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Research Article

CHROMATOGRAPHIC DETERMINATION OF PHENOLIC PROFILE FROM *PUNICA GRANATUM* FRUIT PEELS

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ABSTRACT

Polyphenols are the important active compounds present in the fruits peels of *Punica granatum*. The objectives of this study were to isolate and identify the phenolic compounds by UV- spectrophotometry, thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) and liquid chromatography - mass spectrometry (LC- MS) methods for qualitative and quantitative analyses of phenolic compounds in fruit peels of *P. granatum*. For separation of bioactive compounds, the crude ethanolic extracts of *P. granatum* were fractionated by using column chromatography. These fractions were coded as PG I, PG II, PG III, PG IV and PG V for *P. granatum*. These fractions were analyzed by thin layer chromatography (TLC) and showed that PG II fraction had an interested compound. The highest total phenolic and flavonoid content were observed in the PG II fraction of *P. granatum* (617 ± 0.017 mg/g and 546.33 ± 0.032 mg/g respectively) in comparison to other fractions. The phenolic compounds gallic acid and caffeic have been identified in the PG II fraction of *P. granatum* by using different chromatographic techniques i.e. TLC, HPLC, LC- MS analysis. The overall results of the present studies were indicated that PG II fraction of *P. granatum* was a good source of phenolic compounds. The present analytical study proved a potential application to identify and quantify the phenolic compounds in the plant fractions. Hence, it could be pursued further for obtaining phytomedicine.

Keywords: HPLC, LC-MS, *P. granatum*, Gallic acid

INTRODUCTION

Herbal products i.e. plants extract, either as pure phytoconstituents or as standardized extracts, offer infinite prospects for new drug discoveries because of the unmatched accessibility of chemical diversity[1,2]. Nowadays, there is rising evidence that plant phenolic compounds as well as flavonoids are distinctive nutraceuticals and supplementary treatments for various diseases. Phenols can modulate carbohydrate, lipid metabolism, attenuate hyperglycemia, dyslipidemia, insulin resistance, alleviate oxidative stress and prevent from diabetic complications[3]. *Punica granatum* (PG) commonly known as pomegranate is a small tree belonging to the Punicaceae family. Pomegranate is grown in India, Iran, USA and most near and Far East countries. Pomegranate juice and wine have become gradually more popular because of the attribution of imperative biological dealings to this plant, as well as cardiovascular protection[4,5,6]. In traditional Ayurvedic medicine, all parts of pomegranate are used for the treatment of various disorders[7]. In recent times, pomegranate derived products used in weight loss soap, hormone replacement therapy, resolution of allergic symptoms, oral hygiene, ophthalmic ointment, cosmetic beautification and enhancement and as an adjunct therapy to increase bioavailability of radioactive dyes during diagnostic imaging[8,9]. Due to the increasing demand, herbal extract usually arise as a combination of several types of bioactive constituents with different polarities, their separation still remains an immense challenge for the procedure of identification and characterization of phytochemicals or bioactive compounds. The pure compounds are then utilized for the determination of structure and biological activity. The aim of this study was to isolate, identify and quantify the phenolic constituents from *P. granatum* fruit peels which may be responsible to the treatment diseases. However, for the foreseeable future, long- term tolerance studies are needed before being recommended for human use.

MATERIALS AND METHODS

Collection and Identification of plant

Fresh fruit peels of *Punica granatum* were collected from the juice shop (National Handloom Juice Centre, Jaipur) during the month of May, 2012. The sample specimen was identified based on the taxonomical characteristics and registered by Herbarium" Department of Botany, University of Rajasthan, Jaipur. Registration number allotted to *P. granatum* was RUBL – 21111.

Preparation of Crude Extract by Soxhlet Extraction Method

P. granatum fruit peel powder (35 g) was filled in the thimble and extracted successively with 95 % ethanol (ethanol: distilled water; 95: 5) solvent in soxhlet extraction unit for 48 hours[10].

Qualitative Phytochemical Screening

Various qualitative phytochemical tests were performed for determination of presence or absence of bioactive constituents according to the method of Practical Pharmacognosy by C. K. Kokate, (2004)[11]. These tests have been done in the earlier study of Sharma *et al.*, (2015)[10].

Fractionation of bioactive compounds by column chromatographic techniques

Separation of the bioactive compounds from extract of *P. granatum* using column chromatographic technique was carried

out with a glass column of internal diameter 2.0 cm and length 75 cm (Borosil). Solvent system ethyl acetate and di- ethyl ether (1:1) were selected for isolation of phenolic compounds according to the method of Meena and Patni, (2008) with slight modification[12].

Thin Layer Chromatographic Analysis

After collection of different fractions, these fractions were applied on to the TLC plate for observation of number bioactive compounds. About 20 µl fractionated solutions of selected plant were applied on TLC and the plate was run in to the chloroform: methanol (9:1) solvent system. The plate was kept in UV 254 and 366 nm and results were observed. The plates were then spray with 0.1 % ferric chloride reagent; iodine vapors separately and the color of the spots were identified. An individual Rf value for each spot was measured and compared with standard reference compounds run in same respective solvent systems.

Quantitative phytochemical Screening
Total Phenolic and flavonoids Determination

The amounts of total phenolic and flavonoids contents of fruit peel extract and fractions of *P. granatum* were determined by the spectrophotometric method Katasani (2011) with slight modification[13]. Total phenolic and flavonoids content were determined from extrapolation of the calibration curve, which was made by preparing various concentrations of gallic acid and quercetin solution. The present work has been followed by the study of Sharma *et al.*, (2015)[10].

High Performance Liquid Chromatographic Analysis

Phytochemical analysis of the samples by HPLC was performed according to the method described earlier by Arun *et al.*, (2011) with slight modification[14].

Liquid Chromatography- Mass Spectrometry (LC – MS) Analysis

Liquid chromatography–mass spectrometry (LC- MS) analysis was performed from CDRI (Central Drug Research Institute) Lucknow. LC–MS analysis of the selected fraction of *P. granatum* was performed on water UPLC – TQD mass spectrometer. LC separation was attained by a Waters ACQUITY QSM Postrun, consisting of a wavelength UV detector operated at 200 and 450 nm. A Column (C 18 Diameter 100 cm x 3 mm particle size 2.6µm) was used to separate the compounds. An injection volume of 5 µl and a constant flow of 0.250 ml/min were used for each analysis. The entire flow from the LC was directed into the mass spectrometer. Solvent system for mobile phase was selected according to HPLC gradient method. A binary mobile phase consisting; solvent A: Acetonitrile + water (5:95); solvent B: Acetonitrile; solvent C: Methanol; solvent D: 5 mM ammonium acetate pH 6.5 was used. The linear gradient profile was as follows: 90% D (1 min), 60 % D (6 min), 60 % D (8 min), 40 % D (12 min), 20 % D (14 min), 20 % D (16 min), 90 % D (17 min) and 90 % D (20 min). Electrospray mass spectra data were recorded in the negative ionization mode for a mass range from m/z 150 to 2000. Data was acquired by the Masslynx data system for both the MS and UV data.

RESULTS AND DISCUSSION

The qualitative phytochemical screening of ethanolic extract of *P. granatum* fruit peels has been summarized in the Sharma *et al.*, (2015) which revealed the presence of phenolic compounds[10]. In the present study, the ethanolic extract of *P. granatum* was subjected to column chromatography using ethyl acetate and di ethyl ether solvents in 1:1 ratios to yield several sub-fractions. These fractions were coded as PG I to PG V (PG I, PG II, PG III, PG IV and PG V). Similarly, the studies of other workers Meena and Patni, (2008) were coincides with the present study who reported the free and bound flavonoids or phenolic compounds were isolated using di ethyl ether and ethyl acetate solvents[12]. Fractions of *P. granatum* were also analyzed on TLC plates by using chloroform: methanol (9:1) solvent system, against the standards to identify each of the isolated gallic acid (Rf - 0.11± 0.009) and Caffeic acid (Rf – 0.2 ± 0.007) (Figure 1.1). Similarly, the result of the current study was coincided with Entessar *et al.*, (2012), who examined the gallic acid was extracted by fruit rind of *P. granatum* which was identified in TLC chromatographic method and obtained one zone was clearly visible; Rf - 0.9 by using Toluene: ethyl acetate: formic acid system 3:3.5:0.5 as a mobile phase which compared with Rf of gallic acid standard compared[15].

(A)

(B)

Figure 1.1: TLC Chromatogram of Standard Compounds (Caffeic acid and Gallic acid) With FeCl3 spray (A) and Fractions of *P. granatum* (PG I to PG V) with $FeCl_3$ and Iodine vapors spray (B)

Quantitative study of total phenolic and flavonoids content

The results of total phenolic and flavonoid content of ethanolic extract and various fractions of *P. granatum* (fruit peel) were summarized in Table 1.1. The highest total phenolic and flavonoid content was observed in the PG II fraction of *P. granatum* (617 ± 0.017 and 546.33 ± 0.032 mg/g respectively).

Table 1.1: Quantitative Estimation of Total Phenolic and Flavonoids Content of Crude Extracts and Different Fractions of *Punica granatum*

P. granatum	Total phenolic content (mg /gm)*	Total flavonoids content (mg /gm)*
Ethanolic extract	337 ± 0.012	197 ± 0.032
PG I Fraction	583 ± 0.008	471 ± 0.044
PG II Fraction	617 ± 0.017	546.33 ± 0.032
PG III Fraction	218 ± 0.012	152.66 ± 0.032
PG IV Fraction	263 ± 0.014	238 ± 0.032
PG V Fraction	481 ± 0.008	320 ± 0.024

Values are means of three independent determinations ± Standard Error Mean (SEM)

High Performance Liquid Chromatographic Analysis

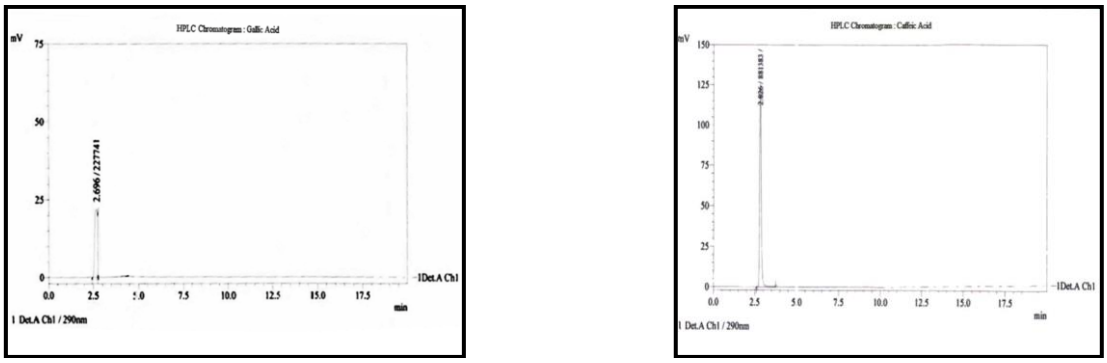
HPLC chromatogram of standard gallic and caffeic acid were shown retention time at 2.69 min ± 0.011 and 2.82 min ± 0.002 respectively (Figure 1.2). Retention time (R_t) of standard gallic acid and caffeic acid as phenolic compounds were coincided with the retention time of A and B compounds in PG II fraction of *P. granatum* (Figure 1.3 and Table 1.2). In this concern, the present finding was coincided with Hmid *et al.*, (2013), who reported the presence of gallic, chlorogenic, caffeic, ferulic, ellagic acids, catechin, epicatechin, phloridzin, quercetin and rutin in *P. granatum* fruit juices[16]. Furthermore, Middha *et al.*, (2013), revealed the presence of some major phenolic compounds such as gallic acid and ellagic acids in addition to punicalagin as a major ellagitannin in *P. granatum* fruit peel which were in agreements with the current studies[17]. On the other hand, the studies of Mansour et al., (2013), also examined the presence of quercetin and vanillic acid in the pomegranate fruit peel of Chinese and Tunisian variety although in the present study, quercetin was not found in *P. granatum* fruit peel[18].

The studies of Ali *et al.*, (2014), revealed that chlorogenic acid was the most abundant phenolic acid in the methanolic extract of *P. granatum* fruit peel which constituted the 11.91% of the total extracted compounds[19]. However, in the present finding, gallic acid was the most abundant phenolic compound in the fraction of *P. granatum* fruit peel.

Table 1.2: HPLC analysis data of Ethanolic Extract and selected Fraction of *Punica granatum*

Plant Material (Extract or Fractions)	No. of Isolated Compounds	Identified Compounds	Retention time (R_t) of Peak (min)	Area	Total phenolic Content (mg / gm)
PG II Fraction	3 compounds	A	2.672 ± 0.016	1065436 ± 0.57	462.87 ± 0.013
		B	2.805 ± 0.011	1086223 ± 0.33	142.77 ± 0.016

*A and B proposed gallic acid and caffeic acid, Values are means of three independent determinations ± Standard Error Mean (SEM)



(A) **(B)**

Figure 1.2: HPLC Chromatogram of Standard (A) Gallic acid (R_t : 2.696 min) and (B) Caffeic acid (R_t : 2.826 min)

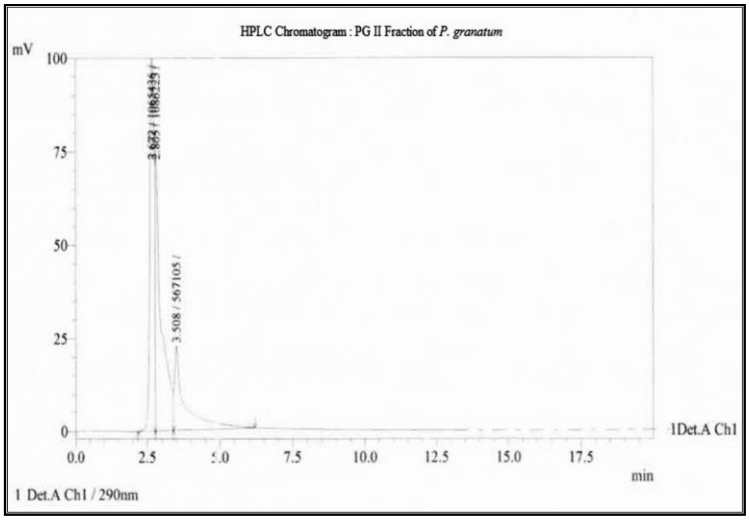


Figure 1.3: HPLC Chromatogram of PG II Fraction of *P. granatum*

Liquid chromatography mass spectrometry (LC- MS) analysis

These data were useful to identify and characterize the LC-ESI MS data obtained in PG II fraction of *P. granatum.* In the present study, the two phenolic compounds from PG II fraction of *P. granatum* were identified by the interpretation of their fragmentation patterns obtained from mass spectra and retention times of peaks (Figure 1.4). The peak of compound 1 at retention time 1.47 min had molecular weight [M-H]- at m/z 169 which was identified as gallic acid and peak of compound 2 at retention time 2.05 min had molecular weight [M-H]- at m/z 179 which was also identified as caffeic acid (Figure 1.4 and 1.5). These compounds were compared with standard gallic acid and caffeic acid. The results of current studies were also in agreement with other researchers Fischer *et al.*, (2011) who reported that different gallic and ellagic derivatives of pomegranate fruits, juices and peels were identified by LC- MS techniques[20]. Similar, results were also coincided with Al – Rawahi *et al.*, (2014) who reported a variety of *P. granatum* peels which cultivated in Oman were also identified the gallic acid, caffeic acid and their other different types of derivatives by LC-MS analysis[21]. Also, Fischer *et al.*, (2011) demonstrated that some anthocyanin compounds lacked in some cultivars[20]. According to Borochov-Neori *et al.*, (2011), some phenolic compounds were extremely dependent on the climate conditions and the proportion of phenolic compounds increased with seasonal warming[22]. The overall results of the present studies were indicated that PG II fraction of *P. granatum* was a good source of phenolic compounds.

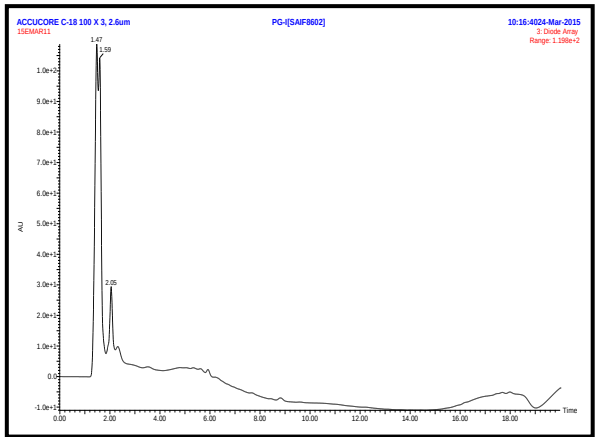


Figure 1.4: LC-MS Chromatogram of PG II Fraction of *P. granatum*

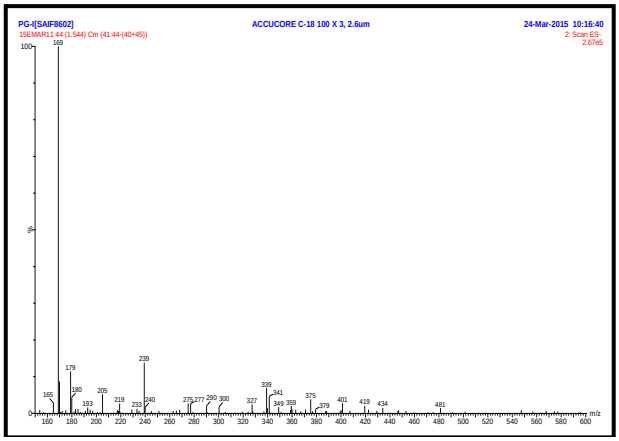


Figure 1.5: Mass Spectra of PG II Fraction of *P. granatum*

CONCLUSION

Due to the increasing attention in natural drug formulations, standardization of plant extracts using chromatographic techniques and phytochemical quantification have been developed. This study revealed that fruits peel extract deserves more intensive study on quantification, isolation of dominant compounds, bioavailability and possible protection against some common ailments, either for future studies or in herbal drug formulations.

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