THE ISOLATION AND STRUCTURAL DETERMINATION OF FLAVONOID FROM THE FLOWERS OF PUNICA GRANATUM L

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Abstract: ***The present work deals with the isolation and structure elucidation of new flavones from methanolic flowers extract of Punica granatum. Isolation of the compound was carried out by chromatographic techniques, followed by different spectral analytical methods. The spectral data of UV-Vis, FT-IR, ^{1}H NMR, ^{13}C NMR and GC-MS of the isolated compound is in support of presence of flavone nucleus. The compound has been characterized as 3, 7, 8, 4' -tetrahydroxy-3'-myrt-8-enylflavones (Punicaflavone). The isolation of the above considered flavonoid would be helpful to practice plant-based pharmaceutical research to treat various difficulties linked with human diseases.***

Keywords: Punica granatum, UV, FT-IR, NMR, MS, Punicaflavone

INTRODUCTION

Flavonoids have gained recent attention because of their broad biological and pharmacological activities in these order flavonoids have been reported to exert multiple biological property including antimicrobial, cytotoxicity, anti-inflammatory as well as antitumor activities but the best-described property of almost every group of flavonoids is their capacity to act as powerful antioxidants which can protect the human body from free radicals and reactive oxygen species. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. On the other hand flavonoids such as luteolin and catechins are better antioxidants than the nutrients antioxidants such as vitamin C, vitamin E and β-carotene.

Flavonoids have been stated to possess many useful properties, containing anti-inflammatory activity, enzyme inhibition, antimicrobial activity, estrogenic activity, anti-allergic activity, antioxidant activity, vascular activity and cytotoxic antitumor activity [1]. Flavonoids constitute a wide range of substances that play important role in protecting biological systems against the harmful effects of oxidative processes on macromolecules, such as carbohydrates, proteins, lipids and DNA [2]. In addition, flavonoids are known to inhibit lipid-peroxidation, platelet aggregation, capillary permeability and fragility, cyclo-oxygenase and lipoxygenase enzyme activities [3].Flavonoids are capable of modulating the activity of enzymes and affect the behavior of many cell systems and exerting beneficial effects on body [4]. Flavonoids decrease the risk of coronary heart disease by three major actions: improving coronary vasodilatation, decreasing the ability of platelets in the blood to clot, and preventing low-density lipoproteins (LDLs) from oxidizing [5]. Flavonoids have been reported to exert wide range of biological activities. These include anti-inflammatory, antibacterial, antiviral, anti allergic, cytotoxic antitumor and vasodilatory actions [6].

Punica granatum L. (Punicaceae), known as pomegranate, is a deciduous small tree, up to 8 m in height with attractive reddish scarlet edible fruits. The species originated in Iran, Afghanistan and Baluchistan, found wild in the warm valleys of the Himalayas and is cultivated throughout India [7]. The dried flowers, known as Gulnar, are efficacious to treat haematuria, haemoptysis, diarrhoea, dysentery, nasal hemorrhage [8] and in Unani literature as a remedy for diabetes [9, 10]. Flower juice is recommended as a gargle for sore throat, in leucorrhoea, hemorrhages and ulcers of the uterus and rectum. The root bark and stem bark of the plant are astringent and used as anthelmintic especially against tapeworms. Fruit rind is valued as an astringent in diarrhea and dysentery. The powdered flower buds are useful in bronchitis. The seeds are reputed as stomachic and the pulp as cardiac and stomachic. The green leaf paste is applied to relieve conjunctivitis [11]. In Chinese medicine these flower are also used for the treatment of injuries from falls and grey hair of young man [12]. In addition *Punica granatum* L. is considered as "a pharmacy unto itself" in ayurvedic medicine and is used as an antiparasitic agent, a blood tonic, and to ulcers [13].

However, to the best of our knowledge, flavonoids are useful in pharmaceutical applications for drug discovery and development. Hence, we have isolated, identified and reported the flavonoid from methanolic extracts of flowers of *Punica granatum*. Furthermore, we have been characterized the flavonoid through UV, FT-IR, (13C and 1H) and MS techniques. In our present study reported the isolation and structural elucidation of new the flavone.

MATERIALS AND METHODS

Collection and authentication of plant material

The flowers of *Punica granatum* L. were collected from in and around the Mannargudi, Thiruvarur District, Tamil Nadu, India. They were identified and authenticated by Dr. S. John Britto, Department of Botany, RAPINAT Herbarium and Center for Modular Systematics, St. Joseph's College, Trichurappalli, Tamil Nadu, India.

Preparation of plant material

Collected plant material were thoroughly washed with distilled water and then dried under shade at room temperature for few days. The dried plant samples were ground well into a fine powder using blender. The powdered samples were then stored in airtight containers for further use at room temperature.

Preparation of extract

500 g of powdered plant material (flower) of *Punica granatum* L. was filled in a thimble and extracted exhaustively by Soxhlet apparatus (12h) using 2.0 L methanol solvent at 60°C. The extract obtained was collected and passed through Whatman no.1 filter paper to remove all debris and unextractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent. The solvent from extract was removed under reduced pressure and controlled temperature (40-50 ºC). The yield of the extract was 12.28% w/w. The dry extract was kept in tightly closed container in refrigerator for further analysis.

Screening for phytochemicals

The Phytochemical tests were carried out on the methanol extract of the flowers of *Punica granatum* (Linn.) using standard procedures to identify the constituents as described by Harborne 1983 [14].

Isolation, purification and characterization of compound

The isolation, purification and characterization of bioactive compounds were carried out using repeated silica gel column chromatography and thin layer chromatography (TLC). The purified bioactive compound was characterized by subjected to UV, IR, NMR and Mass spectroscopy studies.

RESULTS AND DISCUSSION

SPECTRAL ANALYSIS

The pure compound obtained was then subjected to spectral analysis for the structural elucidation of the compound. Characterization of compound was carried out by following techniques.

UV-Vis Spectroscopic analysis

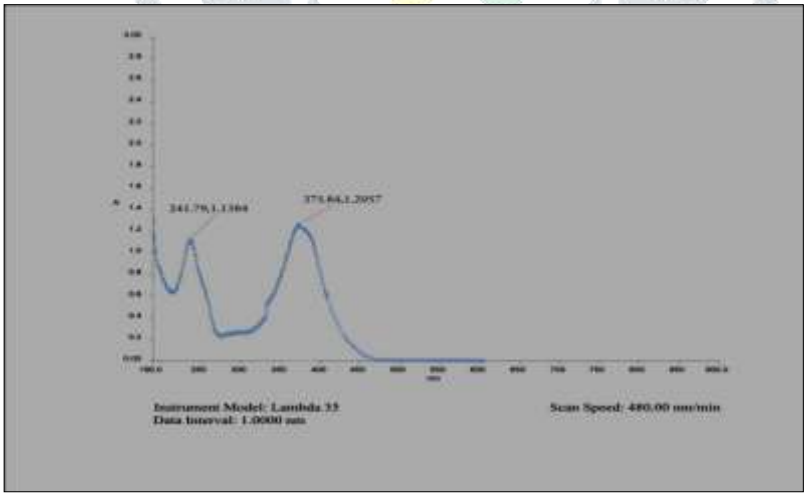
The qualitative UV-Vis spectrum profile of isolated compound was selected at wavelength from 120 to 900 nm due to sharpness of the peaks and proper baseline. The profile showed the peaks at 214 and 375 nm with the absorption of 1.1384 and 1.2957 respectively (Table 1 and Figure 1).

UV-Visible spectrophotometry is one of the most frequently employed techniques in pharmaceutical analysis. Spectroscopic methods have become a powerful tool for secondary metabolite profiling as well as for qualitative and quantitative analysis of the pharmaceutical and biological materials. The application of standardized UV-Vis spectroscopy has years been used in analyses of flavonoids. Visible wavelengths cover a range from 400-800 nm and the near ultra violet region out to 200 nm. Molecules undergo electronic transitions in these ranges of the electromagnetic spectrum [15]. The qualitative UV-Vis spectrum profile of methanolic extract of *Punica granatum* was selected at wavelength from 120 to 900 nm due to sharpness of the peaks and proper baseline. UV- Visible spectroscopy interpretations of *Punica granatum* has one major peak. The profile showed the peaks at 214 and 375 nm with the absorption of 1.1384 and 1.2957 respectively.

Table1: UV-Visible profile of punicaflavone

Wavelength(nm)	Absorption
241.79	1.1384
375.84	1.2957

Figure 1: UV-Visible spectrum of Punicaflavone



FT-IR Spectroscopic analysis

FT-IR spectrum was used to identify the functional group present in the compound. The results of the FT-IR spectrum profile was illustrated in Table 2 and Figure 2. The FT-IR gave broad peak at 3443 cm^{-1} which indicated the presence of O-H stretching. It gave a strong peak at 3195 and3010 cm^{-1} which indicated the presence of O-H stretching and C-H stretching. The peaks obtained at 2105 and 1480 cm^{-1} indicated the presence of C=O stretching. The peak obtained at 780 cm^{-1} indicated the presence of C-H Bend out of plane. The peaks obtained at 1640 and 1010 cm^{-1} indicated the presence of aromatics. The FT-IR spectrum confirmed the presence of alcohols, phenols, alkanes, alkenes, carbonyl and aromatics in the compound.

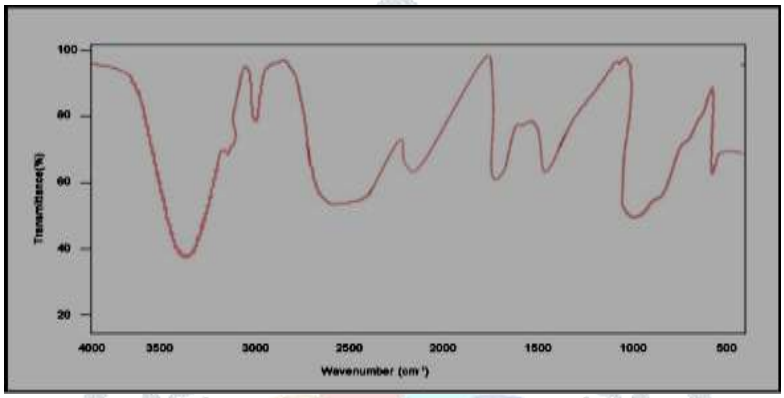
FT-IR was used to analyze and identify functional groups of active compounds based on major peak values. Results were compared using the infrared chart. Flower extract has alcohols, phenols, alkanes, alkenes, carbonyl and aromatics in methanolic extract. The alkanes are found in the plant cuticle and epicuticular wax of many species. They protect the plant against water loss, prevent the leaching of

important minerals by rain and protect against micro-organisms and harmful insects [16]. Alkenes are important in the manufacture of plastics, e.g., polythene and as fuel and illuminant. They serve as raw materials for the manufacture of alcohols and aldehydes. They are used for artificial ripening of fruits, a general anesthetic, making poisonous mustard gas and ethylene-oxygen flame. Aldehydes are used in the production of resins when combined with phenols [17].

Table 2: FT-IR profile of Punicaflavone

S.No.	Wave number cm -1	Bond	Functional groups
1	3443-3195	O-H Stretch, H-bonded	Alcohol, Phenol
2	3010	C-H Stretch	Alkane
3	2548-2105	C=O Stretch	Carbonyl
4	1640-1480	C=O Stretch	Carbonyl
5	1010	C=C Stretch	Aromatics
6	780	C-H bending	Alkene

Figure 2: FT-IR spectrum of Punicaflavone



NMR analysis

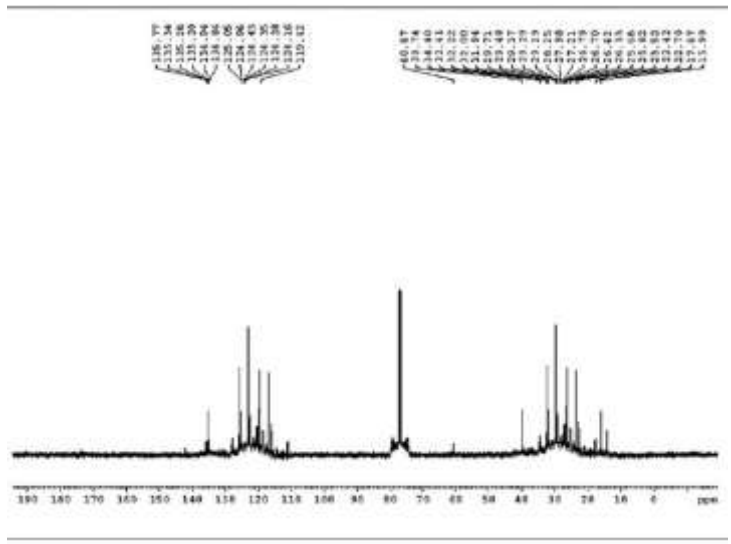
The ^{1}H NMR spectrum of compound displayed the characteristic signals at different chemical shift (δ) showed at 5.34, 5.21, 4.20, 2.10, 1.89, 1.56, 1.37. The ^{13}C NMR spectra showed resonance for 21 carbons differentiated by the distortion less enhancement by polarization transfer experiments, including one carbonyl (C-4, δC 137.0), nine quaternary aromatic signals (δC 137.3, C-2; 125.4, C-3; 122.4, C-5; 120.1, C-7; 118.6, C-9; 117.3, C-10; 111.2), and oxygenated methines (δC 77.5, 39.6,31.4,28.4, 26.5, 24.1,22.6) and methyl (δC 17.4, 15.1, 16.8) groups. Those data indicated compound 1 as having a basic flavone structure. One of the aromatic rings contained only two aromatic proton signals, at δ 5.34 (^{1}H, d, J= 2.1 Hz, H-8) and 4.20 (^{1}H, d, J = 2.1 Hz, H-6), with characteristic meta coupling (J = 2.1 Hz). Since there were no other proton signals on this ring, this suggested the aromatic ring was tetra substituted. The chemical shifts and coupling of the protons at δ 5.34 and 6.20 and the chemical shifts of the three carbon signals in the range of 119-135 ppm, implied a 1,3,5-trioxygenation pattern around the aromatic ring.

The ^{13}C NMR data, 21 carbon atoms, in agreement with one carbonyl, two aromatic rings (5 methines and 7 ternary carbons), one tetra substituted double bond and 12 aliphatic carbon atoms (1 methylene, 1 methyl and 10 methines). The NMR spectrum (recorded in methanol-d4) confirmed the presence of five aromatic protons, 12 non-exchangeable protons consistent with a sugar moiety, and a methyl group.

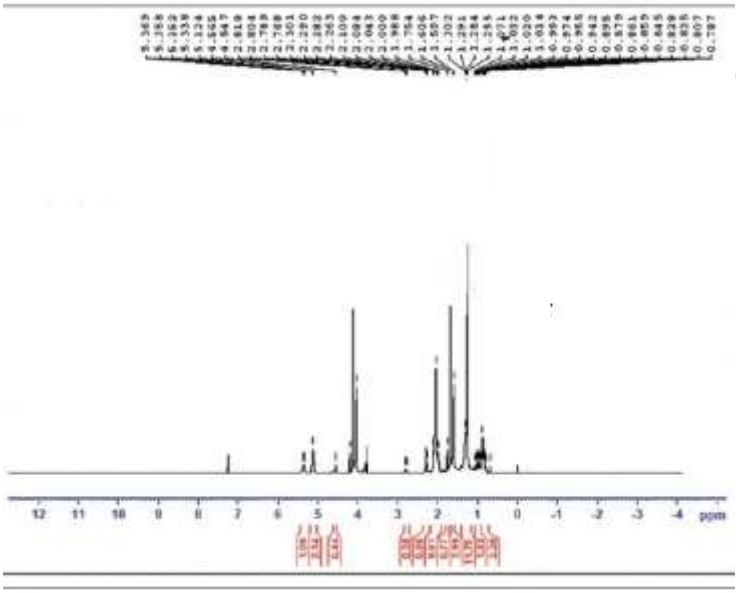
The second aromatic ring showed an ortho coupled doublet at δ 5.34 (1H, d, J = 8.5 Hz, H-5'), a doublets at δ 6.20 (^{1}H, d, J = 8.5, 2.1 Hz, H-6') and an ortho coupled doublet at δ 7.23 (^{1}H, d, J= 2.1 Hz, H-2'), consistent with a 1, 3, 4-substitution pattern. The carbon resonances at δc 120.1 (C-3') and 137.3 (C-4') indicated a dioxygenated phenyl ring. The spectrum showed correlations of the protons at δ 4.20 (H-6') and 5.34 (H-2') to δc 158.0. This carbon chemical shift is characteristic of a C-2 double bond of ring C of a flavonoid skeleton and the correlations were consistent with a 3',4'-dioxygenated phenyl ring (ring B) being connected to C-2. The results are shown in Figure 3.

Together^{1}H and ^{13}C NMR is used in determining a substance's molecular structure. It is often used in conjunction with other spectrometric techniques such as FT-IR and Mass Spectrometry. NMR is primarily related to the magnetic properties of certain atomic nuclei; notably the nucleus of the hydrogen atom, the proton, the carbon, and an isotope of carbon. NMR spectroscopy has enabled many researchers to study molecules by recording the differences between the various magnetic nuclei, and thereby giving a clear picture of what the positions of these nuclei are in the molecule. Moreover, it will demonstrate which atoms are present in neighboring groups. Ultimately, it can conclude how many atoms are present in each of these environments Several attempts have been made in the past by using preparative or semi preparative thin-layer chromatography, liquid chromatography, and column chromatography to isolate individual phenols, the structures of which are determined subsequently by NMR off-line [18].

Figure 3: NMR STUDY SPECTRUM OF PUNICAFLAVONE



Proton Nuclear Magnetic Resonance (^{1}H NMR)



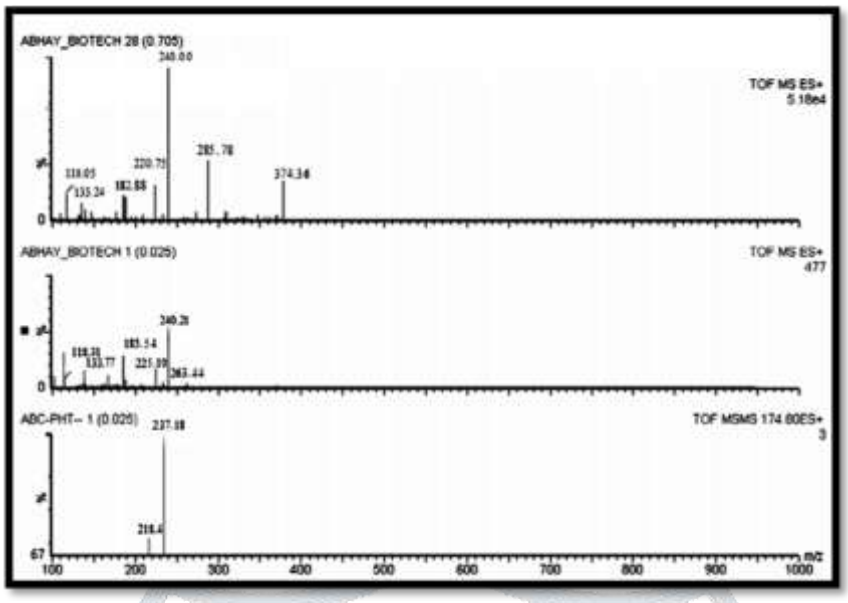
Carbon Nuclear Magnetic Resonance (^{13}CNMR)

Mass spectrometric analysis

In MS analysis, the parent molecular ion peak [M^{+1}] peak at m/z 420 suggesting one of the possible molecular formula as $C_{25}H_{24}O_6$.In the present finding dihydroxylated A ring and another hydroxyl group and monoterpenic moiety in ring B. The intense ion peaks supported the presence of tetra cyclic ring in a mono-type monoterpene (Figure 4).

Mass spectrometry allows the determination of the molecular mass and the molecular formula of a compound, as well as certain structural features. A small sample of the compound is vaporized and then ionized as a result of an electron being removed from each molecule, producing a molecular ion (a radical cation). Many of the molecular ions break apart into cations, radicals, neutral molecules, and other radical cations [19]. The bonds most likely to break are the weakest ones and those that result in the formation of the most stable products. These fragments of the molecules are detected individually on the basis of their mass-to-charge ratios [20]. The details of exactly how these positively charged fragments are separated and detected differ according to the specific design of the mass analyzer portion of the instrument. In any case, the information acquired and displayed by the data system (the so-called mass spectrum) allows the analyst to reconstruct the original molecule and thereby identify it. Besides the significant applicability to molecular compound identification, mass spectrometry also finds application in elemental analysis, such as to determine what isotopes of an element might be present in a sample [21]. The antioxidant activity of flavonoids depends strongly on the number and positions of hydroxy groups are presumed to increase the antioxidant capacity. The presence of the hydroxy group on 3, 7, 8, 4' depicted to enhance the antioxidant activity of isolated compound.

Figure 4: Mass Spectrum of isolated compound

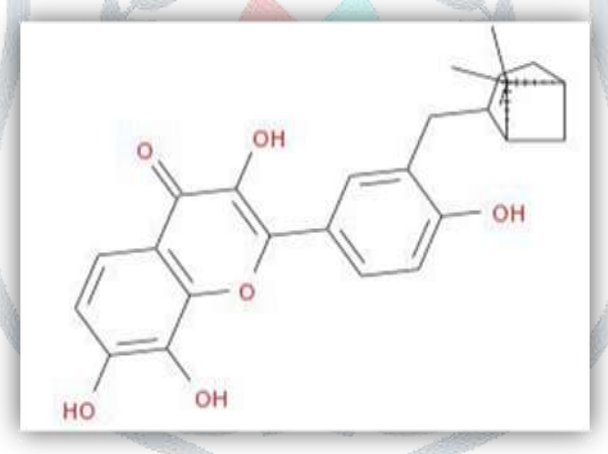


STRUCTURAL ELUCIDATION OF ISOLATED COMPOUND

The compound was isolated from the methanol extract of the flowers of *punica grantum* by a combination of chromatographic techniques. The structure of this isolated compound was identified by NMR spectroscopy and Mass spectrometry as punicaflavone. From the entire above chemical and spectral analysis we conclude that the isolated compound as a flavone, 3,7,8,4'-tetrahydroxy-3'-myrt-8-enylflavones (Punicaflavone), whose structure is given below in Figure 5.

Properties of Punicaflavone

- Structural formula



- Molecular formula : $C_{25}H_{24}O_6$
- Molecular mass (m/z) : 420
- Colour : Red colour mass
- Nature : Crystalline powder
- Solubility : Freely soluble in methanol
- Chemical name : 3, 7, 8, 4' -tetrahydroxy-3'-myrt-8-enylflavones (Punicaflavone)

CONCLUSION

Hence from the above discussion, it was concluded that the isolated molecule contains flavone moiety attached to substituted hydroxyl group at C-3′, C-7′and C-4′. The reported experimental results and ^{13}C-NMR, ^{1}H-NMR and the Mass spectra reported in the present study led us to formulate the molecular formula of the compound as $C_{25}H_{24}O_6$, bearing the IUPAC nomenclature3,7,8,4'-tetrahydroxy-3'-myrt-8-enylflavones (Punicaflavone).

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