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Phytochemical studies on cyanidin 3-O glucoside•HCl from the ripened fruit pulp of Terminalia catappa

Kranti Kamble
Research Scholar, Department of Pharmaceutical Sciences, Institute of Chemical Technology, Mumbai, Maharashtra, India
Email: phg18kg.kamble@pg.ictmumbai.edu.in, krantik244@gmail.com

Dr. K. S. Laddha
Research Guide, Department of Pharmaceutical Sciences, Institute of Chemical Technology, Mumbai, Maharashtra, India
Corresponding author email: ks.laddha@ictmumbai.edu.in, ksladdha@yahoo.co.in

***Abstract*---**Context: *Terminalia catappa* (Combretaceae) is a large tropical tree grows mainly in the tropical regions of Asia, Africa, and Australia. The fruits a drupe 5-7 cm long and 3-5.5 cm broad, green at first, then yellow, and finally red when ripe, containing a single seed. Ripened fruit pulp contains anthocyanins. Objectives: To extract and isolate active anthocyanin from fruit pulp of *Terminalia catappa*. And characterize isolated cyanidin-3-0-glucoside by spectral analysis.Methods: An easy and efficient technique for isolating and extracting anthocyanin from *Terminalia catappa* ripen fruit pulp. Besides the total phenolic, flavonoid, anthocyanin, and reducing sugar content of the extract, the antioxidant capacity of *T.catappa* fruit pulp was evaluated and by DPPH, H2O2 methods. Characterization of cyanidin-3-o-glucoside done by UV, IR spectral analysis. While single crystal structure and elemental detection done by EDS analysis. And further structural confirmation done by LCMS analysis.Result: Fresh extract contain 26.17 % and isolated compound contain 83.79% total anthocyanin content, the total phenolic content was found to be 1.58 % in fresh extract as compared with gallic acid standard. 34.48 % total reducing sugar content and 54.50 % total flavonoid content observed. In vitro antioxidant activity was observed by DPPH method was 55.88% while 73.79 % by H2O2 method of analysis. Structure analysis and characterization was done by UV,IR and LCMS.Conclusion: Successfully extracted and isolated new method for anthocyanin from *Badam* fruit. Also study reveals the in vitro antioxidant activity of both extract and isolated too.

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***Keywords*---**anthocyanin, cyanidin-3-o-glucoside, total phenolic content, antioxidant activity, Terminalia catappa.

Introduction

Terminalia catappa (TC) is commonly known as Indian almond a flowering tree belonging Combretaceae family. One of the natural resources from which natural dyes can be derived and substituted for the use of synthetic colors. *Terminalia catappa* is an aesthetic tree and Indian almond usually referred to as "desi badam" (a well-known plant in the Ayurvedic system). It turns from green to yellow to bright crimson to dark purplish-red at full maturity during the maturation process. Fruits begin to produce around the age of three, and you can consume the wholesome, delicious seed kernels right away after they are extracted. Additionally, natural products have been isolated from the plant *T. catappa*, which contains flavonoids (rutin), gallic acid, hydrolyzed tannins, punicalin, chebulagic acid, as well as triterpenoids (ursolic acid, Asiatic acid) (Venkatalakshmi, V. 2016, Thomson, L. A., 2006, Anand, A., 2015). Anthocyanins are water-soluble pigments that, depending on their pH, may appear red and purple in acidic while blue towards less acidic or basic condition. The general anthocyanidins are the basic structures of the anthocyanins. The anthocyanidins (or aglycons) consist of an aromatic ring [A] bonded to an heterocyclic ring [C] that contains oxygen, which is also bonded by a carbon–carbon bond to a third aromatic ring [B].The distribution of the six more common anthocyanidins in fruits and vegetables is: Cyanidin 50%, Delphinidin 12%, Pelargonidin 12%, Peonidin 12%, Petunidin7% and Malvidin 7%(Castañeda-Ovando, A., 2009). *Terminalia catappa* (TC) fruits contains anthocyanin - cyanidin 3-o-glucoside as a major constituent(Kaneria, M. J., 2018)while TC leaves contains cyanidin 3-o- β-D-glucopyaranoside(Garzón, G. A., 2009).Their stability is affected by several factors such as pH, storage temperature, chemical structure, concentration, light, oxygen, solvents, and the presence of enzymes, flavonoids, proteins and metallic ions(Rakkimuthu, R., 2016). The aim of present research work was to extract, isolate and characterize cyanidin-3-O-glucoside from hull part of *Terminalia catappa*fruit. And characterize the isolated active principal by spectral analysis and structural elucidation by UV, IR, LCMS analysis and followed by its antioxidant activity by DPPH and Hydrogen peroxide methods.

Material and Methods

Raw material and chemicals

Terminalia catappa fruits; an Indian almond fruit were collected from the university campus of the Institute of chemical technology, Mumbai in the month of March to July and stored at 4° C. All the solvents were purchased from SciTech scientific, Mumbai (Chauhan, A., 2022).

Extraction and isolation

Weigh about 500 gms*Terminalia catappa* fruits were clean with water, and air dry(Panda, S., 2022). Cut fruit hull part (mesocarp and exocarp) into small pieces

and grind it. To the fruit pulp (about 390gms) 750 ml solvent methanol was added along with 0.1% methanolic HCl and sonicate for 30min. in intervals, followed by overnight cold maceration. The next day, filter the mass through muslin cloth and collect the filtrate. The collected material was again kept for maceration further 24 hr, to settle down hydrolysable tannins and other acids, and fibers in the extract. Collect the supernatant and separate settled acid by centrifugation at 5000RPM for 10 min. The solvent was recovered by vacuumed pressure at or below 40ºC to get semisolid mass. Collect the semisolid dark purple red colored extract. The concentrated extract was used for further studies(Sekar, A., 2022). For isolation of active anthocyanins components and anthocyanin in extract, column chromatography was done with 100% methanol as mobile phase by using acidic alumina (pH 3.5 – 4.5). Evaporate the collected material at or below 40º C to get crystals of salt of cyanidin-3-glucoside as major anthocyanin. Confirmation of cyanidin 3-O- glucoside by thin layer chromatography (TLC) on silica gel G-254 plate.

Characterization of isolated compound

The pure isolated compound was characterized by different spectroscopic techniques (i.e. UV, IR). Spectroscopic analysis was carried out using a double-beam JASCO V-530 Ultraviolet-Visible Spectrophotometer. Followed by elemental detection by EDS analysis. Structural characterization was done by LCMS analysis.

Total sugar content

Sample material measuring 100mg was taken into a boiling tube and hydrolized by keeping it in boiling water bath for three hours with 5mL of 2.5N HCl and allowed to cool under room temperature. Later it was neutralized with solid sodium carbonate until effervescence ceases and check it with pH paper, then made up volume to 100mL and centrifuged if needed. The supernatant was collected and taken 0.5 and 1mL aliquots for analysis. Standard were prepared by taking 100 mg of D-glucose in 100 mL water and in test tube taking 0.2; 0.4; 0.6; 0.8; 1 mL of reference standard and ‘0’ serves as blank. Then, made up volume to 1mL in all tubes including sample tubes by adding distilled water and added 4mL of anthrone reagent. The test tubes were heated for eight minutes in a boiling water bath and allowed to cool rapidly and read the green to dark green colour at 630 nm. The content of all the test tubes were subjected to cooling on ice before adding ice-cold anthrone reagent.

Standard graph was drawn by plotting the concentration of standard on X-axis versus absorbance on Y-axis. From the graph, the amount of carbohydrate present in the sample tube was calculated using following formula

$$\text{Amount of carbohydrate present in 100mg of sample} = \frac{mgofD-glucose}{volumeoftestsample} X\ 100$$

Total anthocyanin content

The differential assay method for anthocyanin is based on measuring the absorbance at two different pH values, previously reported by Lee et.al. It is based on structural transformation of anthocyanin chromophore as function of pH. In order to determine the total monomeric anthocyanin content at pH 1.0 and 4.5 measured at both 516 nm and 700nm wavelength. Samples were diluted in two buffer solutions: potassium chloride buffer 0.025M (pH 1.0) and sodium acetate buffer 0.4 M (pH 4.5) and then absorbance was measured simultaneously at 516nm and 700nm after 15 minutes of incubation at room temperature. Absorbance reading were made at room temperature against distilled water as blank. A JASCO V530 UV-Vis spectrophotometer was used for measurements. The monomeric anthocyanin pigment concentration was calculated according to the following equation

$$\textbf{Totalanthocyanincontent (mg/l)} = \frac{\boldsymbol{AxMWxDFx1000}}{\boldsymbol{\varepsilon xl}}$$

Where A= (A516-A700) pH 1.0 – (A516-A700) pH 4.5, MW is molecular weight, DF is dilution factor, ε is the molar absorbance of cyanidin -3- glucoside; and l is the path length (1cm). The total monomeric anthocyanin was expressed in terms of monomeric cyanidin.

Total phenolic content

Preparation of Standard Gallic Acid and sample for Calibration Curve. Total phenolic contents (TPC) in the fruits extract was determined by Folin–Ciocalteu colorimetric method as described by (Garzón et al., 2009),(Ondo J. P., 2013).with some modifications. Standard gallic acid solution was prepared by dissolving 10 mg of it in 10mL of methanol (1 mg/mL). Various concentrations of gallic acid solutions in methanol (1, 2, 3, 4, 5 and 6 µg/mL) were prepared from the standard solution. Followed by 2 ml Folin– Ciocalteu reagent (0.2 N diluted in MeOH). A reagent blank using MeOH instead of sample was prepared. After 5 min incubation at room temperature, 1 ml sodium carbonate solution (7 %) was added. Samples were incubated at room temperature for 1 h and the absorbance was measured at 765 nm versus the prepared blank. And follow the same procedure same as standard for crude extract of *Terminalia catappa* fruit.

Total flavonoid content

The total flavonoid content of the sample was determined by (Phuyal, N., 2020)method using the aluminium chloride colorimetric technique. Quercetin was used as a standard to make a calibration curve for the measurement of total flavonoid concentration. The standard quercetin solutions were made by one capsule of 100 mg of quercetin in 100 mL of methanol and further serial dilution of quercetin with methanol (5-200 g/mL) after the stock quercetin solution was made by dissolving. Separately, 0.6 mL of 2 percent aluminium chloride was combined with 0.6 mL of diluted standard quercetin solutions or extracts. The

mixture was then left to sit at room temperature for 60 minutes. At a wavelength of 420 nm, the reaction mixtures' absorbance was compared to a blank.

Antioxidant activity

DPPH scavenging activity

Preparation of DPPH Solution (0.1M):- DPPH solution (0.1 M) was prepared by dissolving 0.39 mg of DPPH in a volumetric flask, dissolved in methanol, and the final volume was made 100 ml, prepared purple-coloured DPPH free radical solution was stored at −20°C for further use. The DPPH scavenging activity was determined as described by (Phuyal, N., 2020). Sample was compared with standard Gallic acid (10-60 µg/ml).The crude methanolic extract 3.9 ml ripened fruits of *Terminalia catappa* was mixed with 1 ml of a DPPH solution (0.1mM in methanol) in a test-tube, final volumemake up with methanol up to 5ml. And the absorbance was measured at 517 nm after 30 minutes of incubation at room temperature. DPPH radical scavenging activity was calculated as follows:

$$\mathbf{DPPHradicalscavaingactivity} = \mathbf{1} - \left[\frac{\boldsymbol{A517(Sample)}}{\boldsymbol{A517(Blank)}}\right]\boldsymbol{X100}$$

H_2O_2 scavenging activity

A modified method by(Bhatti, M. Z., 2015) was used to estimate antioxidant activity by H2O2. This approach was used to assess the extract's capacity to scavenge hydrogen peroxide (H_2O_2). Following the addition of 1 mL of H_2O_2 (2mM) solution, an aliquot of 0.2 mL and 0.8 mL of extracts (approximate 10 to 100 mg/g of TC extract) was placed into the test tubes. The volume make up-to 5 mL with 50mM phosphate buffer (pH 7.4). After 10 minutes of reaction time, the reaction mixture was vortexed, and its absorbance at 230 nm was determined. The positive control used was ascorbic acid. The following equation was used to determine the extracts capacity to scavenge H_2O_2.

$$\mathbf{H2O2scavaningactivityinpercentage} = \frac{\mathbf{(A0 - A1)}}{\mathbf{A0}}\mathbf{x100}$$

where: A0 = Absorbance of control (Ascorbic acid) , A1 = Absorbance of sample.

Results and Discussion

Thin layer chromatography

After frozen, anthocyanins that were chromatographed in an acidic environment mostly maintain their red color. Blue appears when the environment is alkaline. The thin layer chromatography was useful tool for qualitatively analyzed. The Fig.1 represents presence of cyanidin 3-0-glucoside in comparison with standard CYN-3-0 Glucoside.

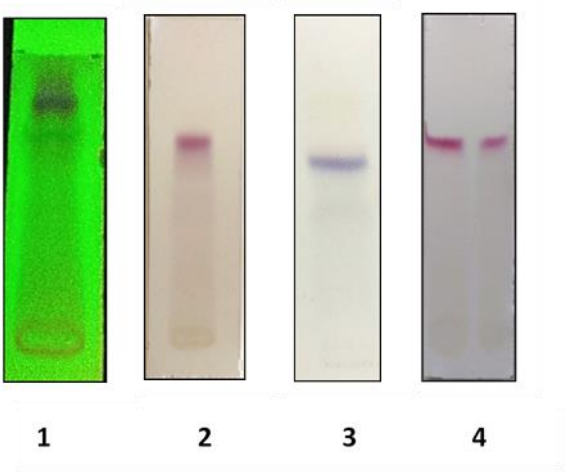


Fig. 1. Thin layer chromatogram for methanolic extract of *Terminalia catappa* and isolated cyaniding 3-O-glucoside. (Mobile phase:- Butanol: Methanol: Water (1:0.5:0.5)

The extract of TC indicates presence of active anthocyanins along with flavonoids, while the isolated matches with standard. The solvent system was self-developed for its proper separation and identification with Rf reported in book of plant drug analysis by H. Wagner and S.Bladt(Lee, J., 2005).

Track No	Sample	R_f
1	Extract of TC at UV 254	0.71
2	*Terminalia catappa*extract Visible light	0.68
3	Isolated Cyanidin-3-O- glucoside	0.68
4	CYN STD compared with isolated cyanidin-3-o-glucoside	0.68 & 0.68

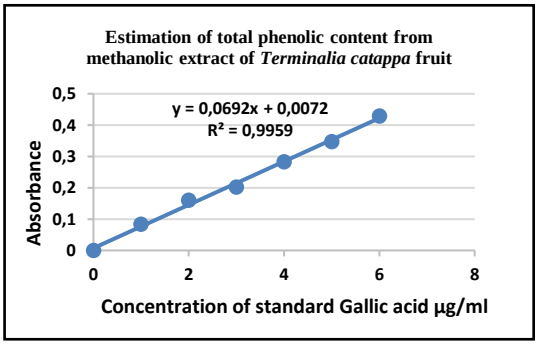
Table 1. TLC of different forms of *Terminalia catappa* and its corresponding Rf values

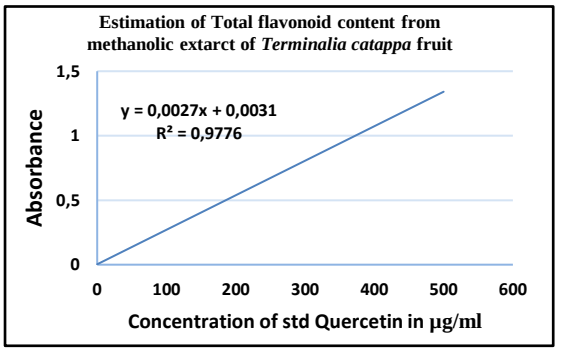
The extract of TC under UV-254, track 1 showed presence of quercetin as flavonoid towards solvent front along with cyanidin-3-O-glucoside. Fig.1 track 3 and 4 are isolated and in comparison with standard one. So we have confirmed that, the TC methanolic extract contains cyanidin-3-O-glucoside as prime anthocyanin.

The characterization of extract and isolated CYN-3-O-glucoside.HCl

As the *Terminalia catappa* fruit present for preliminary phytochemical testing for anthocyanins, flavonoid, polyphenols so further quantitatively these parameters were analyzed. Also checked for reducing sugar content. We have checked the presence of glucoside by TLC and quantitatively by reducing sugar content by anthrone method. Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green colored product with an absorption maximum at 630nm. Table 2 indicates the

quantitative data for total flavonoid, total anthocyanins, total phenolic and reducing sugar content etc.

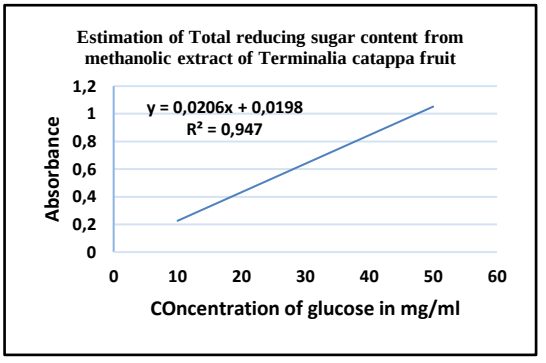


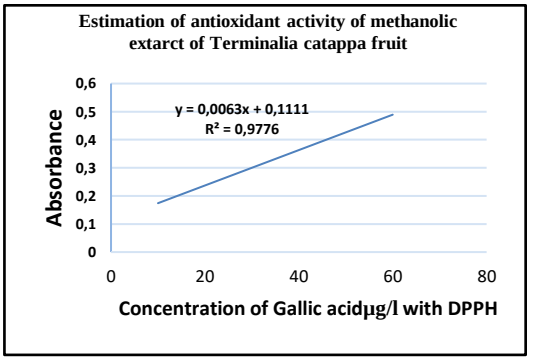


A **B**

Fig.2. Estimation of Total Phenolic content (A) and Total flavonoid content (B)

Generally, phenolic components of plant showed antioxidant activity(Krishnaveni, M., 2014). Majorly polyphenols are flavonoid, tannic acid and anthocyanins. The methanolic extract of TC was showed the higher phenolic content what will gives its bioactivity, hence it is to be expected that it will perform well in terms of antioxidant and antibacterial properties. Fig. 2 and 3 gave details about estimations of phenolic content, flavonoid content, sugar content and DPPH antioxidant activity.





A **B**

Fig.3. Estimation of Total reducing sugarcontent (A) and antioxidant activity (B)

Quantitatively all the parameters calculated by graph and by y=mx+c. Table 2 indicates the data generated from the graphs and equations.

Sr. No.	Characterization tests	Quantitative analysis
1	Total anthocyanin content	26.17 mg/L CYN equivalent of TC fresh extract
2	Total flavonoid content	21.62 mg/ 100g of Quercetin standard
3	Total phenolic content	2.670 mg/ 100 g GAE

4	Total reducing sugar content	36.74 mg/100g D-glucose carbohydrate
5	DPPH scavenging activity	77.88 %
6	H2O2 scavenging activity	72.65 % (compared with ascorbic acid)

Table 2. Quantitative parameters for different characterization tests on *Terminalia catappa* fruit extract

We showed that *Terminalia catappa* exhibits significant free radical scavenging potential activity by both the methods, like DPPH and H2O2. As 2, 2-diphenyl-1-picrylhydrazyl (DPPH), which is based on the ability of DPPH to react with proton donors such as polyphenols present in TC. Also it breaks down fast into oxygen (O2) and water (H2O), and it may also produce hydroxyl radicals (OH) (Ayina, K., 2015, Anuforo, P. C., 2018). The ability of TC methanolic extract was to neutralize hydrogen peroxide may be linked to the presence of phenolic groups of anthocyanins (Cyanidin 3-o-glucoside), which could donate electrons to the compound and convert it to water (H2O).

In Table 2 data was given regarding quantitative parameters for antioxidant activity by these two methods 77.88 % by DPPH radical scavenging while 72.65 % (compared with ascorbic acid) by using H2O2. Anthocyanin possesses antioxidant activity due to presence of polyphenolic nature of structure. All the three rings contains OH group, along with glycoside as glycone unit.

Elemental detection by EDS analysis

EDS is a method for identifying the elemental constituents of the samples and assessing their relative abundance. We have carried out elemental detection from Sprint Testing laboratory, Mumbai. The data demonstrated in Fig. 3 and table 3 explain for Cyanidin-3O-glucoside isolated crystals: energy-dispersive X-ray analysis. Carbon, oxygen, and chloride elements were detected by HCl. The internal standards for carbon were CaCO3, oxygen, and chlorine were SiO2 and KCl, respectively. However, the outcome suggests that cyanidin 3-O-glucoside chloride salt, by calculating its relative number of atoms present. (Fig.3)

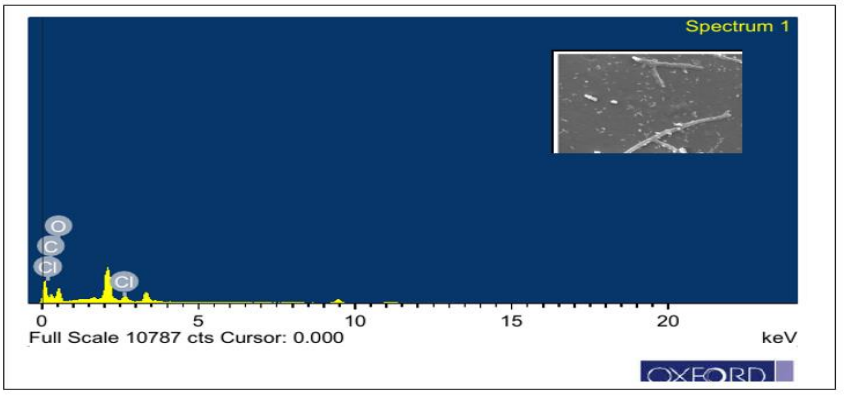


Fig.3. EDS analysis for identification of elements present in isolated cyanidin-3-O-glucoside from TC extract

Element's name	Element %	Atomic %
C K	41.59	49.72
O K	54.06	48.52
Cl K	4.35	1.76
Total	100	100

Table 3 Percentage of elements present in isolated CYN-3-O-glucoside.HCl

Spectral analysis of TC extract and isolated CYN-3-O-glucoside.HCl

Figure 4 represents the IR spectra of TC extract in methanol (A) and isolated cyanidin 3-o-glucoside (B), showed 3194, 3299 (O-H) stretching of hydrogen bond, 2836, 2946 & 2834 (C-H) aliphatic stretching, 1641 &1652 ; 1446 &1405 (C=C) stretching aromatic, 1107 & 1012; 1110 & 1014 (C-O) stretching alcohol & phenols.

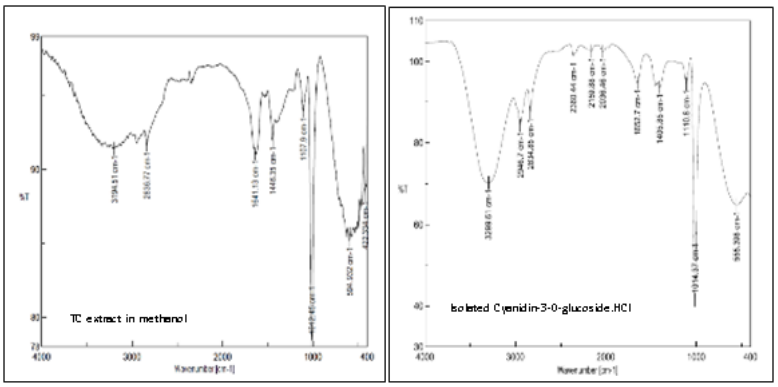
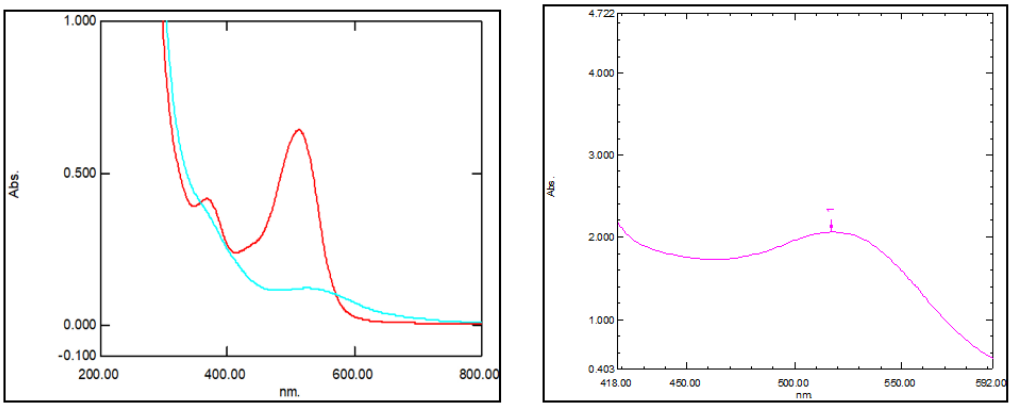


Fig. 4 IR spectra for methanolic extract of *Terminalia catappa* and isolated cyaniding 3-O-glucoside

The IR data interpreted for presence of polyphenolic nature, in both A & B of Fig.4. On other side, Fig. 5 shows the UV-Vis absorption spectra of the crude anthocyanin extract of *Terminalia catappa*. The UV-Vis spectra clearly demonstrate the pH dependence of anthocyanins. Cy-3-glucoside (Cy-3-glu) is an example of the structural alterations that are depicted in Fig. 5. pH below 2, the flavylium ion of cyanidin-3-O-glucoside predominates and exhibits a red colour, whereas at pH > 2, a series of chemical changes that are partially reversible occur which can be proven by UV spectra.



A **B**

Fig. 5 Spectral characteristics of Cyanidin-3-0-glucoside.HCl from methanolic extract of *Terminalia catappa* in buffers at pH 1.0 and 4.5 (A) and Methanolic extract of TC

The positive charge on the interior ring of anthocyanins causes them to strongly absorb light between 385 and 550 nm at pH 1.0, with a maximum absorption at around 520 nm. Lee et al was previously reported the pH differential approach is predicated on the idea that polymeric or degraded anthocyanins have little to no absorbance in pH 4.5 buffer whereas monomeric or "pure" anthocyanins have a lot. (Favaro, L. I., 2018, Fedenko, V. S., 2017). In comparison with this, our study showed absorbance at the pH 1.0, anthocyanins more even though almost less at pH 4.5, monomeric anthocyanins take on the hemiketal form i.e. degraded anthocyanins in the polymeric form are resistant to colour change with change in pH. Therefore, polymerized anthocyanin pigments are not measured by this method because they absorb both at pH 4.5 and 1.0. Only a small differences are in the flavylium form which will only slightly increase the absorbance.

Structural Confirmation by LC-MS analysis

The Fig.6 represents LCMS analysis was performed at BITS pilani, GOA. The LC-MS studies for the anthocyanins were all first carried out in positive ion mode. Since anthocyanin may be found in acidic solutions as flavylium ions, these ions were discovered as a $[M]^+$ rather than as an adduct ion peak $[M + X]^+$ operating in positive ion mode. The given data represented isolated cyanidin-3-O- diglucoside HCL was confirmed by the molecular mass fragmentation. The prominent *m/z* peak for cyanidin-3-o-glucoside was at 449 was previously by Heejung Yang &Trikas et.al.(Yang, H., 2017, Trikas, E. D., 2016).The ESI-Fragmentations of our results were at 449 denoted for aglycone part of anthocyanin i.e. cyanidin while for sugar substitution at 485. As the cyanidin was along with traces amount of Quercetin fragment ion at 256 and 146. (Table 4)

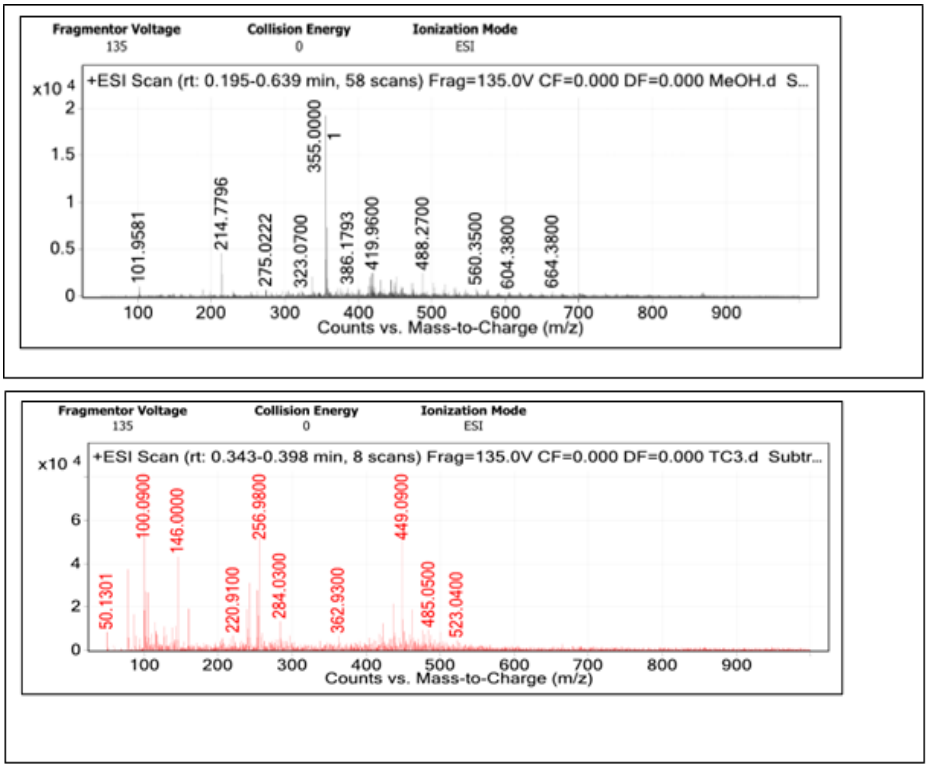


Fig. 6 LCMS peak fragmentation of isolated cyanidin 3-o-glucoside from TC

m/z	Abundance
78.1	37525.54
100.09	75107.31
102.0999	27240.16
105.9999	26827.59
146	43332.38
256.98	52334.38
449.09	59260.86

Table 4. Relative abundance of LCMS analysis of CYN-3-O-glucoside.HCl

Conclusion

The results of analysis of *Terminalia catappa* ripened fruits reported in Fig no .1 and table no 2. Data was demonstrated the qualitative and quantitative presence of cyandin-3-o-glucoside. Further characterization was found that TC has CYN-3-O-glucoside.HCL a predominant anthocyanin, along with traces of quercetin. It had stronger antioxidant activity due to polyphenols. The total anthocyanin content was 26.17 mg/L CYN equivalent of TC fresh extract. Other Gallic acids, punicalagin were separated during last step of extraction and isolation. The other characteristic tests showed in table no 2. TC exerts good antioxidant activity. The flavilliyum ion differs its UV absorption by slight change in p^H (p^H1.0 and p^H 4.5) denoted in Fig 4. Finally structure was confirmed by FTIR and LCMS. The developed method of extraction, isolation and characterization had advantages

like simple, reliable and from agro-waste to best by technically. The use of cyanidin-3-O-glucoside was in food and nutraceuticals.

Conflict of interest

The authors have no conflicts of interest to declare.

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