Research Article

Isolation and HPLC quantitative analysis of flavonoids from flower extract of *Punica granatum* L.

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Abstract

Objective: The present study deals with the phytochemical screening of *Punic granatum* flowers, an important medicinal plant in all regions. This study involves the preliminary screening. Further HPLC flavonoid profile of the methanolic extract had been studied. **Methods:** The retention behavior of flavonoid isolated in methanolic extract of *punica granatum* flowers in high performance liquid chromatography (HPLC) system has been studied on an Inertsil C18-RP column using HPLC solvents were phosphate buffer (v/v) as aqueous solvent (A) and CH_3CN as an organic solvent (B). **Results:** The preliminary screening test results in the detection of bioactive principles and the retention time is identified in the HPLC chromatogram, as flavonoid (Rt=21.8±0.14). **Conclusion**: The preliminary phytochemical analysis of plants will help in the identification of bioactive components present in each species, the knowledge of which could help in comparing the differences between species and also in drug discovery. The study will help in future for identifying this plant for further research.

Keywords: Phytochemicals, *Punica granatum,* flavonoid, HPLC

Introduction

Plants which have one or more of its organ containing substances that can be used for the therapeutic purpose are called medicinal plants (Sofowora, 1993). The medicinal plants are useful for healing as well as for curing of human diseases because of the presence of photochemical constituents (Nostro et al., 2000). Phytochemicals are naturally occurring in the medicinal plants, leaves, vegetables and roots that have defense mechanism and protect from various diseases. Phytochemicals are primary and secondary compounds. Chlorophyll, proteins and common sugars are included in primary constituents and secondary compounds have terpenoids, alkaloids and phenolic compounds (Krishnaiah et al., 2007). Medicinal plant parts are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins (Packer et al., 1999).

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 (Satyavati et al., 1990) The dried flowers, known as Gulnar, are efficacious to treat haematuria, haemoptysis, diarrhoea, dysentery, nasal hemorrhage (Nadkarni, 2002) and in Unani literature as a remedy for diabetes (Jurjani et al., 1878; Majoosi et al.,1889).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 (Anonymous,1969) The aqueous-ethanol (50%, v/v) extract of the flowers leads to significant blood glucose lowering

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effect in normal, glucose-fed hyperglycemic and alloxan-induced diabetic rats (Jafri et al., 2000). In Chinese medicine these flower are also used for the treatment of injuries from falls and grey hair of young man (Lansky et al., 2007). In addition *Punica granatum* is considered as "a pharmacy unto itself" in ayurvedic medicine and is used as an antiparasitic agent, a blood tonic, and to ulcers (Naqvi, 1991).

The medicinal importance of a plant is due to the presence of some special substances like flavonoids, glycoside, resins, phenolic compounds and Tannins etc. Considering all these facts, presents investigation is designed to find out phytochemical investigation of *Punic granatum* a plant which evokes various therapeutic effects and HPLC analysis of isolated compounds. Due to the fact that plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities, their separation still remains as big challenge for the process of identification and characterization of them. It is a common practice in isolation of these bioactive compounds that a number of different separation techniques such as Thin-Layer Chromatography (TLC), column chromatography, flash chromatography, Sephardim chromatography and High-Performance Liquid Chromatography (HPLC), should be used to obtain pure compounds (Naqvi et al., 2005). HPLC is a versatile, robust, and widely used technique for the isolation of natural products, HPLC is a chromatographic technique that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture (Cannell, 1999; Piana et al., 2013). Currently, this technique is gaining popularity among various analytical techniques as the main choice for fingerprinting study for the quality control of herbal plants (Fan et al., 2006).

Materials and methods

Collection and authentication of plant material

The flowers of *Punica granatum* were collected from in and around the Mannargudi, Thiruvarur DT, Tamil nadu, India. They were identified and authenticated by Dr. S. John Britto, Department of Botany, Rabiant Herbarium and center for Modular Systematics, St. Joseph's College, Tiruchirappalli, 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

The residue was exhaustively extracted in a Soxhelt apparatus for at least 12 h with alcohol (methanol) and extract was used for experiment. 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 extract was kept in tightly closed container in refrigerator till further analysis.

Phytochemical screening of Methanolic Crude Extract

The Phytochemical tests were carried out on the extracts using standard procedures to identify the constituents as described by Harborne (1983).

Isolation of flavonoid by column chromatography

This was done to isolate and purify the constituents present in the extracts.

Packing of Column: Dried glass column was held in place by retort stand and was sealed with glass wool. The column was packed with n-hexane and silica gel as adsorbent and the column was tapped in order to avoid air-bubbles. The residual water phase was extracted with equal volume of ethyl acetate to draw all the flavonoid content from the organic compounds. 5ml of the extract was introduced into column then solvent mixture (eluent) in proper ratio was added into the column. Several fractions were obtained, concentrated and their purity was determined by using thin layer chromatography. The impure fractions were further rechromatographed using a different solvent mixture. The methanolic extract (5g) was chromatographed over Silica gel column (200g: 60-200 mesh) and eluded with solvent mixture of CH2Cl2 /CH3OH / H2O (70:30:1.V/V) 350ml, 150ml and 5ml of water respectively. Flavonoid was successfully isolated from the extract. The fractions were further screened for flavonoids.

Thin Layer Chromatography

TLC was used to ascertain the number of constituents present in the extract and to determine their purity.

Procedure: Thin layer chromatography was performed using standard methods (Harborne, 1998). Small quantities of samples (2 mg/ml) were dissolved in their respective solvents. Flavones (100µM) standard was dissolved in methanol. The dried residue dissolve in methanol and using the silica gel as stationary phase and mobile phase contain chloroform-methyl alcohol (19:1), performed thin layer chromatography. 50g of silica gel powder was weighed into a conical flask, 100ml of distilled water was added and the resulting solution was shaken vigorously in order to avoid lumps. The white smooth paste mixture was spread over the glass plate and was allowed to solidify. The coated glass plates were put inside oven for 1-2 hours at 110°C to

ensure further solidification. Spotting of the plates was done with aid of capillary tubes to introduce few drops of the dissolved sample extract unto the coated plate, allowing each drop to dry before adding another drop.

Developing of the Plates

After the solvent had travelled some distance across the plate, the plate was removed and allowed to dry and then viewed. The separated components appeared as colour spots using the coloring agent. Blue bands of 3, 7, 8, 4-tetrahydroxy-3'-myrt-8-enyl-flavone (Punica flavone) on the chromatograms were located with Rf value 0.38. The obtained chromatograms were observed under ultraviolet light at 254nm. The retention values were calculated by making use of the distance moved by the solvent and the distance moved by the component.

Rf = Distance travelled by the component / Distance travelled by the solvent

The Rf value of the different spots that were observed was calculated. The colors that were produced were scratched and suspended in the mobile phase separately for 3–4 days. After that mobile phase was sucked out, vacuum evaporated and the residue left was collected till sufficient amount for dosing and spectrophotometric analysis was obtained. The selected purified material was subjected to its HPLC chemical analysis.

Qualitative test for Flavanoids

Shinoda Test

To a small amount of test solution in alcohol, magnesium ribbon was added followed by addition of drops of concentrated hydrochloric acid; formation of pink color confirms the presence of flavonoids.

Alkaline Reagent Test

To the extracts add a few drops of sodium hydroxide solution, yellow color were obtained which turns to colorless on addition of few drops of dilute Hcl.

Zn-HCl reduction Test

To the small amount of extract add a mixture of Zn- dust and concentrated hydrochloric acid. Heat the solution after few minutes, color of the solution changes to red.

Determination of total flavonoid content

Principle: The total flavonoid content of the plant extract was estimated according to Aluminium chloride method. Flavones was used as reference compound to produce the standard curve and expressed as mg/g of plant extract.

Procedure: The flavonoid content was determined by aluminum chloride method using flavones as standard. Extracts and flavones were prepared in methanol (1 mg/ml). 0.1 ml of extract was mixed with 0.9ml of distilled water in test tubes, followed by addition of 75 µl of a 5% sodium nitrite solution. After 6 min, 150 µl of a 10% aluminum chloride solution was added and the mixture was allowed to stand for further 5 min after which 0.5 ml of 1M sodium hydroxide was added to the reaction mixture. The reaction mixture was brought to 2.5 ml with distilled water and mixed well. The absorbance was measured immediately at 510 nm using a spectrophotometer. The determination was performed in three replicates. The absorbance was measured using various concentrations of (20-140 µg). Blank consist of all the reagents, except for the extract or flavones standard solution is substituted with 0.1 ml of methanol.

High performance Liquid Chromatography

Preparation of standards

1.2 mg of standard (Flavone) was taken in 5ml of methanol (HPLC grade). From which

20µL were injected in HPLC system for making standard curve.

Preparation of sample

10 mg of dry 50% alcoholic extract was dissolved in 10 ml extraction solvent (HPLC grade) to get 1 mg/ml solution, filtered through 0. 45 µm Millipore and injected to waters HPLC system.

Procedure: HPLC analysis was carried out on injection valve with a 20 µl, a UV variable wavelength detector (set at 280 nm) sensitivity was 0.001, 5 µm RP-18 column (30°C). The HPLC solvents were phosphate buffer (v/v) as an aqueous solvent (A) and CH3CN as an organic solvent (B). The analytes were eluted gradientally at a flow rate of 1.2 ml/min. Chromatograms were generated on software. The HPLC instrument was operated at room temperature (23 ± 2°C). Each diluted extract 20 µl was injected in to the HPLC three times and the average peak area was reported and used for quantification.

As shown in the figure, the HPLC analysis of the fractions revealed that the purities of the compounds were 98.2%. HPLC chromatogram of (A) crude extract fractions [peak 1 - punicanyl benzoate, 2 - granatumol; 3 - grantumoside; 4 - ellagic acid; 5- catechin; 6- isoquerecitrin; 7-punicaflavone; 8- beta-sitosterol; 9- daucosterol]. (B)- Isolated targeted flavonoid compound (peak 7). (C) -Standard flavone compound.

Statistical Analysis

SD.: standard deviations. [b]LR: linear range. Linear regression formula: y = A + Bx. where

y = peak areas ratio. [c]B: slope. [d]A: intercept. x: µg. [e]R:

correlation coefficient. [f]N: number of samples. Compounds A: Flavone isolated from extract; Compound B: Flavone standard.

Results

The preliminary Phytochemical tests of methanolic flowers extract of *P. granatum* (L.) were screened for the presence of active principles such as carbohydrates, glycosides, fatty acids, protein and amino acids, saponins, tannins, phenolic compounds, β-sitosterols, triterpenoids, anthocyanins and flavonoids using standard procedures (Table 1) and qualitative test for flavonoid (Table 2).

Table 1. Phytochemical analysis

Plant constituents	*Punic granatum* extract
Proteins	+
Carbohydrates	+
Alkaloids	-
Steroids	-
Triterpenoids	+
Glycosides	+
Saponins	+
Flavonoids	+
Tannins	+
Polyphenols	+
Anthocyanins	+
Fatty acids	+
β-sitosterol	+
Amino acids	+

+ Presence, – Absence

Table 2. Qualitative test for flavanoids

Test	Flavonoid content
Shinoda Test	Positive
Alkaline Reagent Test	Positive
Zn-HCl reduction Test	Positive

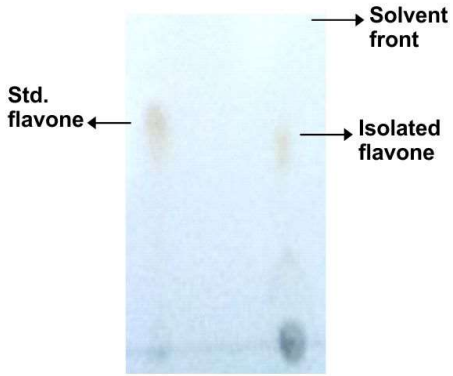

Figure 1. Thin layer Chromatography

Retention factor which is the distance moved through the stationary phase to that of mobile phase. Column chromatography gave different fractions and these fractions were concentrated and their purity was determined by using thin- layer chromatography (Figure 1 and Table 3). The total flavonoid content was found as 0.64%.

Table 3. Rf values and weight % for isolated compounds

Fractions	Rf values	% weight
Isolated flavone	0.38	0.15
Flavone Standard	0.40	0.22

The fractions separated by column chromatography were analyzed by HPLC, under the optimum analytical conditions, and the chromatograms are presented in this figure. As shown in the figure, the HPLC analysis of the fractions revealed that the purities of compound were 98.2%. HPLC chromatogram of (B) - Isolated targeted flavonoid compound (C) - standard flavones compound (Table 4 and 5, Figure 2).

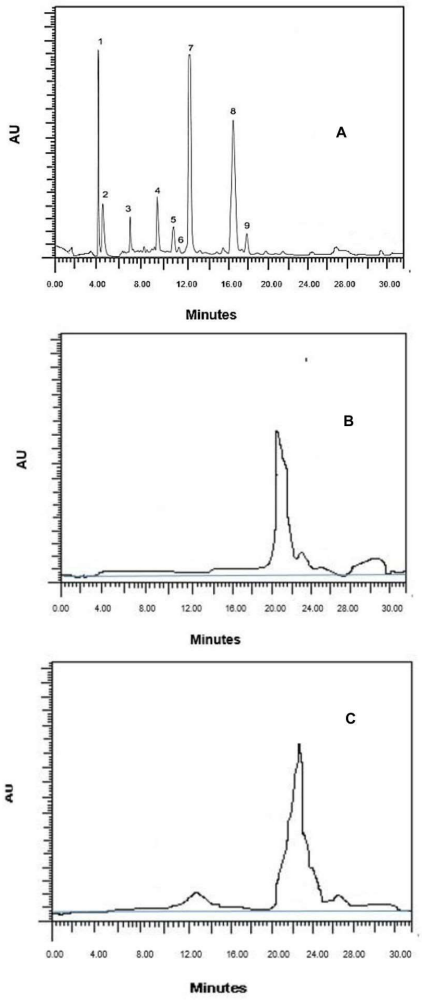

Figure 2. High performance liquid chromatogram for the isolated flavonoid compound

Results are expressed as mean ± SD. The quantification of compounds was made by comparison of their retention time

with those of authentic standards and by standards addition in the same samples analyzed using HPLC. The photodiode array detector was employed in the samples in the determination of compounds and in the evaluation of interference in all peaks of the chromatograms. The identified flavone fraction content was found 11.49 ± 1.9.

Table 4. Specific conditions during HPLC analysis

Instrument	SHIMADZU HPLC SYSTEM WITH LC 10A PUMP		
Column	Inertsil C-18		
Flow rate	1.2 ml/min		
Detection wavelength	280nm		
Mobile phase	Pump A	Phosphate buffer	
Gradient time program	Pump B	Acetonitrile	
	Time (minutes)	Conc. of B	Conc. of A
	0.01	05	95
	4.00	30	70
	8.00	55	45
	12.00	55	45
	16.00	30	70
	20.00	05	95
	24.00	30	70
	30.00	stop	-
Injection volume	20µl		
Detector	SPD-M10Avp photodiode array detector at 280nm		

Table 5. Retention time and regression data of calibration curve for quantitative determination by HPLC

Components	Retention time (min±SD)	LR[b]	B[c]	A[d]	R[e]	N[f]
A	21.8 ± 0.14	5.18-23.6	500747.06	- 34321.75	0.8956	5
B	22.0 ± 0.09	5.09-20.4	500738.13	34016.57	0.8961	5

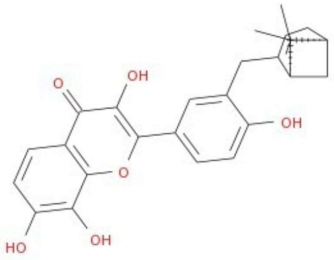

Figure 3. Isolated flavones compound: 3, 7, 8, 4'-tetrahydroxy-3'-myrt-8-enyl-flavone (*Punicaflavone)* (Molecular formula: C25H24O6)

Discussion

In this study we used C18 analytical column with 4.6mm×250 mm i.d., 5µm in length, to determine the flavonoid contents of flower extracts of *P.granatum.* The results from HPLC profile for flowers extract of *P.granatum* showed a lot of peaks at the retention time between 0 to 30 minutes. After 30 minutes, compound within this range of polarity have already been flushed out which indicates the polarity of the solvents have increased by increasing the percentage of the acetonitrile used. After 20% acetonitrile were used, there were no peaks appeared anymore to indicate that all compounds were being flushed out from the column. The peak spectrum of isolated flavones compound in 10% of acetonitrile showed the moderate peak spectrum at 2 to 3, 4 to 5 and 5.5 to 6 minutes. While, at 6.5, 10 to 12, 15.5 to16, 16.5, 17, 18.5, and 19 to 22 minutes showed the highest peak spectrum. The result showed that the crude extract of *P.granatum* may have some compounds which were not flushed out by the acetonitrile after 30 minutes and above. It has proved that in using acetonitrile, some compounds showed the low peak spectrum at 30 minutes and after 30 minutes there is no peak shown and it indicates that all compounds were being flushed out from the column. The results are shown in HPLC chromatogram A, B and C. The results from HPLC crude extract profile for flowers of *P.granatum* showed a lot of peaks at the retention time between 0 to 30 minutes using phosphate buffer and acetonitrile as solvent. The atomized wavelength for this analysis uses the 280 nm absorbance. This is because the wavelength of 280 nm is the best as this wavelength needs for detection of organic compound. By choosing the flowers of the plants in this study, investigation has found that it contained diverse groups of chemical compound. The effect of solvent on the extraction of flavonoid, the methanolic extracts of *P.granatum* was used to identify the flavonoids (Punicaflavone). The method developed for HPLC fingerprinting provided a quick analysis of the crude extracts of the *P.granatum*. The compounds were identified by comparison with the chromatogram of the standards obtained under the specified experimental conditions. The present method was a quick and accurate method for the separation of flavonoid with a run time of 30 minutes.

Conclusion

Quantitative analysis of punica flavonoids and was achieved for the first time in this work. The result of this study demonstrated that flowers of *P. granatum* extract does contain bioactive compounds according to a few peaks pattern that were shown in HPLC profiling analysis result by using phosphate buffer and acetonitrile as solvents. These peaks confirmed the presence of the potential

chemical content of *P. granatum.* HPLC is a versatile, reproducible chromatographic technique for the estimation of secondary metabolites in the plants. It has wide applications in different fields in term of isolation, quantitative and qualitative estimation of active molecules. Discovering the beneficial health effects of flavonoids and the "going-back-to-nature" trend motivates the development of more efficient and fast procedures for their identification and quantification, with HPLC remaining the most powerful technique for their separation from the complex mixtures.

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