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SEPARATION OF POLYPHENOLS FROM HIGH YIELD POLYPHENOLIC EXTRACT (HYPE) OF *PUNICA GRANATUM* BY COMBINED CHROMATOGRAPHIC TECHNIQUES AND THEIR CHARACTERIZATION BY LC-MS/MS

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ABSTRACT

In this study, we developed a preparative isolation process of the plant polyphenols obtained from the HYPE of Pomegranate peel (*Punica granatum*) which is the major part of agrowaste. Polyphenols namely catechin, myricetin and quercetin were isolated and further were characterized by using LC-MS/MS. Polyphenols were extracted from the enzyme pre-treated matrix with the help of pectinolytic and cellulolytic enzyme preparation was taken for a pre-developed gradient system. Phase- A = Acetonitrile Phase -B = Acetonitrile: water (10:90) with buffer to maintain pH = 3.25 (O-phosphoric acid). Flow rate was set at 1.0 ml/min. Preparative column with 50micron particle size reverse phase was used. The detection wave length was selected 272nm after being confirmed by a PDA detector. Under the above conditions a satisfactory separation of the targeted compounds was achieved with the standard catechin, myricetin and quercetin in crude extract. Polyphenols analysis by LC-ESI-MS/MS were carried out using an Agilent 1100 series LC and LC/MSD Trap VL mass spectrometer (Agilent Technologies, USA) equipped with electrospray ionization (ESI) interface. A good separation of polyphenols were achieved and isolated fraction of quercetin and myricetin were collected without any interference but catechin fraction further purified and analysed by MS/MS libraries. Our study demonstrates an effective isolation of

polyphenols from pomegranate agro waste by gradient followed by an isocratic system. The present study may lay a foundation for large scale preparation of these plant polyphenols specially quercetin and myricetin from pomegranate waste material.

KEYWORDS: polyphenols, quercetin**,** catechin, myricetin, preparative chromatography, LC-MS/MS.

INTRODUCTION

Flavonoids are large group of compounds that ubiquitously exist in natural products and have been considered as active ingredients of many medicinal plants. Chemically they are polyphenols with diphenylpropanes (C_6-C_3-C_6) skeletons. They have been demonstrated to possess a number of biological activities and thus received much attention in phytotherapy research. All these polyphenols possess catechol group which make them a potential anti-oxidant. Flavonoids consist two aromatic ring linked via three carbon chain, which in most of cases is oxygenated heterocycle ring. Flavonoids can be divided into several subfamilies according to the degree of oxidation of the oxygenated heterocycle, being flavanols, flavanones, flavones, flavonols (essentially, flavan-3-ols), isoflavones, and anthocyanidins, the most relevant for human diets. Different parts of Pomegrante (***Punica granatum***) are widely used for different medicinal purposes. Pomegranate peel which constitutes major part of the agrowaste an act as potential source of polyphenols. The natural products can be separated by conventional separation methods such as silica gel, microporous resin, Sephadex LH-20, and high performance liquid chromatography (HPLC). The preparative isolation depends on the extraction process as well as purification process before isolation.

Very little literature was reported about polyphenols isolation from pomegranate species. In this study a preparative isolation process is reported for three polyphenols like catechin, quercetin and myricetin and characterizing it with the help of LC-MS/MS.

MATERIALS AND METHODS

Materials, Chemicals and Reagents

All the biomarkers were purchased from Sigma (USA). All technical grade solvents were procured from SD fine chemicals Mumbai (India). Peels of *P.granatum* were obtained from local markets, Mumbai. All HPLC grade and technical grade solvents were procured from SD fine chemicals (India). Water was purified by Milli-Q water Purification system (Millipore, MA, and USA).

Extraction of polyphenols from an enzyme pre-treated matrix

Around 50 gm peel (40#) was taken in acetate buffer (pH=4.6) and stirred with 25000 ppm of pectinolytic and cellulolytic enzyme preparation at 40^0C for 4 hr. The solution was evaporated under vacuum to prepare an enzyme pre-treated matrix. Then dried residue of powder and enzyme were taken in Soxhalation process of extraction for 4 h and filtered and concentrated under vacuum at 40^0C.

HPLC analysis of crude extract

The crude methanolic extract of *Punica granatum* peels with pre treated with pectinolytic and cellulolytic enzyme preparation was taken for a pre-developed gradient system mentioned in Table-1[3]

Phase- A = Acetonitrile Phase -B = Acetonitrile: water (10:90) with buffer to maintain pH = 3.25 (O-phosphoric acid). Flow rate was set at 1.0 ml/min. Preparative column with 50micron particle size reverse phase was used. The detection wave length was selected 272nm after being confirmed by a PDA detector. Under the above conditions a satisfactory separation of the targeted compounds was achieved with the standard catechin, myricetin and quercetin in crude extract.

LC instruments and analytical condition

Polyphenols analysis by LC-ESI-MS/MS were carried out using an Agilent 1100 series LC and LC/MSD Trap VL mass spectrometer (Agilent Technologies, USA) equipped with electrospray ionization (ESI) interface. In order to obtain optimum ionizing conditions, using the reference solution, both Atmospheric Pressure Chemical Ionization (APCI) and electrospray ionization interface were tested in positive and negative ion modes by scanning between m/z 200-550 per second. The column temperature was maintained at 25^0C. Quantification was achieved using selected ion monitoring system (SIM) mode of ion. The flow rate was .5ml/min

Separation of phenolic compounds from High Yield Polyphenolic Extract (HYPE) of an agro waste of *Punica granatum* by Preparative methods

Under the optimized conditions peak fractions were collected in one step elution more than 2h for 7 days. The preparative fractions were collected and concentrated and further analysed LC-ESI-MS/MS. Three fractions I, II and II were collected at 7.3 min (catechin), 9.3 min (myricetin), and 10.2min (quercetin). These concentrated fractions were collected after distillation and freeze dried and analysed by pre-developed gradient LC-ESI-MS/MS

method.[2] The mobile phase consisting of a gradient system, A = Methanol: Water with 5mM ammonium formate buffer (9:1) B = Water: Methanol, buffer 5Mm ammonium formate (8:2). Gradient elution was employed and was used as follows: started with 10%B and increased linearity to 90%B within 3 min, and then increased linearity to 100%B within 7 min and at last re-equilibrate linearity to 10%B with in 10 min.

RESULTS AND DISCUSSION

Table 1: Gradient method employed.

Timing	Mobile Phase ratio (A:B)	Response
0-5 min	A(100):B(0)	Linear gradient
5-15 mins	A(60):B(40)	Linear Gradient
15-20 mins	A(100):B(0)	Re-equilibration

Extraction of polyphenols from an enzyme pre-treated matrix

Table 2: Comparison of yield of extract after various extraction processes.

Extraction process	Yield of extract / 25gm of peel powder
Maceration	2.56 gm
Maceration with enzyme	2.96 gm
Extraction after soxhalation with enzyme	4.95 gm

Total 10 gm of extract was taken for preparative isolation process.

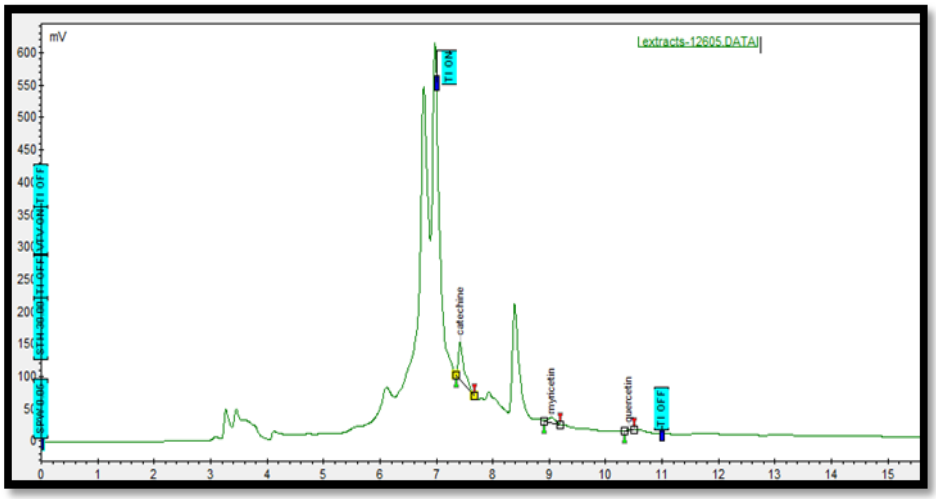


Figure 1: HPLC analysis of crude extract.

HPLC chromatogram for preparative separation of polyphenolic fractions.

Separation of phenolic compounds from High Yield Polyphenolic Extract (HYPE) of an agro waste of *punica granatum* by Preparative methods

A good separation of polyphenols were achieved and isolated fraction of quercetin and myricetin were collected without any interference but catechin fraction further purified and analysed by MS/MS libraries.

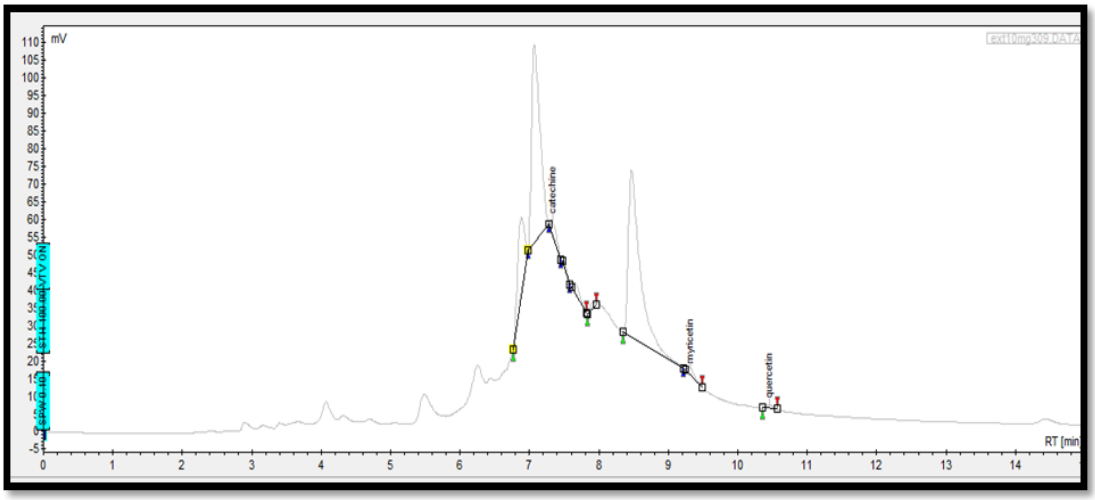


Fig 2: HPLC chromatogram for preparative separation of polyphenolic fractions during isolation.

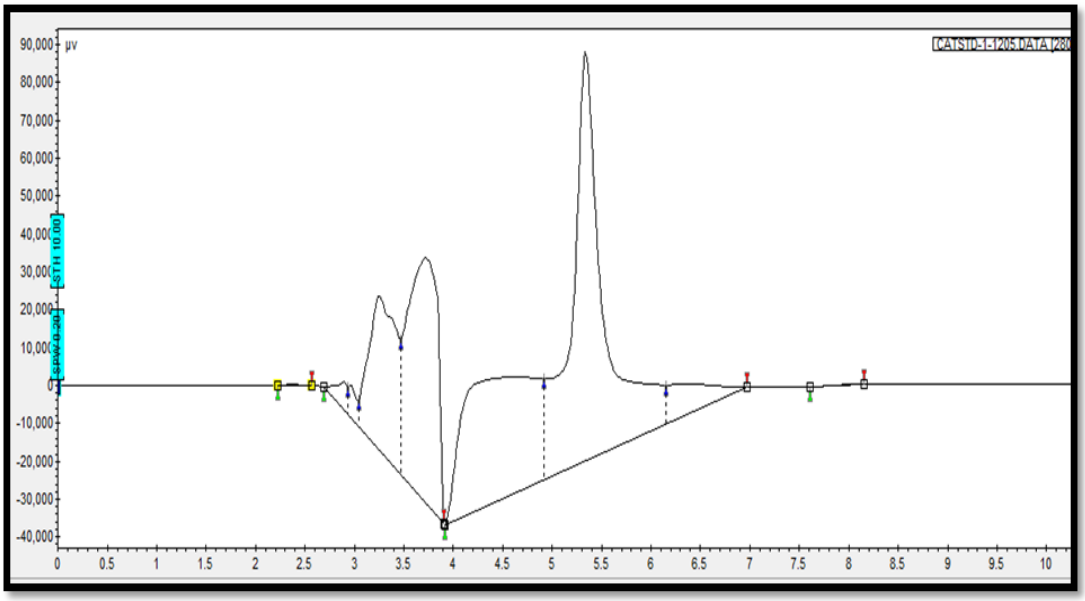


Fig 3: Catechin fraction was further isolated by an isocratic system (A).

Recording of mass spectra

Mass spectra were recorded for each of the polyphenols. The characteristic peak was observed in negative ion mode for all three polyphenols. Data has showed below.

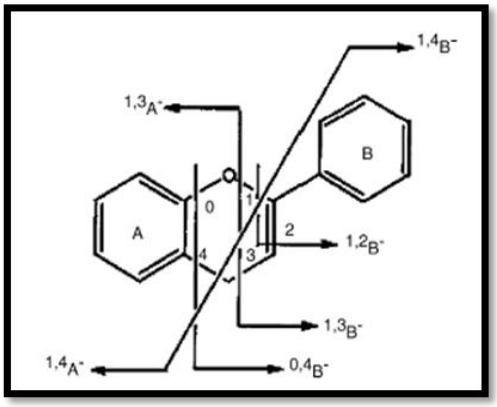


Fig 4: Fragmentation pattern for flavonoids.

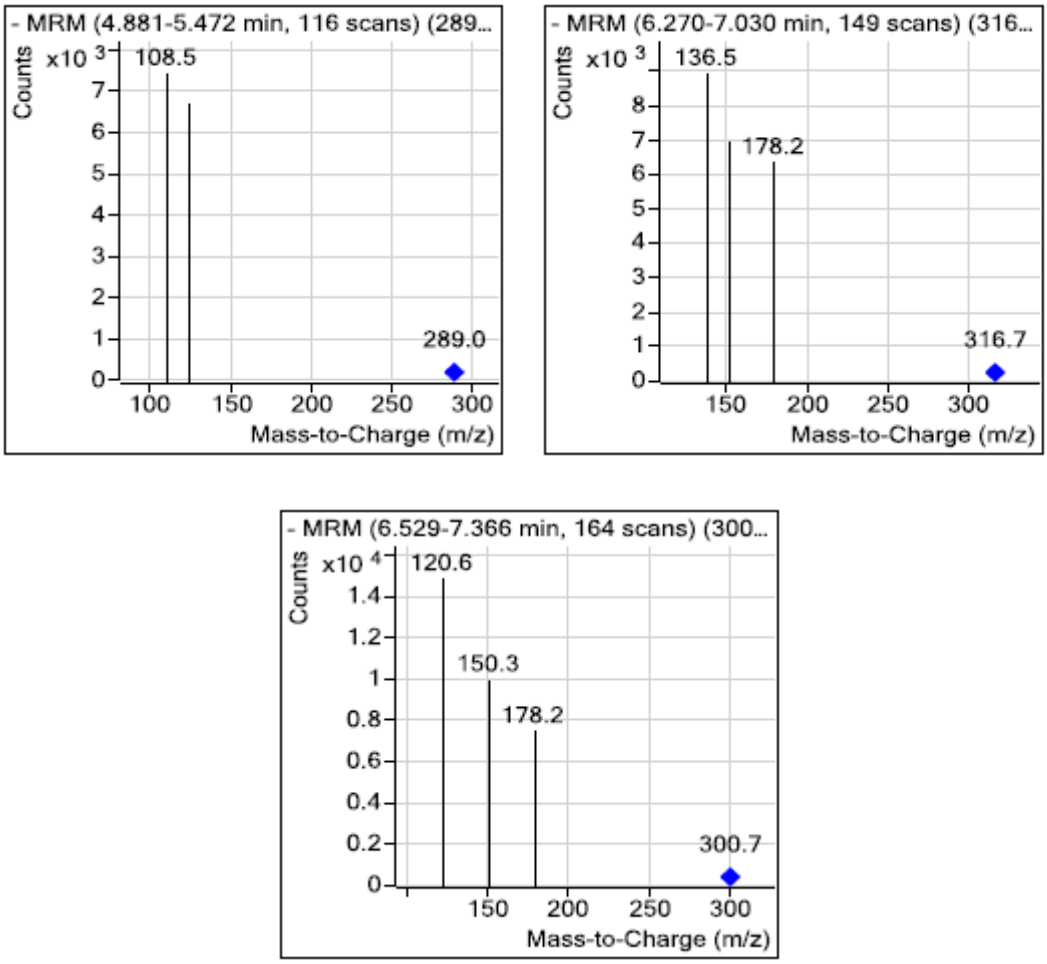
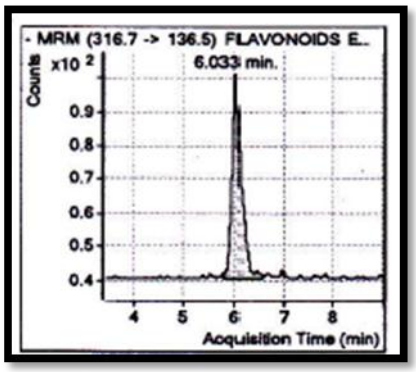


Fig 5: Fragments of catechin, myricetin and quercetin recorded by LC:MS:MS.

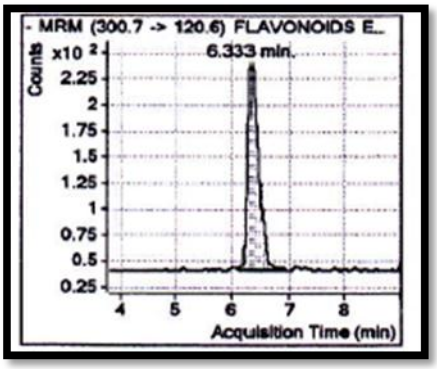
Application of phenolic compound fingerprinting in isolated fractions of HYPE from *Punica granatum* by MS/MS libraries

Based on the optimized LC-ESI-MS/MS conditions established for the phenolic compounds using standard solutions, the isolated fraction of HYPE of *Punica granatum* was

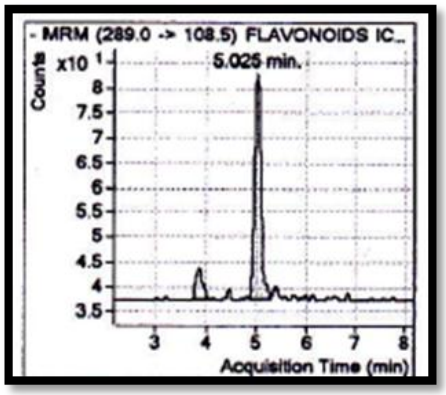
analysed[deep paper] Using our standard library information it was identified as the isolated polyphenols. The $[M-H]^-$ peaks and the subsequent peaks of MS/MS data were seen to coincide with the data of catechin, myricetin and quercetin and was thus identified as isolated pure biomarkers.



Fraction II - Myricetin



Fraction-III- Quercetin



Fraction-I Catechin

Fig 6: TIC of isolated polyphenols.

To determine the most effective ionization mode for the phenolic standards ESI source in positive or negative ion mode were investigated. The results indicated that the ESI source at negative-ion mode with MS/MS activation energy of 1.0 V was best for the analysis of low molecular phenolic compounds which coincide with the previous reports [136, 139]. Information about ESI-MS/MS has already updated in above. At the same time compiled MS/MS libraries of phenolic standards was applied to isolated compounds from High Yield Polyphenolic Extract (HYPE) of an agro waste of *punica granatum* by a RP gradient HPLC-MS/MS.

Table 2: Different molecular ion peaks.

MS/MS fragments	Catechin Fraction-I	Quercetin Fraction –III	Myricetin Fraction- II
$[M-H]^-$	289	301	317
$[M-H]^-$ - 180.5	108.5		
$[M-H]^-$ - 122.8		178.2	
$[M-H]^-$ - 138.8			178.2
$[M-H]^-$ - 151.3		150.3	
$[M-H]^-$ - 180.5			136.5
$[M-H]^-$ - 180.4		120.6	
Amount isolated from 10 gm of extract	200µg	300 µg	22.55 ng

CONCLUSION

Our study demonstrates an effective isolation of polyphenols from pomegranate agro waste by gradient followed by an isocratic system. Using these techniques three polyphenols namely catechin, quercetin and myricetin were isolated and analysed. The present study may lay a foundation for large scale preparation of these plant polyphenols specially quercetin and myricetin from pomegranate waste material.

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