyses were performed in triplicate.

Solvent study

In order to obtain the ACNs extract with maximum concentration, different acidified (HCl, 0.1% v/v, for equal visual color strength) extraction solvents like water, methanol, ethanol and binary mixture (1:1) of acidified methanol with water and ethanol were investigated.

Determination and quantification of total anthocyanin contents

Total ACN contents in *G. asiatica* samples were determined by UV-Visible Spectrophotometer (Biochrom Libra S22) following previously reported a simple and cost effective AOAC pH differential method.[16] It is well-known that ACNs undergo reversible structural transformations with pH alteration, manifested by amazingly different absorbance spectra. At pH 1.0 the coloured oxonium form while at pH 4.5 colourless hemiketal form is prevailed. The pH-differential method, is based on that principle[8] and permits accurate and rapid measurement of total ACNs content, even in the presence of other interfering agents.

To estimate the concentration of monomeric ACNs, the absorbance of methanolic crude extracts, diluted in potassium chloride (0.025M, pH 1.0) and sodium acetate buffer (0.4M, pH 4.5), was measured in curette of 1cm path length at 520 and 700nm and ambient temperature (23°C) against a blank containing acidified methanol and aforementioned buffers. The resultant absorbance (A) was calculated as follows:

$$A = \left(A520\ nm - A700\ nm\right)\ pH\ 1.0 - \left(A520\ nm - A700\ nm\right)\ pH\ 4.5$$

The monomeric ACN concentrations were calculated, according to the following formula

$$Anthocyanin\ content(mg\ /\ ml = A \bullet MW \bullet DF \bullet 1000\ /\ \left(\varepsilon \bullet 1\right)$$

Where the molecular weight (MW = 630.98) and molar absorptive (ε=26,900) of cyanidin-3-O-rutinoside, and dilution factors (DF) of concerned samples were used.

Stability study of anthocyanin

Temperature and light are among the most important factors that influence the ACNs stability and deterioration.[8] The thermal stability was investigated in the range of 10-55°C in dark and light for 144h (6days) using temperature control New Brunswick Innova 4200 Incubator-Shaker (Gaithersburg, Maryland) equipped with incandescence light bulb, In typical procedure,[8] four screw-topped glass vials containing samples of ACNs extract enclosed with aluminium (to protect from light) and sealed with para film, were placed in the dark at temperatures mentioned above. Meanwhile, four other samples without covering of aluminium foil were exposed to light at same temperature range. The stability was studied by recording UV-visible spectral variation at 520 and 700nm.

HPLC-DAD analysis and quantification of individual anthocyanins

Chromatographic analyses were performed following a reported method,[17] using auto sampler UV 000LP series HPLC (Thermo electronic corporation) equipped with diode array detector. Ten micro liter sample was injected and separated with Hypersaline gold (Thermo scientific) analytical C18 column (250×4.6mm, 5µm) using a mixture of methanol and formic acid solution (5% v/v) as eluent at the ratio of 15:85 with flow rate of 1ml min^{-1}. The detection wavelengths were set to 520, 530 and 540nm.

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LC-MS/MS confirmation of individual anthocyanins

The confirmation and identification of individual ACNs were established with Agilent (model 6460) triple quadruple liquid chromatography/mass spectrometer (LC-MS/MS). The analysis was made in ESI interface with nebulizer gas pressure 45 psi with flow rate of 12ml min^{-1}. The capillary voltage was kept at 3500V and the tandem mass spectrometry scan was accomplished in the range of 350 to 1500m/z.

Statistical analysis

Analysis of variance (ANOVA) was used to analyze data by Tukey's post-test, data was considered significant at $P<0.05$ using SPSS version 18.0 (SPSS, Inc., 2009, Chicago, IL, USA). The association among variables was evaluated by Pearson's correlation.

Results and discussion

Solvent effect on anthocyanin extraction

Solvent extraction is considered as the most employed method to prepare fruit samples. In this connection, we tried acidified (HCl, 1% v/v) water, methanol, ethanol and binary mixture (1:1) of acidified methanol with water and ethanol.

The best extraction efficiency was demonstrated by methanol with 1193.8µg/g extract ($P<0.05$) as it is evident from the bar graph. Binary solvents i.e. 50% v/v acidified methanol and ethanol also showed satisfactory performance Figure 1 and no remarkable difference ($P>0.05$) was observed between the extraction capability of binary and single solvent systems.

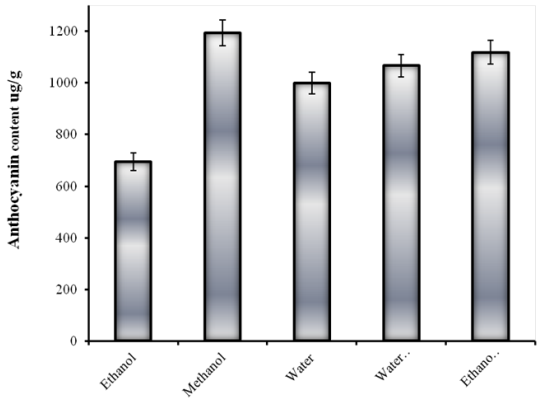

Figure 1 Solvent extraction efficiency for anthocyanin extracted from *G. asiatica.*

The quantification of anthocyanin in *G.asiatica* fruit was done by plotting the standard calibration graph of Keracyanin chloride (Cyanidin-3-O-Rutinoside). In Figure 2 the peak at similar wavelength as shown by Cyanidin-3-O-Rutinoside confirmed that anthocyanins from *G.asiatica* fruit pigment contain the same structural unit as cyanidin.

Thermal Stability of anthocyanins at different storage conditions

Total eight samples of ACNs extracts were subjected to temperature ranging 10–55°C, the results exhibit that the degradation of ACNs, exposed to light, was 28.55% at temperature between 10 to 25°C and 60% between 25 to 55°C ($P<0.05$) as it can be seen in (Figure 3(A)). ACNs, placed in dark, also showed almost the same stability at temperature 10 to 25°C ($P>0.05$), however, a noticeable reduction in stability occurred with 50% degradation by increasing temperature from 25 to 55°C as it is obvious from Figure 3(B). It has been reported by other authors also temperature elevation leads to the acceleration of ACNs destruction.[5]

In addition, ACNs in dark were found to be comparatively more thermally stable (for 96h) at 40°C which is in agreement with the degradation behavior of ACN extracts of black grape skins at identical temperature.[18] Thus light was found to be deleterious to ACNs. Horbowicz and his colleagues also have documented a short half-life (197days) for grape ACNs in light and long half-life (416days) in dark at 20°C.

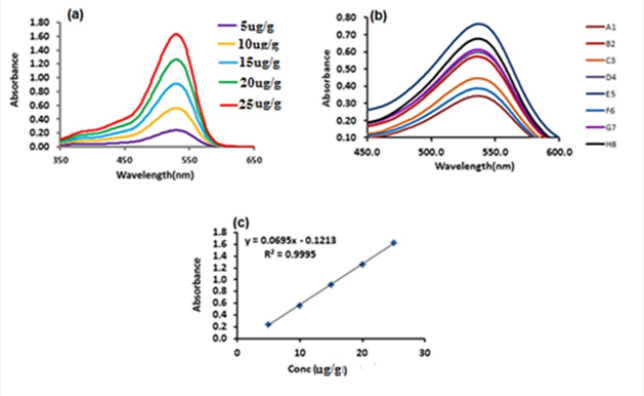

Figure 2 UV-Visible spectra of (A) Keracyanin chloride (Cyanidin-3-O-Rutinoside) from 5 to 25µg/g (B) Anthocyanin extract of different G.asiatica samples (C) Linear calibration graph of Keracyanin chloride (cyaniding-3-O-rutinoside) standard.

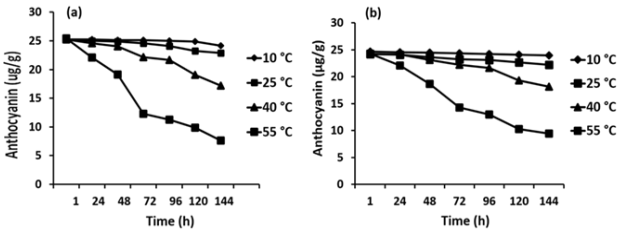

Figure 3 Stability of G.asiatica anthocyanin extract in (A) presence of light and (B) dark with variable temperature and time.

Peak identification and quantitation

The comparison of Analytes retention times and mass spectral data with those of standards and literature revealed that in the present study, for the first time seven ACNs of G. asiatica fruits have been identified and quantified (Figure 4).

The tabulated LC-MS/MS data of ACNs composition in eight different samples of G. asiatica extracts show three categories of ACNs, on substitution basis as non-acylated anthocyanin, acylated anthocyanin and pyranoanthocyanin Table 1. Cyanidin-3-O-(6"acetyl glucoside) was the major ACN comprising 44-63% (695µg/g) of total ACNs composition followed by 3-30% (163.6µg/g) peonidin-

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3-O-glucoside and pelargonidin-3-O-(6"-acetyl glucoside) 8-14% (140.4µg/g). Small quantities of delphinidin-3-O-glucoside, pelargonidin-3-O-malonyl glucoside, peonidin-3-O-(6"-acetyl glucoside) and malvidin-3-O-glucoside pyruvic acid were also detected as shown in Table 1. *Xianli et al.*[3] *and* Fan□Chiang et al.[14] *have reported that* Cyanidin-3-O-(6"acetyl glucoside) was the main ACNs found in *Black raspberry (669mg/100g) and black barriers (*137mg/100g). In addition has also reported the presence of cyanidin 3-glucoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside and pelargonidin 3-rutinoside and pelargonidin 3-acetylglucoside in strawberry samples collected from Salamanca, Spain.

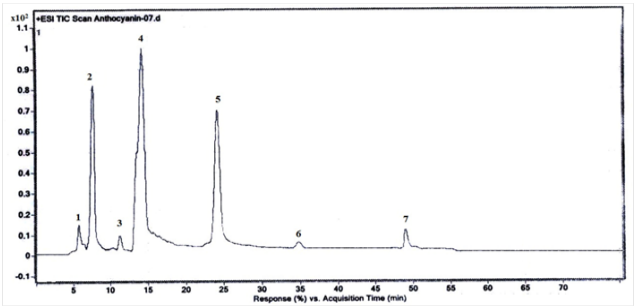

Figure 4 Total ion chromatogram of G.asiatica freeze dried extract showing anthocyanins separation (1) Delphinidin 3-O- glucoside (2) Peonidin 3-O-glucoside (3) Peonidin-3-O (6"-acetyl glucoside) (4)Cyanidin-3-O(6"acetyl glucoside) (5) Pelarigonidin-3-O-6"acetyl glucoside (6) Pelarigonidin-3-O-malonyl glucoside) (7) Malvidin-3-O-glucoside pyruvic Acid.

Here it is worth mentioning that some authors have proposed that ACNs are therapeutically more efficient in mixture form. The data of LC/PDA/ESI-MS and anthocyanin composition in eight different samples of G.asiatica extracts is summarized in Table 1.

Fragmentation patterns of G.asiatica anthocyanins

Molecular weights and fragmentation behaviour of ACNs were determined by LC-MS/MS as previously reported[18] and it was found that amongst the seven identified ACNs, three were nonacylated (peak 1, 2 and 5), other three were acylated (peak 3, 4 and 6) and one was Pyranoanthocyanin (peak 7) as depicted in Table 2.

Peak 1 displayed molecular ion peak [M]+ of delphinidin-3-O-glucoside at 465m/z and fragment ion peak [M-162]+at 303m/z due to the removal of glucoside unit. Peak 2 had molecular cation of peonidin-3-O-glucoside at 463 m/z and fragment ion [M-162]+ at 301m/z for peonidin anthocyanidin backbone. Similarly, peak-5 showed molecular cation of pelarigonidin-3-O-malonyl glucoside at 519m/z along with fragment ions at m/z 433 [M–186]+ and 271m/z due to the cleavage of malonyl and glucoside groups respectively [Figure 5].

Acylation of anthocyanin sugar moieties brings about an increase in molecular weight and decrease in polarity. Therefore, in peak -3 the molecular ion of peonidin-3-O-(6"-acetyl glucoside) appeared at 505m/z, higher than its nonacylated counterpart, with fragment ion [M+204] of acetyl glucoside at 301m/z. Peak -4 yielded M+ of cyanidin-3-O-(6"-acetyl glucoside) at 491m/z and fragment ion of acetyl glucoside at 287m/z. Another acylated anthocyanin,[19,20] pelargonidin-3-O-(6"-acetyl glucoside) was specified by peak-6 with molecular ion peak [M]+ at 475m/z and fragment ion [M-204]+ at 271m/z (Figure 6).

Peak 7 demonstrated [M]+ of malvidin-3-O-glucoside pyruvic acid (Figure 7) at 561m/z and two main fragments at 493 and 331m/z (due to the removal of malvidin) with the difference of 162 due to the loss of glucoside group.

Table 1 Anthocyanin composition in different samples of *Grewia asiatica*

Anthocyanin (µg/g)	GA1	GA2	GA3	GA4	GA5	GA6	GA7	GA8
Delphinidin 3-O-glucoside	28.6±1.1^{a}	37.2±1.3^{b}	36.3±1.5^{b}	55.5±2.1^{c}	50.5±2.5^{c}	13.1±0.6^{d}	85.7±4.1^{e}	34.5±1.6^{b}
Peonidin 3-O- glucoside	132.7±6.6^{a}	80.5±4.0^{a}	60.7±3.0^{c}	63.7±3.1^{c}	37.2±1.8^{d}	116±5.7^{e}	334.2±16.6^{f}	320.6±15.8^{f}
Pelarigonidin-3-O-malonyl glucoside	45.0±2.24^{a}	62.4±3.1^{b}	27.9±1.3^{c}	76.3±3.8^{d}	85.6±4.2^{e}	36.5±1.8^{f}	17.6±0.8^{g}	19.9±0.9^{g}
Cyanidin-3-O(6"acetyl glucoside)	605±30.0^{a}	584.1±29.1^{b}	636±31.2^{a}	671.4±33.21^{c}	760.8±38.0^{d}	636.9±31.8^{a}	470.5±23.2^{e}	504.4±25.1^{f}
Peonidin-3-O (6"-acetyl glucoside)	51.6±2.5^{a}	107.5±5.3^{b}	86±4.1^{c}	69.1±3.4^{d}	73.7±3.6^{e}	44.7±2.2^{a}	33.9±1.7^{e}	34.5±1.7^{e}
Pelarigonidin-3-O-(6"acetyl glucoside)	78±3.8^{a}	160.6±8.02^{b}	118.3±5.9c	126.2±6.2^{d}	126.4±6.31^{d}	126.4 97±4.8^{f}	118.2±5.90^{c}	158.6±7.9^{b}
Malvidin-3-O-glucoside pyruvic acid	51±2.5^{a}	72.9±3.6^{b}	76.5±3.8^{b}	52.0±2.4^{a}	59.7±2.9^{a}	69.5±3.4^{b}	61.7±3.1^{c}	79.1±3.9^{b}

Data is presented as Mean±standard deviation; different superscript in a column shows significant differences at P<0.05.

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Table 2 Mass spectral data of anthocyanins in phalsa (Grewia asiatica)

Peak No:	Retention time (min)	[M]+(m/z)	MS/MS (m/z)	Anthocyanin
1	5.716	465	303 (Dp)	Delphinidin3-O- glucoside
2	7.495	463	301 (Pn)	Peonidin 3-O- glucoside
3	11.143	505	301 (Pn)	Peonidin-3-O (6"-acetyl glucoside)
4	14.104	491	287 (Cy)	Cyanidin-3-O(6"acetyl glucoside)
5	24.161	519	433/271 (Pg)	Pelarigonidin-3-O-malonyl glucoside
6	34.814	475	271 (Pg)	Pelarigonidin-3-O-(6"acetyl glucoside)
7	49.116	561	493/331 (Mv)	Malvidin-3-O-glucoside pyruvic Acid

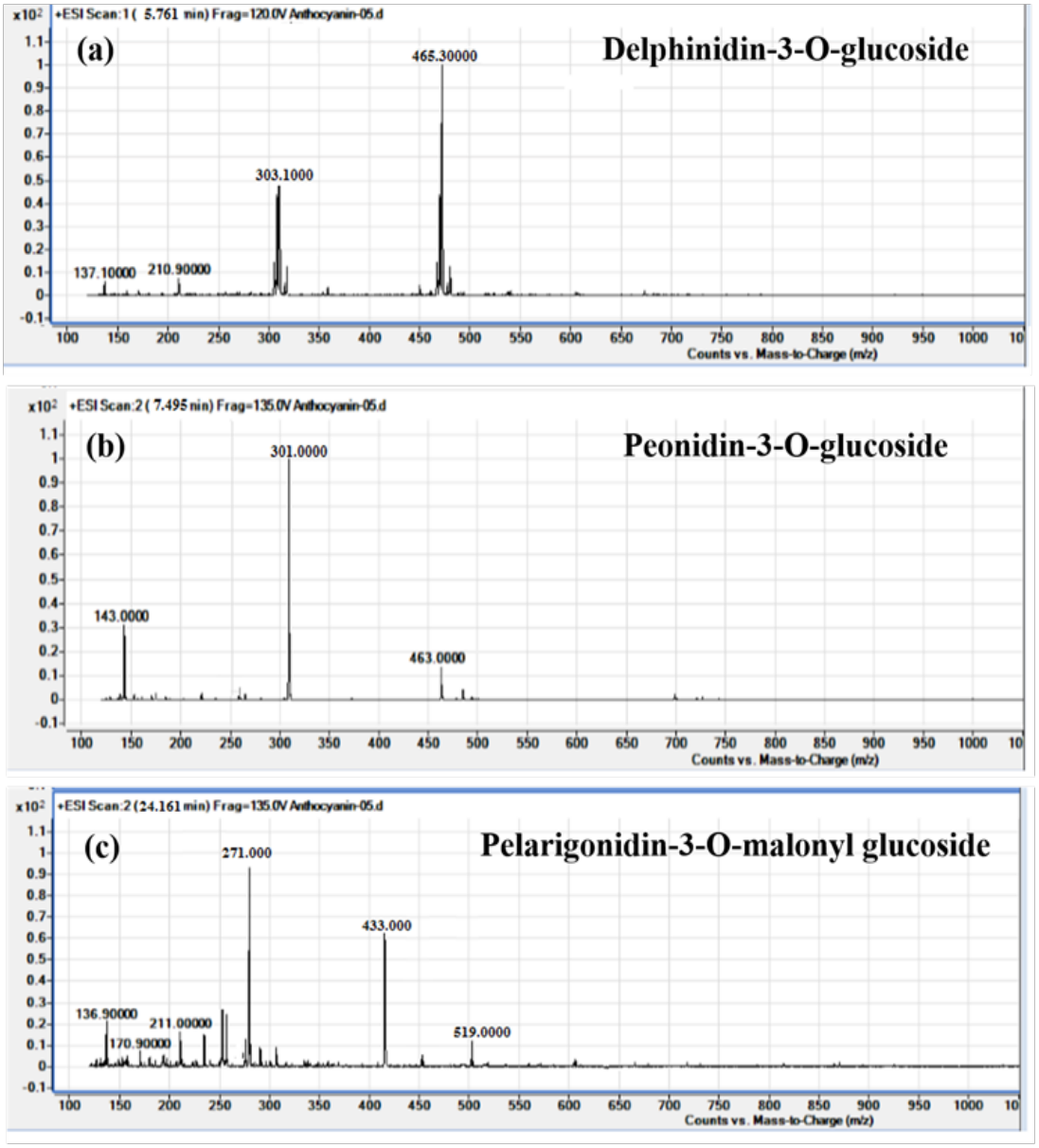

Figure 5 Mass fragmentation of non-acylated anthocyanins, (A) delphinidin-3-O-glucoside, (B) peonidin-3-O-glucoside and (C) pelarigonidin-3-O-malonyl glucoside.

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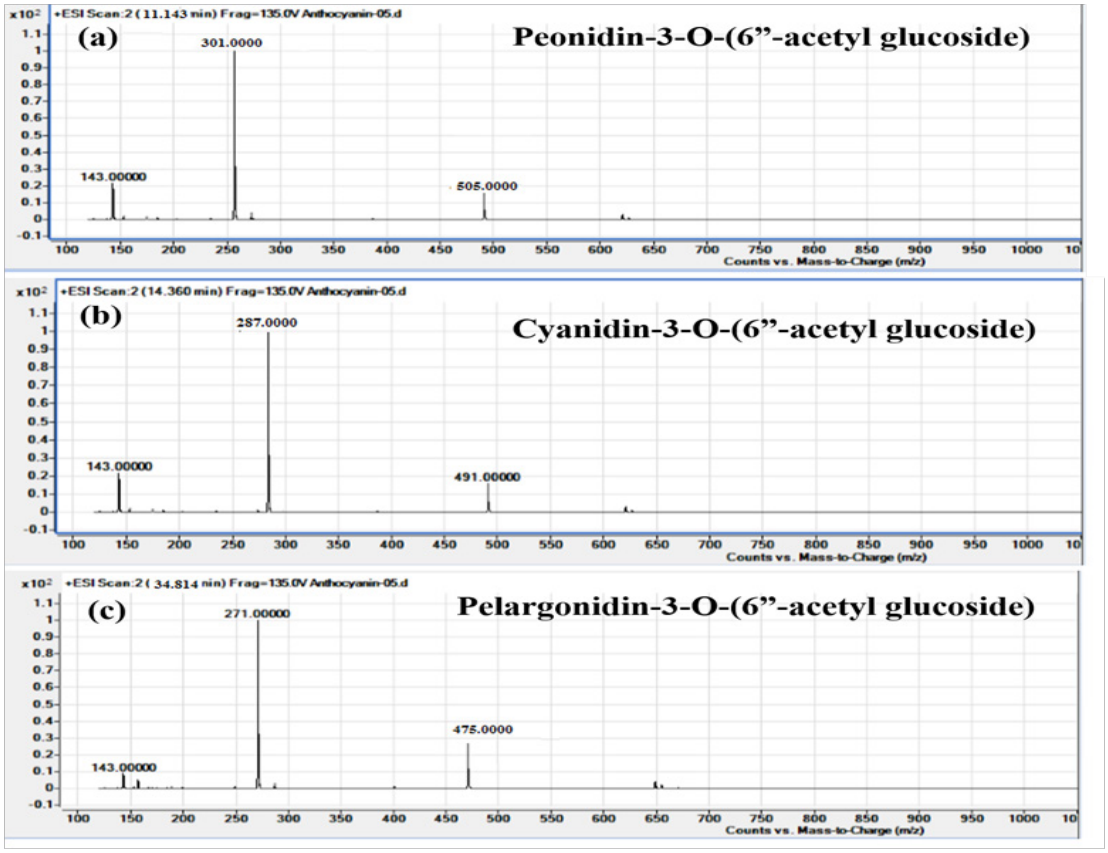

Figure 6 Mass fragmentation of acylated anthocyanins, (A) peonidin-3-O-(6"-acetyl glucoside), (B) cyanidin-3-O-(6"-acetyl glucoside) and (C) pelargonidin-3-O-(6"-acetyl glucoside).

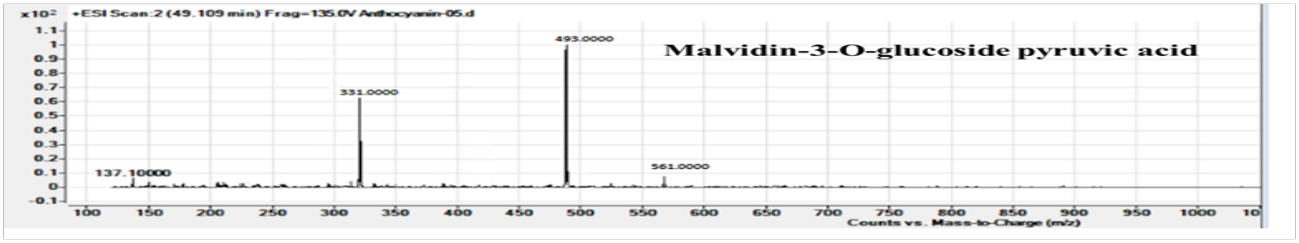

Figure 7 Mass fragmentation of Malvidin-3-O-glucoside pyruvic acid.

Conclusion

The present study reveals that Phalsa (Grewia asiatica) is a promising indigenous fruit in terms of anthocyanins content. *The results showed that* cyanidin-3-O(6"acetyl glucoside) was the *abundant anthocyanins comprising* 44-63% *of total anthocyanin* followed by peonidin 3-O- glucoside (3-30%) and pelarigonidin-3-O-(6"acetyl glucoside) (8-14%). Other anthocyanins identified were delphinidin 3-O- glucoside, pelarigonidin-3-O-malonyl glucoside, peonidin-3-O (6"-acetyl glucoside) and malvidin-3-O-glucoside pyruvic acid *contributing 16-25% of total anthocyanin.* Acidic methanol was found as a best solvent for *extraction of* anthocyanins from *Grewia asiatica. In addition* extracted anthocyanins *were* more stable in dark than exposed to ultra violet light. In dark extract was stable at temperature range of 10-40℃. Overall current study indicates that *Phalsa (Grewia asiatica) is a promising indigenous fruit and has potential to be explored as food colorant and nutraceuticals using food grade solvent extraction strategies.*

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Conflict of interest

All authors declare that they have no conflict of interest.

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