RESEARCH ARTICLE

Extraction, Isolation, and Characterization of Pelargonidin 3,5-O-Diglucoside Salt from Pomegranate Flowers

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Received: 20th December, 2022; Revised: 09th February, 2023; Accepted: 18th May, 2023; Available Online: 25th June, 2023

ABSTRACT

This study aimed to understand the simple and reliable process for obtaining pelargonidin 3,5-o-diglucoside from pomegranate flowers. The process of isolating, characterizing, and exploring a constituent's potential for natural origin is the first step in the development of new pharmacological frameworks. The investigation covers preliminary screening, titrable acidity, total phenolic, total anthocyanin, total reducing sugar content, and spectrum analysis by UV, IR, spectrofluorometric analysis, and EDS. The DPPH technique is then used to assess antioxidant activity. the 3, 5-O-diglucoside of pelargonidin. The cold maceration method removed HCl from pomegranate flowers using a green solvent methanol containing 0.1% methanolic HCl. Anthocyanins, flavonoids, and tannins are found in the methanolic pomegranate flower extract as secondary metabolites, according to preliminary screening. Total phenolic content (1.40–6.19 mg/g) of gallic acid as standard), total anthocyanin content 0.03 to 0.68 mg equivalent to cyanidin 3-glucoside (CGE)/g. UV-vis spectra showed λ_{max} in the visible region at 502 nm. It showed strong DPPH radical activity at 85.80%, equivalent to the ascorbic acid standard. Energy dispersion X-ray analysis checked for elemental detection. The titrable acidity was found to be 1.216 in terms of citric acid. LCMS analysis for structural confirmation. Thus, the anthocyanins from agro-waste of pomegranate flowers could be the best way to extract and isolation it for commercial, pharmaceutical, and nutraceutical use.

Keywords: Anthocyanin, DPPH activity, Flavonoid content, Maceration, Pelargonidin-3,5-o-diglucoside, *Punica granatum L.*

International Journal of Drug Delivery Technology (2023); DOI: 10.25258/ijddt.13.2.07

How to cite this article: Kamble KG, Laddha KS. Extraction, Isolation, and Characterization of Pelargonidin 3,5-O-Diglucoside Salt from Pomegranate Flowers. International Journal of Drug Delivery Technology. 2023;13(2):494-500.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

The pomegranate, or *Punica granatum* L., is a member of the Lythraceae family. Maharashtra is home to commercial pomegranate farms. Blooms can be induced at any time based on the seasonality of the rainfall. Agro-waste can result from overweight to dwarf shrubs and blooms dropping off due to constant fruit production throughout the year. The plant is useful for its medicinal and nutritional qualities.[1-4] Because of its capacity to impart vivid colors ranging from red to blue, anthocyanins are a superior alternative to synthetic dyes in culinary applications. Six distinct anthocyanins have been identified in pomegranate juice, with three found in the fruit and three in the flower.[5-7] The fruit includes pelargonidin, caffeic acid and ferulic acid.[8-11] Anthocyanins form glycosidic linkage to flavonoids like kaempferol covalently.[12] Flowers typically contain complex mixes of sugars, flavonoids, and anthocyanins; sometimes, categorizing is difficult. When O-glycosylated at C3, anthocyanidins range in hue from orange to red.[13, 14] Pomegranate fruit has numerous nutritional, biological, and pharmacological significance, including anti-proliferative, anti-cancer, cardioprotective, and anti-diabetic properties.[15] In general, pelargonidin glycoside was extracted with ethanol acidified with hydrochloric acid and other acids, or by maceration with acidified acetic acid and methanol, according to experimental design.[16-18] Nevertheless, scientific investigations and dependable methods of extraction and isolation of anthocyanins have been lacking, despite the fact that anthocyanins have a relatively low yield in practice.

This study set out to determine the most efficient and straightforward strategy for extracting, separating, and characterizing pelargonidin-3,5-O-diglucoside salt from pomegranate flowers for use as a biofuel feedstock. Anthocyanin was extracted and purified from pomegranate flower by-products using a green solvent developed specifically for this purpose. Finally, spectrum data obtained via spectroscopic methods was used to establish the characterization of their chemical backbone. Quantitative and qualitative content identification of polyphenols, anthocyanins, reducing sugar, etc., were determined using spectral and LCMS analysis, which accompanied the phytochemical study.

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METHODS AND MATERIALS

Plant Material, Extraction, and Preliminary Phytochemical Screening of Extract

Young orange-red colored flowers of pomegranate procured from a farm located at Sangola, Maharashtra, in April-May. Clean and stored at 4°C. Weigh accurately about 250 gms of flowers, cut into small pieces and add 250 mL methanol with 50 mL of 0.1% methanolic HCl[18] (green solvent) sonicate for 30 minutes and keep for cold maceration at 4°C overnight. Tannins (Punicalagin) were settled down at the bottom of the beaker and gently removed from the supernatant. The finished extract was centrifuged at 3000 rpm for 15 minutes to separate the supernatant. Using column chromatography, the extract was refined. Using a rotatable evaporator, the produced extracts were concentrated to dryness at 40°C under decreased pressure and then stored at 2°C in airtight glass vials with screw-on caps.

Spectral Analysis

Light absorption is used to determine a color's value. While FTIR provides information about each functional group by absorbing the spectral frequency of the IR spectrum, the IR spectrum is absorbed by FTIR. Shimadzu also studied FTIR spectra (model IR Affinity-1S, Kyoto, Japan) at 4 cm^{-1} intervals over the 400–4000 cm^{-1} wavelength range**.** Fluorescence measurements of the pelargonidin 3, 5-diglucoside HCl crystals, and pomegranate extract were carried out on a spectrofluorimeter Jasco FP-8200 Quartz Cuvette with 1.0 cm × 1.0 cm.

Titrable Acidity

Fresh pomegranate flower extract (10 g) should be diluted with 10 mL of sterile water. In order to determine the percentage of citric acid present, take 10 mL (10 g) of fresh methanolic extract from PMG flowers, Combine 2–5 drops of phenolphthalein indicator with 10 mL of distilled water, titrate against 1N potassium hydroxide, and then express the titratable acidity as a colour scale from red to light brown to colorless. Titratable acidity can be calculated as follows.[19,20]

$$\text{Titrable acidity} = \frac{\text{mL of alkali (B.R)x normality x Eq. wt. of citric acid}}{\text{Weight of sample in mL x10}}$$

Total Phenolic content

As per S. Chandra, it was evaluated with a modified Folin-Ciocalteu (FC reagent) test. Multiple amounts of gallic acid (from 0 to 100 mg/mL) are used as standards. A crude pomegranate flower extract was mixed with FC reagent, taking up 0.2 mL of volume (2 mL, 1:10 diluted with distilled water). Sodium bicarbonate solution (1.5 mL) and distilled water (to fill the rest) were added after waiting 5 minutes. A spectrophotometer reading of 725 nm was taken after letting the solutions sit for 90 minutes at room temperature. The total phenolic content was reported as the amount of gallic acid equivalents (GAE) per gramme of dried extracts.[21,22]

Total Anthocyanin Content

Total anthocyanin content was calculated using a modified version of the approach described by Xuan Tien Le *et al*. Separate the crude extract in halves and dilute it with different buffers, one with 0.025 M KCL (pH = 1) and the other with 0.4M acetate (pH = 4.5). The concentration of each sample was modified with buffers to yield an absorbance reading between 0.1 and 1.0. We measured the mixture's maximum absorbance at 710 nm and its maximum absorbance at vis-max with a UV-vis spectrophotometer. Followed a formula to determine the overall anthocyanin concentration:[23,24]

$$\text{Total anthocyanin content} = \frac{A \times MW \times DF \times V \times 1000}{a \times l \times m}$$

where A represents the absorbance, MW represents the molecular weight of cyanidin-3-glucoside (449.2 g/mol), DF represents the dilution factor, V represents the volume of the solvent in mL, a represents the molar absorptivity (26,900 $L.mol^{-1}.cm^{-1}$), and l represents the length of the cell path (1-cm).

DPPH Antioxidant Activity

A modified method by Marjoni MR[25] and TutyAnggraini[26,27] was used for the DPPH antioxidant activity of pomegranate flower extract. One gram of fresh flowers was added to 10 mL of (0.1%) methanolic HCl and crushed. After incubating the crude extract in methanol and DPPH solution for 30 minutes, the absorbance at 517 nm was measured (1 mM in methanol). To extrapolate unknown amounts of the extract using the calibration curve method ascorbic acid was utilized as a reference with concentrations of (10, 30, 50, 70, 90, and 120 g/mL).[28]

Total Reducing Sugar Content by DNSA Method[29,30]

Fructose standard stock was prepared concentration of 250 µg/mL and working standards of 10 to 50 µg/mL from this stock solution. The test sample was prepared by dissolving 0.2 and 0.8 mL of fresh pomegranate flower extract (equivalent to 100 mg of dry extract). The 3 mL sample was mixed with 1-mL of DNSA reagent and the final volume was 10 mL with distilled water. Once the reaction was complete, the mixture was boiled for five minutes in boiling water to stabilize the final product. Absorbance spectra were taken at 540 nm in a UV-vis spectrophotometer using 1-mm quartz cuvettes (Shimadzu, Japan). For the purpose of the blank test, one mL of distilled water was used in place of the test sample.

Table 1: Phytochemical screening of secondary metabolites from pomegranate flower extract

S.no.	*Functional group/ Preliminary phytochemical group*	*Reactor*	*Description*
1.	Anthocyanins	With HCl and NaOH	+ + +
2.	Flavonoids	HCl and magnesium turning	+ + +
3.	Alkaloids	Mayer &Dragendorffs	-
4.	Glycosides	Liberman-Buchard	-
5.	Tannins	Lead acetate, $FeCl_3$	++
6.	Terpenoids	Borntragger Test	-
7.	Steroids	Liberman-Buchard	-

LCMS Analysis

The modified method by Mary H. Grace[31] was used for the present study. A total of 5 mg/mL of extracted and purified pelargonidin 3,5-diglucoside HCL from pomegranate flowers was dissolved in a solvent mixture consisting of 65% methanol, 35% water, and 5% formic acid. Before analysis, sample solutions were frozen at - 20°C. LC-MS testing was performed at the BITS, Goa location. For confirmation of pelargonidin 3, 5-diglucoside in pomegranate flower extract. LCMS conditions are as below, elution was done by a gradient system of the binary mobile phase of (A) water with 1% formic acid (v/v) and (B) acetonitrile with 1% formic acid (v/v); at 10% B in 0 minutes, 10% B in 1-minutes, increasing to 50% B in 5 minutes, decreasing to 10% B in 5.1 minutes and 10% B in 7 minutes; flow rate 350 µL/min.; injection volume 1-µL; auto-sampler temperature 4°C. An instrument used 6400 agilent technology series triple quadrupole B.08.00 (Data Acquisition B8023.5 SP1).

RESULTS

Preliminary Screening

Farmers discard the pomegranate flower once the season expires, so the use of agro-waste to make products is the study's goal. The extract of *Punica granatum L* contains a high amount of flavonoids, anthocyanins, and tannins (Table 1). Thus, using green solvent methanolic HCL, successful extraction was done by cold maceration method. The extraction process was done using green solvent and checking its secondary metabolite constituents. Simple chemical tests determine the phytochemical analysis of pomegranate flower extract to find out the secondary metabolite content. The tests reveal the presence of anthocyanins, flavonoids, polyphenols, and tannins while it gives negative results for steroids, glycoside, and alkaloids. Table 1 shows the screening result.

Identification of pelargonidin 3, 5-O-diglucoside by Thin layer chromatography

To establish the presence of active phytoconstituent qualitatively thin-layer chromatography analysis was utilized. Pomegranate extract was found to have a pH of 1.10. For the development of TLC, prepare a 0.5% solution of isolated pelargonidin 3, 5-O-diglucoside in methanol. Pelargonidin 3, 5-o-diglucoside was qualitatively analyzed by TLC using a previously extracted stock of extract and compound following purification by column chromatography. M. Filip earlier reported a chromatographic spot for pelargonidin from strawberry juice on a cellulose plate that showed Rf toward the solvent front. On the contrary, we saw the Rf at 0.63, which was caused by the presence of the diglucoside moiety in the pelargonidin molecule.[32,33]

Estimation of titrable acidity of Pomegranate flower extract

Measure about 10 mL (10 gms) of fresh methanolic extract of PMG flowers, Combine 2 to 5 drops of phenolphthalein indicators to 10 mL of pure water, and titrate against 1N potassium hydroxide, and express the titratable acidity against

TLC profile

1. Pelargonidin 3,5- diglucoside.HCl (Before column)
2. PMG extract
3. Pelargonidin 3,5- diglucoside.HCl (After column purification n) R_{f}-0.63

Condition of TLC: - Mobile phase: - n-Butanol: Methanol: Water (10:5:1)

Figure 1: TLC profile

the percentage of citric acid, with the endpoint of red to light brown to colorless. Individual acids cannot be identified by routine titration. As a result, the major acid is frequently used to express titratable acidity. Citric and malic acids may be present in significant levels, and the dominant acid may change with development. And the percentage of that acid varies with the maturity of the flower to become fruit. The pH of raw extract was 1.10, and titrable acidity was found to be 1.216 in terms of citric acid percentage. The reason of high acidity and low pH could be due to citric acid and malic acid production.

Spectral Analysis

(UV, IR, and Fluorimeter) Anthocyanins are generally exerted spectra at or above 460 nm. Pelargonidin 3, 5- diglucoside HCl exerts characteristics UV-vis spectra with λ_{max} in the visible region (Figure 1) at 502 nm while other common anthocyanins appear at 520, 535 nm. However, the maximum visible wavelengths of the individual aglycone vary, for pelargonidin as an aglycone has a maximum visible wavelength of 520 nm, whereas the maximum visible wavelengths of any anthocyanin glucosides were roughly 10 to 15 nm lower. Similar to the statement, our observations determined UV spectra for pelargonidin 3, 5-O-diglucoside was 504 nm, (Figure 2), and also chromatographically R_f was above 0.5 i.e. 0.63 (Figure 1). various chromatographic and spectroscopic methods can be used to describe anthocyanins. Among these, UV-vis spectral information has preliminary significant put a spotlight on the structural characteristics and identity of such compounds.

Isolated pelargonidin 3,5-O-diglucoside FTIR. FT/IR-4600 type A with TGS detector measured HCl and methanol extract of pomegranate blossom at 4 cm^{-1} interval over the 400 to 4000 cm^{-1} wavelength range. The active functional group in anthocyanins is polyphenols. Pelargonidin 3,5-O-diglucoside HCl in methanol and crystal form in Figure 3 shows prominent spectra for 3550 to 3200 O-H stretching, 1648 to 1638 C=C stretching, strong, broad O-H stretching alcohol intermolecular bonded, and 1420 to 1330 medium O-H bending alcohol. The IR data confirmed the presence of aromatic groups from the UV data, which also revealed the aromatic C=C and C-H bonds at a spectral range of 1638 to 1648 and 669 cm^{-1}, respectively.

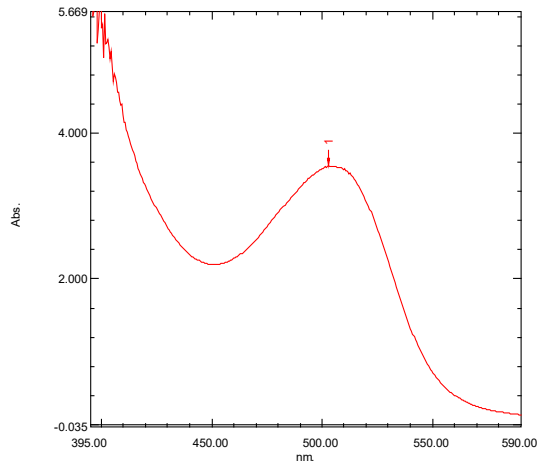

Figure 2: UV-spectra of isolated Pelargonidin 3,5-O-diglucoside.HCl

Anthocyanins were confirmed according to FTIR spectra and comparison between extract and isolated.

Fluorimeter analysis

The fluorescence excitation spectra of pigments found in PMG extracts and isolated compounds have been measured at a beginning range of 200 to 700 nm with intervals of 0.5 nm, and the emission wavelength is 520 nm. The spectral investigation was performed in two steps i.e. in pomegranate flower extract and isolated pelargonidin 3,5-O-diglucoside HCl. The PMG extract (in methanol) color was yellowish-orange while the isolatedcrystals were dark orange. Thus the isolated compound shows more instances of orange color. In the presence of certain physicochemical conditions, one can make the assumption that a number of additional anthocyanins also emit fluorescence. In Figure 4, the characteristic excitation spectra illustrated that the color intensity varies at peak height. The obtained spectrofluoroscence investigation explained the fluorescence emission and excitation spectra at 520 nm were different for fresh PMG flower extract and isolated pelargonidin 3,5-O-diglucoside, at visible wavelength.

Pomegranate extract in methanol showed higher color intensity when compared to methanol as a blank, but it was found to be more intense in color when compared to isolated and purified 250 ppm solution of pelargonidin 3,5-O-diglucosideHCl.The X-axis represents intensity, whereas the Y-axis represents wavelength [nm]. The spectrofluorometric method can be more sensitive than absorbance and provide useful information about particular molecules. G. Agati 2013 reported that anthocyanin and its derivatives in red wine samples, help to distinguish monomeric/ polymeric substitution by fluorescence method.[34-38]

EDS analysis

EDS is a technique for determining which substances are present in the samples and evaluating their relative abundance. Energy-dispersive X-ray analysis for isolated crystals of pelargonidin3,5- diglucoside. HCl showed the presence of carbon, oxygen, and chloride elements. The internal standard for Carbon is $CaCO_3$, Oxygen is SiO_2 and Cl is KCl was

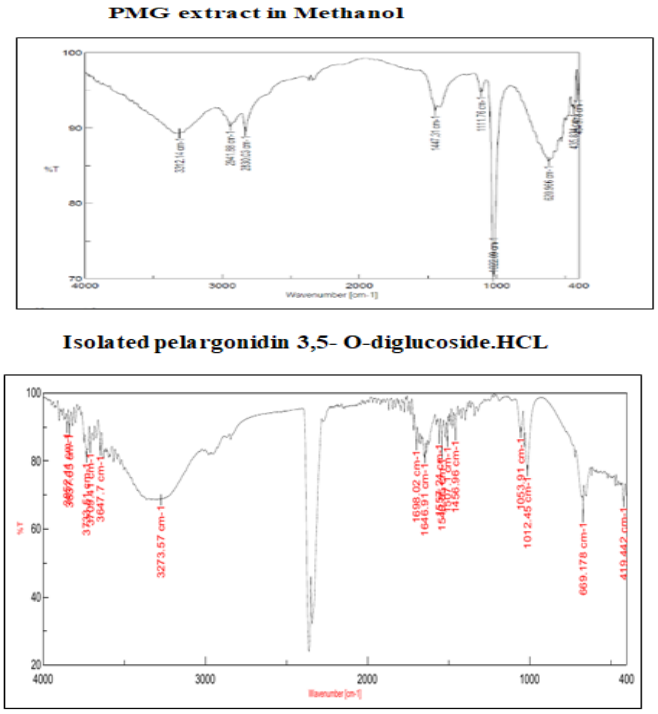

Figure 3: IR-spectra of isolated Pelargonidin 3,5-O-diglucoside.HCl in methanol and crystals

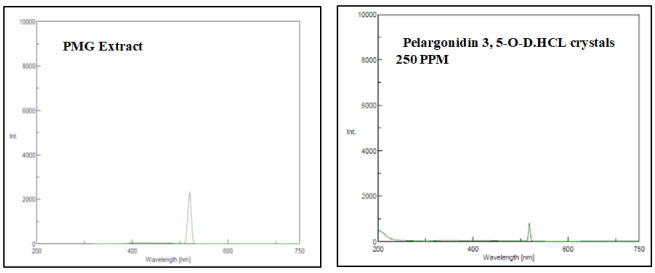

Figure 4: Excitation spectra of isolated pelargonidin 3,5-O-diglucoside in methanol and crystals

used. However, the result indicates the presence of chloride salt of pelargonidin 3,5-O-diglucoside.As the EDS technique applicable for the visualization of particles in liquid form, and also a powerful method for element detection. The isolated crystals may contain hydrochloride salt of pelargonidin. So this method is reliable and easy to analyses presence of salt molecule. From the Figure 5, it was confirmed that isolated compound having chloride ions.

Total phenolic content

Sonicating the extract at 40°C for 15 minutes, then centrifuging at 1,000 rpm for 2 to 5 minutes, yielded a final concentration of about 100 mg. The crystal-clear supernatant was poured into an amber-colored bottle for later study. In contrast, 10 to 50 mg/mL gallic acid standard was prepared in methanol. Calibration curve was plotted ($y=0.1191x + 0.0823$, R^2 0.9851). The experimental trials were carried out with different concentrations of hydrochloric acid, citric acid, etc. But we obtained the good extraction yield (0.6–0.8 mg/100 gms fresh flowers) by 0.1% methanolic HCL and anthocyanin separated

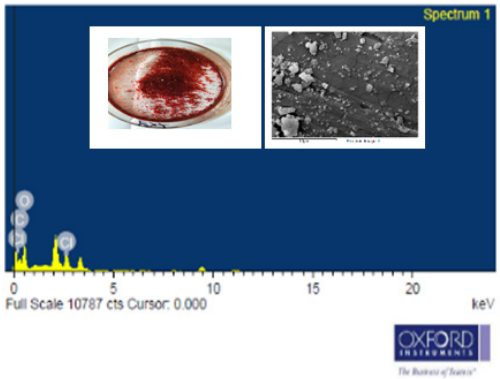

Figure 5: EDS spectrum and elemental data analysis of Pelargonidin 3,5-O-diglucoside.HCl crystals

out. Walid Elfalleh previously estimated the polyphenol content from pomegranate peel was more than flowers. In comparison, we get a significant phenolic content from fresh pomegranate flowers. Figure 6 indicates total phenolic content by Folin-Ciocalteu assay at 765 nm was found to be 1.40 to 6.19 mg/gram of flower extract compared with the gallic acid standard. Thus while extraction and during the overnight maceration stage punicalagin, and tannins were settled down to the bottom of the beaker. Punicalagin, quercetin, and other polyphenols are majorly present in *Punica granatum L* at any flowering or fruit stage. Our data explain, that punicalagin obtained was light yellowish amorphous powder and showed black color with 5 % alcoholic ferric chloride.

Total anthocyanin content

The total monomeric anthocyanin content contained in pomegranate flowers ranges from 0.03 to 0.68 mg, comparable to cyanidin 3-glucoside (CGE) per gramme.

Total Sugar content

The DNSA reagent method at 540 nm determined that the total sugar concentration ranged from 9.11 to 17.84 mg/100g, which is equivalent to fructose sugar. The calibration curve was plotted in Figure 7, which shows the equation as y=0.0185x+0.2716, R2 0.9884. The level of fermentation can be inferred from the amount of reducing sugar that has been measured quantitatively. The strategy depends on the detection of free carbonyl C=O groups in reducing sugars as the target of its investigation. Oxidation is the first step in the

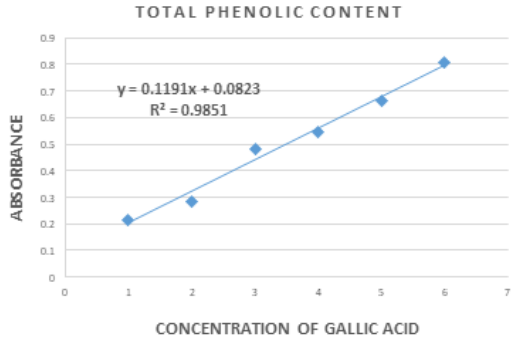

Figure 6: Estimation of total phenolic content (mg GAE/g) from pomegranate flower extract

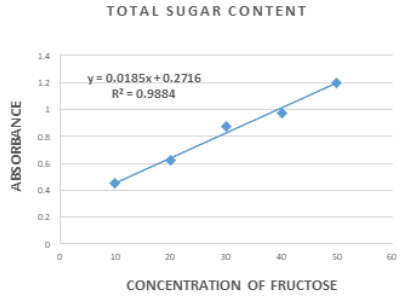

Figure 7: Total reducing sugar content estimation (mg fructose/g) from pomegranate flower extract

transformation of fructose and glucose into their respective ketonic and aldehyde functional groups. Yellow was the original hue of 3,5-dinitrosalicylic acid, but an orange-red color resulted from the oxidation of 3-amino-5-nitrosalicylic acid in an alkaline media.

Antioxidant Activity by DPPH

Figure 8 displays the scavenging activity data for DPPH radicals. The research found that the pomegranate flower extract scavenged free radicals (85.80 ± 0.12). Ascorbic acid (10–120 µg/mL) was used as a reference point to draw the conclusion that flower extract had good scavenging action. It was determined by plotting the calibration curve (y= 0.0031x +0.1324, R2 0.9824). To instead, test pelargonidin for its antioxidant properties using the DPPH scavenging technique. There was a linear correlation between absorbance and the concentration of standard ascorbic acid and a sample of pomegranate flower extract 1 g/10 mL of solution at first, as the concentration of standard get increase then the overall line trend was proportionally increased. From the slope, for sample pelargonidin 3,5-glucoside's DPPH radical scavenging activity value was 85.80 ± 0.12 ascorbic acid/100 g FW. Free radical scavenging is a broad mechanism of antioxidants. Hydrogen donated by anthocyanins can combine with free radicals to generate the reduced state.

LC-MS Analysis

A hybrid Q-TOF mass spectrometer was utilised throughout the analysis process. The result obtained by the analysis showed fragmentation prominent at 595 which is specific for molecular mass of pelargonidin. Samples were prepared in LC grade methanol. To understand chemical nature of the

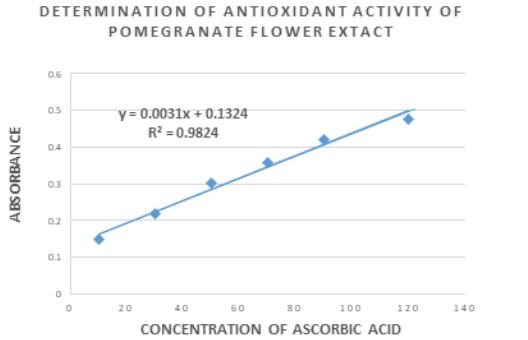

Figure 8: DPPH free radical antioxidant activity of pomegranate flower extract

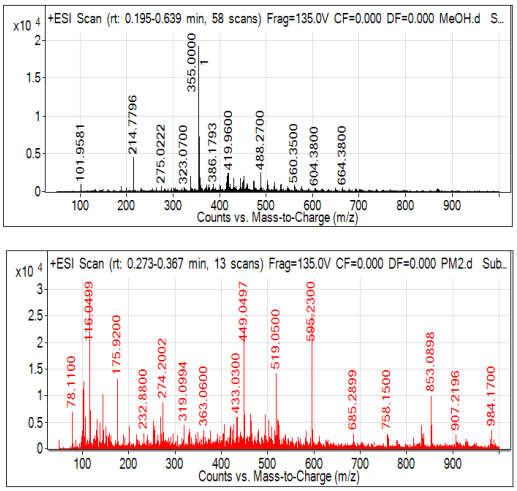

Figure 9: ESI Mass spectral analysis of pelargonidin-3,5-O-diglucoside HCL compared with methanol as blank

Table 2: LC-MS Peak list of pelargonidin 3,5-O- diglucoside HCL

m/z	*Abundance*
78.1002	7872.03
102.02	9073.22
104.24	11831.69
116.0702	8483.29
216.89	9029.3
449.0701	14565.25
503.12	8443.61
519.05	12224.04
595.2	23031.63
596.27	8631.26

molecule, LCMS analysis was carried out, Figure 9 and Table 2 represents the chromatogram and corresponding m/z peak list of molecules. It indicates the presence of two glycoside moieties (519, 685) along with prominent pelargonidin peaks as aglycone (595). This data help to understand the nature of isolated anthocyanin/anthocyanidin molecule in pomegranate flower.

Isolated pelargonidin 3,5-O- diglucoside HCL was confirmed by molecular mass fragmentation by LCMS analysis. The maximum absorbance of all identified peaks was 495 to 505 nm, which is typical of pelargonidin salt. Jeremy S. Barnes demonstrated fragmentation ESI of pelargonidin 3,5-diglucoside and cyanidin 3,5-glucoside but we reported diglucoside linkage of pelargonidin with "O" and in salt form of aglycone part attached to glycone. Finally, the isolated molecule was structurally elucidated by LC-MS analysis. The ESI scan was compared with methanol as blank. Sugar substitutions at two positions yielded fragments m/z at 519.0500 and 685.2899 glycoside moieties, respectively. While 449.049 and 595.2300 pelargonidin aglycone part. Data were compared with previously reported anthocyanins from different sources and showed pelargonidin 3, 5 diglucoside prominent peak at 508 nm. Here, we have interpreted a similar ESI scan at 504 nm. The theoretical mass of pelargonidin 3,5-diglucoside was 595.16.

CONCLUSION

The results of the current study point to a quick and reliable procedure for isolating, extracting, and characterizing pelargonidin-3, 5-O -diglucoside. The present study reveals, counter to what has been previously reported, it is possible to turn agricultural waste into the best product. According to the data obtained, pelargonidin-3,5-O-diglucoside hydrochloride salt crystals successfully extracted, isolated, and characterized from ripened pomegranate flowers showed strong antioxidant activity and might also be employed in foods, nutraceuticals, and medicines.

ACKNOWLEDGMENT

The authors wish to thank Dr. Kumar from BITS Goa and Vishal from Sprint Labs.

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