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Estimation of parent flavonoids from supramolecular lipid complex of *Punica granatum* extract in rat serum by LC-ESI-MS/MS

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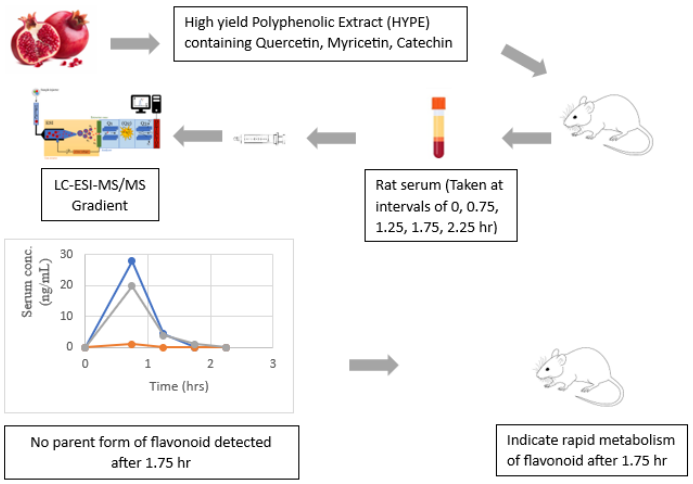
Abstract : This research seeks to investigate the role of free flavonoids in anti-inflammatory activity. *Punica granatum* peel, also known as pomegranate is a rich reservoir of various secondary metabolites, especially flavonoids. The three parent flavonoids namely catechin, quercetin, and myricetin, were extracted from High Yield Phenolic Extract (HYPE) of *Punica granatum* agrowaste using an isocratic system. For bioenhancement, HYPE was subjected to a process of conversion into supramolecular phospholipid complexes and administered to rats via the peroral route. Serum samples were analyzed using a pre-developed LC-ESI-MS/MS method to improve the sensitivity of detection. At 25^0C column temperature, quantification was carried out using the Selected Ion Monitoring System (SIM). Gradient elution was employed and the flow rate was maintained at .5ml/min. After administering the drugs, blood samples were collected at specific time intervals of 0, 0.75, 1.25, 1.75, and 2.25 hours. The concentrations of free flavonoids from the HYPE and its phospholipid complexes were detected as 0.355, 0, and 0.525 ng/ml for catechin, myricetin, and quercetin respectively at the end of 1.75 hours. However, no further detection of these compounds was observed thereafter, indicating that they had been metabolized. Therefore, anti-inflammatory activity after 2nd hour is contributed by the metabolite of flavonoids and not their free form.



Keywords: *Punica granatum*; flavonoids; catechin; quercetin; myricetin; phospholipid complexes; LC-MS/MS; detection sensitivity; bioanalysis

1. INTRODUCTION

Flavonoids are the most abundant group of naturally occurring phenolic compounds that are found in various parts of plants, including roots, bark, heartwood, leaves, flowers, fruits, and seeds. They can exist in two forms: in a free state or bound to sugar molecules as glycosides. Structurally, flavonoids consist of a 15-carbon skeleton, with two benzene rings connected by a linear three-carbon chain, which is often represented as the C_6 - C_3 - C_6 system.

A typical parent flavonoid refers to the free aglycone form. Previous research indicates that the peel of *P. granatum* contains a diverse array of compounds including gallic acid, chlorogenic acid, rutin, ferulic acid, caffeic acid, p-coumaric acid, punicalagin, ellagic acids, ellagitannins, gallotannins, hydroxycinnamic acids, catechins, hydroxybenzoic acids, and anthocyanins (Akhtar et al. 2015; Kharchoufi et al. 2018; Manuel et al. 2022). The specific flavonols identified in the peel of this plant for the given work are catechin, quercetin, and myricetin due to the well-established anti-inflammatory activity profile of the flavonoids (Chen X et al. 2005; Yang C-Y et al. 2020; Terao J et al. 2011). However, the contribution of metabolites and its free form pertaining to anti-inflammatory effect need to be investigated further.

The log P values for quercetin, myricetin, and catechin are reported as 1.59, 1.23, and 0.51 respectively (Gonzales et al. 2014). This information reveals the varying hydrophobicity of these compounds, which presents a challenge for their simultaneous detection. Furthermore, previous HPLC methods faced limitations in detecting these free flavanoids in rat plasma after oral administration (Xie L et al. 2011).

Selective Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS/MS) has emerged as a reliable technique for detection of various phytochemicals and their plant metabolites in the biological matrix. In this study, with the help of LC-ESI-MS/MS methods, the detection of flavonoid aglycones was made possible even in lower concentrations in rat serum ranging from 0.1-30 ng/ml.

The following research work aims to find the effect of free flavonoids on anti-inflammatory activity.

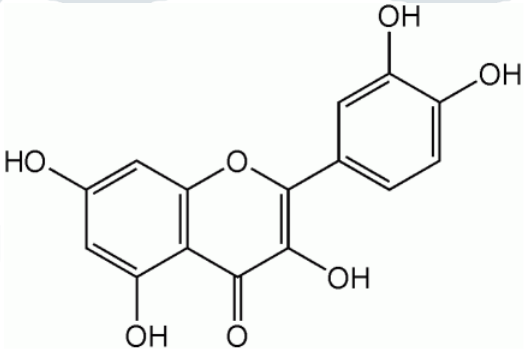


Fig 1: Catechin

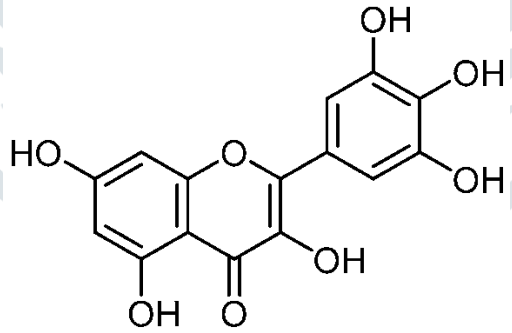


Fig 2: Myricetin

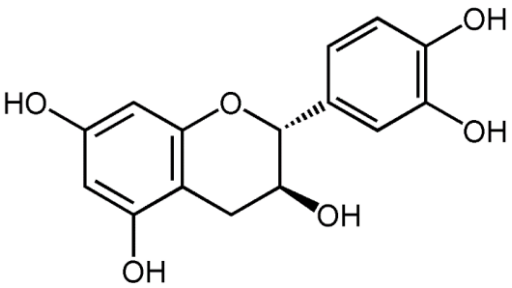


Fig 3: Quercetin

2. MATERIAL AND METHODS

2.1 Materials, Chemicals and Reagents

All the biomarkers were purchased from Sigma (USA). All technical grade solvents were procured from SD Fine Chemicals (India). Peels of *P.granatum* were obtained from JKH Exporter, Mumbai based herb supplier. Water was purified by a Milli-Q water Purification system (Millipore, MA, and USA).

2.2 Animals

Male and female Wistar rats (200-220gm) were purchased from the Haffkin Institute. They were acclimatized for 3 days before starting the experiments. They were fed with food and water *ad libitum* and fasted overnight before drug administration. This study was approved by the Institutional Animal Ethics Committee (protocol number VS-IAEC-2014-015, approved on December 5, 2014).

2.3 LC instruments and analytical conditions

Polyphenols analysis by LC-ESI-MS/MS was 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 reverse phase Qualisil BDS-C_{18} column (4.6mm φ x 25. mm) packed with 5µm diameter particles was used. The column injection volume was 10 µL. 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 mobile phase consisted 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 within 10 min. The column temperature was maintained at 25^0C. Quantification was achieved using the Selected Ion Monitoring System (SIM) mode of ion. The flow rate was .5ml/min.

2.4 Preparation of free flavanoid mixture and High Yield Polyphenolic Extract (HYPE) of *Punica granatum*

Free flavanoids like quercetin, catechin, and myricetin were taken in the ratio of 2:2:5 to prepare the mixture. High yield Polyphenolic extracts were prepared by reported literature. Around 10 gm of peel was stirred with acetate buffer (pH 4.6) and 5000 ppm of pectinolytic and cellulolytic enzyme preparation at 40^0C for 1hr. The solution was evaporated under vacuum to prepare an ezyme pre-treated matrix. Then dried residue of powder and enzyme were taken in the Soxhalation process of extraction for 2 hr and filtered and concentrated under vacuum at 40^0C.

2.5 Preparation of phospholipid complex of free flavanoids mixture and HYPE of *Punica granatum*

Free flavanoid mixture and HYPE of P. granatum was taken with phospholipid separately as per the reported literature. Biomarkers were taken into a solution of phospholipid (Phosphatidylcholine – 60%) in dichloromethane (DCM) in 2:1 proportion (1 gm 25mg of phospholipids and 450mg of free flavonoid mixture). After relaxing for 60 mins for certain time the mixture was filtered and kept for evaporation under vacuum. Then the residue was re-dissolved in the DCM and added slowly to a non-solvent n-hexane. The resultant mixture was maintained at cooling in refrigerator for overnight.

2.6 Preparation of serum samples

Plasma samples were obtained via retroorbital puncture from different group of animals (mentioned in table) in parallel design, and centrifuged target aliquot. Concentrations of free flavanoids in serum were determined before and after β-glucuronidase treatment. After hydrolysis a mixture of 200 µL of plasma sample contained with 20 µL of 10 mm ascorbic acid as masking agent (replacing internal standard to improve accuracy) and acidified with 60 µL of 0.6 M acetic acid was vortex- mixed for 5 min and then added 2 ml acetone and cold centrifuged for 10 min at 12,000 g. An aliquot of 10 µL of the supernatant (serum) was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in the mobile phase and injected into the LC/MS/MS system.

2.7 Freeze thaw stability of biomarkers in plasma and serum sample

The stability of three polyphenols was studied in plasma and the samples after storing at -20ºC for 24 hrs and thawed unassisted at room temperature. When completely thawed the sample was refrozen within 24 hrs. The cycle repeated two times and the sample was analysed after the third cycle.

3. RESULTS AND DISCUSSION

3.1 Freeze thaw stability of plasma and serum sample

Results of the stability study indicate the polyphenols were more stable in serum than plasma. Almost no parent polyphenols were detected from plasma samples after 24 hr. However, in the serum sample, the concentration was determined with RSD < 5% which indicates the polyphenols were stable in the serum sample. Ascorbic acid was added to improve stability as an anti-oxidant which prevents degradation of polyphenols and improves their quantity after extraction and the accuracy of the methods. Previously reported literature had used no internal standard for catechin quantification but a protein precipitation procedure was followed by acetonitrile to prepare plasma sample (Wermeille et al. 1983; Khaled et al. 2003; Winter et al. 1962). However, for quercetin, reported literature stated using 6, 7, -dimethoxy-coumarin, and apigenin as an internal standard (Yang et al. 2020; Terao et al. 2011). Due to the variety of polarity of catechin, quercetin, and myricetin, no internal standard was used except a masking agent ascorbic acid to improve the extraction yield of these polyphenols from serum.

3.2 Analysis of parent flavonoids in blood sample

Blood samples collected at different intervals after administering the drugs were analysed by the pre-developed and validated LC-MS/MS method. (Tarun et al. 2014) The results of the study revealed the fact that quercetin, catechin, and myricetin get rapidly metabolized within 1.25 hr after per oral administration of their mixture to rats and thus parent biomarkers are not detected after 1.25 hr of administration. Results are listed in **Table 3.2.1**

Table 3.2.1 Quantity of free flavanoids in serum from the phytochemical mixtures and its phospholipid complexes

Time (hrs)	**Catechin** (ng/mL) From phyto-chemical mixtures	**Myricetin** (ng/mL) From phyto-chemical mixtures	**Quercetin** (ng/mL) From phyto-chemical mixtures	**Catechin** (ng/mL) From lipid complex of phytochemical mixtures	**Myricetin** (ng/mL) From lipid complex of phytochemical mixtures	**Quercetin** (ng/mL) From lipid complex of phytochemical mixtures
0	0	0	0	0	0	0
.75	2.43	**11.7**	**8.3**	**27.95**	1.01	19.8
1.25	3.8	2.24	1.8	4.36	0	3.831
1.75	**5.6**	0.412	1.1	0	0	1.1
2.25	2	0	0.102	0	0	0

The above results give an insight that the pharmacokinetic study of parent biomarkers can only be possible with LC-ESI-MS/MS methods and the bioenhancement study of these biomarkers after forming their phospholipid complexes requires the metabolite study as they get rapidly metabolized in the liver. A similar result was also obtained with oral administration of High Yield Polyphenolic Extract (HYPE) of an agro waste of *Punica granatum* with no detection
after 1.75 hr. Results are listed in Table **3.2.2**

Table 3.2.2 Quantity of free flavanoids in serum from the HYPE and its phospholipid complexes.

Time (hours)	**Catechin** (ng/mL) From HYPE	**Myricetin** (ng/mL) From HYPE	**Quercetin** (ng/mL) From HYPE	**Catechin** (ng/mL) From the lipid complex of HYPE	**Myricetin** (ng/mL) From lipid complex of HYPE	**Quercetin** (ng/mL) From lipid complex of HYPE
0	0	0	0	0	0	0
.75	**3.44 µg**	**1.0681**	**6.22**	1.1	0.204	1.274
1.25	0.5	.35028	1.1	0.802	0	0.759
1.75	0.1483	0	0.7	0.355	0	0.525
2.25	0	0	0	0	0	0

4. CONCLUSION

The sensitivity of detection was enhanced by utilizing a previously developed LC-ESI-MS/MS method for bio-estimation of the parent flavonoids in rat serum after per oral administration of biomarker mixture, High Yield Polyphenolic Extract (HYPE) of an agro waste of *Punica granatum* and their phospholipid complexes. Interestingly, no parent flavonoids were detectable in the serum after 1.75 hours, suggesting that the pharmacodynamic effects of flavonoids observed after the 3rd hour were solely due to their metabolites. Thus, this study demonstrates that the LC-MS/MS technique can be helpful in the detection of even lower quantities of target compounds in biological fluids.

However, it is imperative to conduct further studies to estimate the specific metabolites resulting from the transformation of the parent compound, particularly considering their pharmacokinetic properties. Our study holds promise for investigating the individual and synergistic effects of polyphenolic phospholipid complexes on various pharmacological activities.

5. ACKNOWLEDGMENTS

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6. DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

7. FUNDING

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