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Original Article

BIO-POTENTIATION AND *IN VIVO* PHARMACOKINETICS STUDIES OF POLYPHENOLS FROM PUNICA GRANATUM BY FORMING PHOSPHOLIPID CONJUGATES FOR BETTER ANTIOXIDANT & ANTI-INFLAMMATORY ACTIVITY

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ABSTRACT

Objective: Polyphenols belong to the chemical class of flavonoids and are widely distributed in vegetables and plants. In this study, we aimed to potentiate the antioxidant and anti-inflammatory effects of polyphenols by forming a phospholipid complex of extracts from *Punica granatum* and also evaluating its *in vivo* pharmacokinetic parameters.

Methods: This method was used for determination and *in vivo* pharmacokinetics studies for the biomarkers and their lipid complexes from *P. granatum* extract. Anti-inflammatory and antioxidant studies ever performed on both uncomplexed and complexed flavonoids. Pharmacokineticbehaviour of biomarker was significantly improved with higher C_{max}, $t_{1/2}$ and shorter t_{max}& AUC_{0-t} and AUC_{0-t} values of glucuronide metabolites indicating the greater bioavailability.

Results: Anti lipid peroxidation inhibitory potential was found greater in case of phospholipid complexes with IC_{50} value 17.41 µg for biomarker complex as compared to biomarker mixture (63.52 µg) & anti-inflammatory activity of 100 mg/kg of agro waste extract complex of *P. granatum* was found very significant at 3rd hour with (21.95±0.071 %) inhibition in rat paw edema which was greater than the extract alone (11.13±0.084 %).

Conclusion: Bio-enhancement of polyphenols was observed and achieved through phospholipids complexes and was demonstrated by *in vitro* and *in vivo* activity testing by forming a supramolecular complex.

Keywords: *Punica granatum*, Flavonoids, *In vivo*, Glucuronide.

INTRODUCTION

Punica granatum is an ancient, unique fruit borne on a small long living tree cultivated throughout the Mediterranean regions including Himalaya. Peels of pomegranate were reported to have flavonoids like catechin, quercetin and myricetin [1]. Flavonoids are a set of secondary plant metabolites important for plant growth and development. Different parts of the plant is under a protective effect against colon and breast cancer, diabetes, hypercholesterolemia atherosclerosis, lupus nephritis, and immune and inflammatory reactions and antitumor activities. Pomegranate a popular plant had been made for the present study as it contains many flavonoids like quercetin, catechin and myricetin which were targeted for evaluating their anti-inflammatory activity and antioxidant activity potential after forming their molecular complex with phospholipids. Due to multiple ring structure flavonoids exhibits problem in systemic absorption through diffusion. Polyhydroxyl functional groups limit the oil solubility of flavonoids. Re systemic metabolism of flavonoids i. e. degradation of phenolic moiety by intestinal bacteria in astrocyte reduces the bioavailability in great extent. Phospholipids complex of flavonoids were said to reduce the above mentioned problem and improves their bioavailability. The present study was carried out to focus on bio enhancement and *in vivo* pharmacokinetics studies of standardized pomegranate peels extracts by enhancing its yield by converting them into lipid compatible complexes by comparing phospholipid complexes of flavonoids mixture of catechin, quercetin and myricetin. Phospholipid complexes of flavonoids are the molecular complexes of flavonoids where flavonoids molecules form a non-covalent bond with a carrier like phospholipids [2]. Many literatures list various potential aspects of flavonoids in preventing inflammatory disorders *in vitro* and *in vivo* [3]. But metabolic pharmacokinetics study demonstrated the fact that not only parent flavonoids are therapeutically active but also their circulating metabolites mainly glucuronide and sulphated form contribute to the activity especially when they reach at the target site and deliver free flavonoids by the action of glucuronidaseenzyme [4]. Flavonoids were said to exhibit two compartmental pharmacokinetics properties. Due to this the part of the administered dose of flavonoids gets shifted from the central component (blood, liver etc.) to peripheral compartments (poorly perfumed tissue like muscle, adipose etc.) which limit their activity.

The present study is an attempt to direct the flavonoids more into central compartments by their supramolecular complex formation in order to enhance their plasma concentration and to enhance the concentration of their circulating metabolites for better bioactivity Also, previous studies also reported about the antioxidant profile of telluric acid and its phospholipids complex which had stated that the complexes were found to be better hepatoprotective than free pelagic acid at the same dose. Allergic acid was reported to be a major component of pomegranate peel extracts [5]. Fenton reaction of phenobarbitone acid (TBA) is reagent and malondialdehyde (MDA) to initiates production of free radicals to evaluate antioxidant property in rat liver homogenates.

MATERIALS AND METHODS

Materials

All the biomarkers were bought from Sigma (USA). All technical grade solvents and thiobarbituric acid (TBA) were procured from SD Fine Chemicals Mumbai (India). Peels of *P. granatum* was available from local markets, Mumbai. Extract of peels of *P. granatum* was prepared as per literature [6].

Animals and preparation of rat liver homogenates

Adult winter rats (200-220g) were purchased from Haffkin Institute, Mumbai. They were acclimatized for 7 days before the experiments. They were fed with food and water *ad labitum* and fasted overnight before drug administration. The livers were excised, perfumed and

homogenized with 120 mM of KCl, 50 mM phosphate buffers, pH 7.4 (1:10 w/v). It was centrifuged at 3000 rpm for 10 mins. The supernatant fraction was kept at -20ºC until the time of use. All procedures involving animals were in accordance with the regulations of the Institutional Animal Ethical Committee and the protocols were approved by IAEC before initiating the study.

Preparation of biomarker mixture, High Yield Polyphenolic extract (HYPE) of *P. granatum*

Biomarkers like quercetin, catechin and myricetin were made in the ratio of 2:2:0.5 to prepare biomarker mixture. Peel powder was stirred with acetate buffer and pectinolytic, cellulolytic enzyme preparation. The solution was evaporated under vacuum. Then dried residue of powder and enzyme was taken in correlation process of extraction by using methanol, filtered and concentrated under vacuum.

Preparation and characterization of phospholipid complexes of flavonoids mixture and the extract of *P. granatum*

Biomarker mixture and HYPE of *P. granatum* was taken with phospholipid separately as per the reported literature [2]. 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 biomarker 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. Phospholipid complex was prepared were marked by ^{1}H-NMR, mass spectrometry and IR spectroscopic technique. A standardized extract of peel 10g was took with 1g of phospholipid in DCM and similar reaction conditions were maintained.

Optimization of LC-ESI-MS/MS condition

Analysis of polyphenols 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 electrostatic ionization (ESI) interface. The mobile phase consisting of a gradient system, A = Methanol: Water (9:1, v/v), B = water: methanol (8:2, v/v) buffer, ammonium formate (pH 3.25; 5 mM) was delivered at a flow rate of 0.5 ml/min. The method was validated by defining the linearity, limits of detection, identification and quantification of analyte's, repeatability, precision, stability and recovery.

Anti lipid peroxidation activity

In vitro antioxidant activity was carried out by TBARS method with slight modification [5]. Lipid peroxidation was initiated by Fenton reaction with the help of ferrous ascorbic system and phenobarbitone acid was used to terminate lipid peroxidation. The complexity of malondialdehyde- TBA complex was measured at fixed wave length of 532 nm. Curcumin was used as a standard. The degree of lipid peroxidation was assayed by estimating the phenobarbitone acid-reactive substances (TBARS) [5, 7]. 3 ml rat liver homogenate (5%) was taken in different 35 mm glass pewter dishes. Different concentrations of plant extract and standard antioxidants were being incubated with homogenate for 10 mins at 37º C. After incubation lipid peroxidation was induced by adding ascorbic acid and $FeSO_4$ (0.5 mM) and (1.6 mM -62 µM) respectively. Petri dishes were further incubated for 30 mins. 100 µl of incubation mixture (5% homogenate in PBS, pH 7.4) was transferred to a tube containing 1.5 ml 10% trichloroacetic acid. After 10 mins, tubes were centrifuged at 5000 rpm for 10 mins. Supernatant was mixed with 1.5 ml TBA (0.67% aqueous TBA in 50% acetic acid, 1:1). The mixture was kept in a boiling water bath for 30 mins. Tubes were cooled and absorbance was taken at 535 nm. The values were evaluated on the basis of a standard curve [7].

Anti-inflammatory activity

The anti-inflammatory potential was assessed by the carrageenan-induced right hind paw; the anti-inflammatory potential was assessed by the carrageenan-induced right hind paw edema method [8]. Briefly, acute inflammation was obtained by sub plantar injection of 0.1 ml of 1% suspension of carrageenan in normal saline, in the right hind paw of the rats 1 hour after the oral administration of test materials. Paw volumes were measured by a digital plethysmometer at 1, 2, 3, 4 and 5 hrs after the administration at 50 mg/kg and 100 mg/kg body weight of biomarker mixture, HYPE of an agro waste of *Punica granatum* and their phospholipids complexes to wiser rats by gavage. Diclofenac at a dose of 40mg/kg body weight was used as standard anti-inflammatory agent. The negative control group received 0.1% Tween-80 in saline solution. The anti-inflammatory effect of the extract was calculated by the following equation:

$$\text{Anti-inflammatory activity (\%) paw edema inhibition} = \left(1 - \frac{D}{C}\right) * 100$$

Where, C=Mean paw volume of control, D=Mean paw volume of testing.

$$\text{Percent inhibition} = \left(\text{control} - \frac{\text{test}}{\text{control}}\right) * 100$$

The results of anti-inflammatory studies obtained were expressed as mean of S. D. The data were analysed using one-way analysis of variance (ANOVA) followed by Dennett's t test to determine the level of significance. A value of P< 0.05 was considered to be significant.

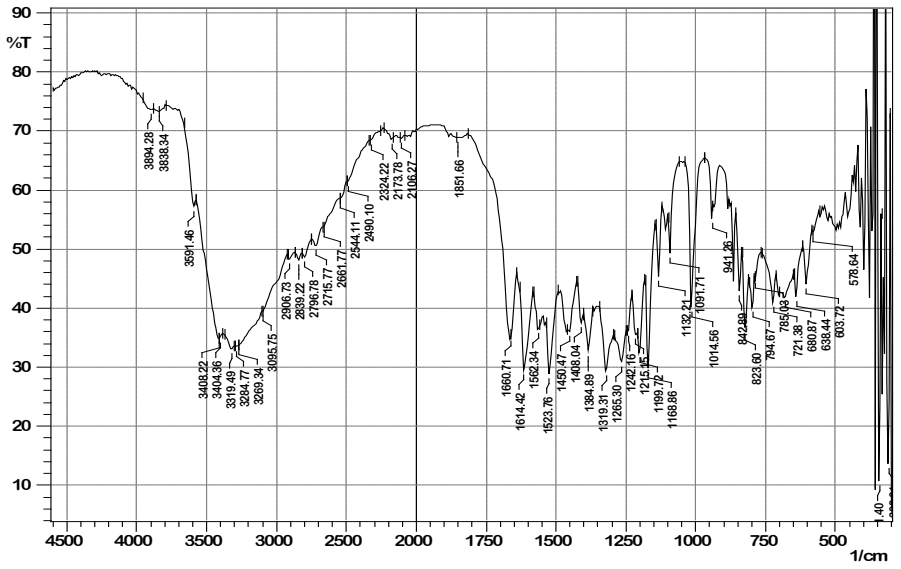

Fig. 1: Myricetin FT-IR spectrum

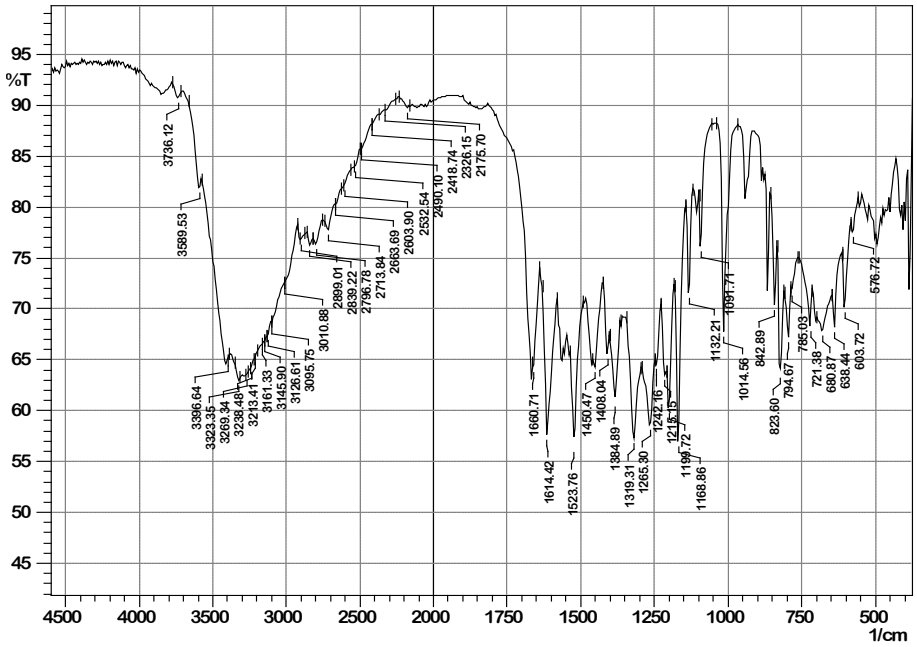

Fig. 2: FT-IR Spectrum of Quercetin

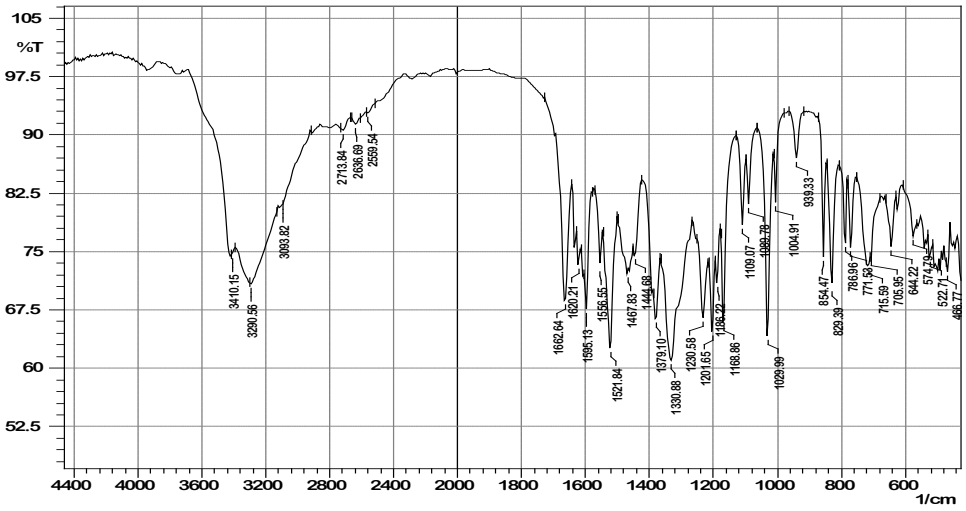

Fig. 3: FT-IR spectrum of Biomarker complex

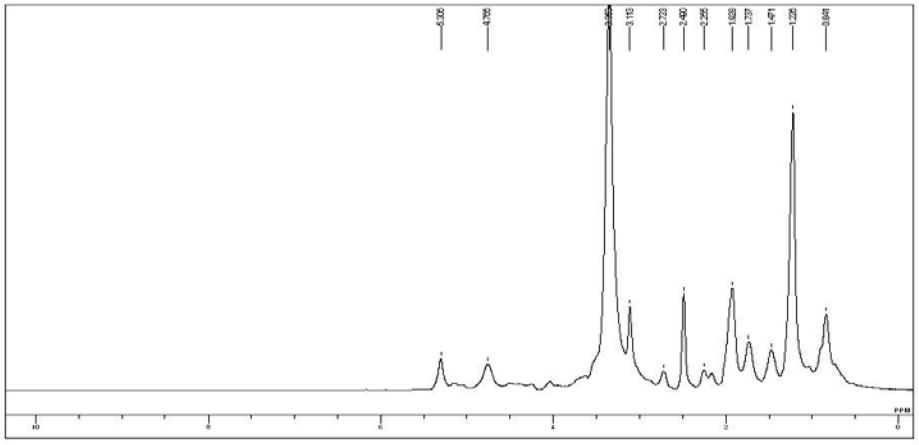

Fig. 4: NMR of phospholipid complex

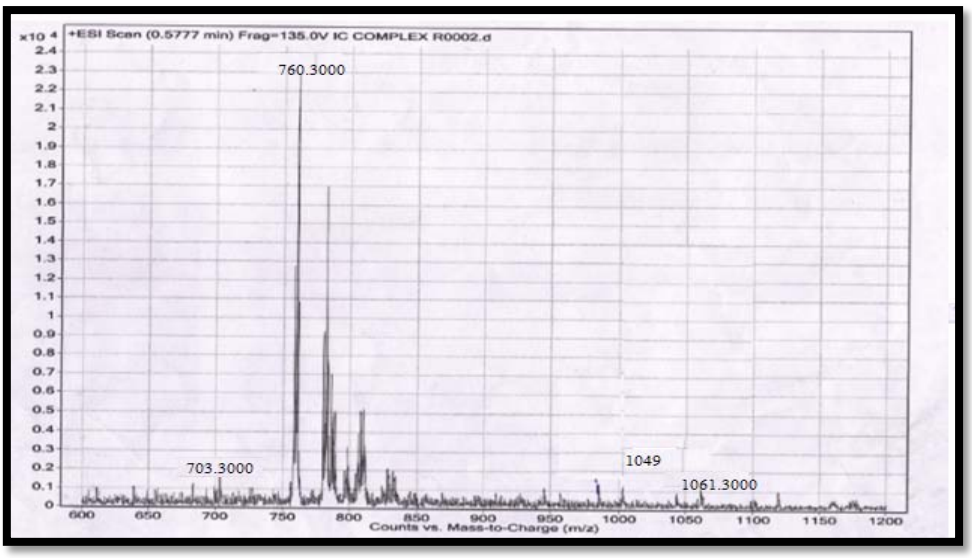

Fig. 5: MS/MS fragments peaks of Phospholipids complex of biomarker mixture

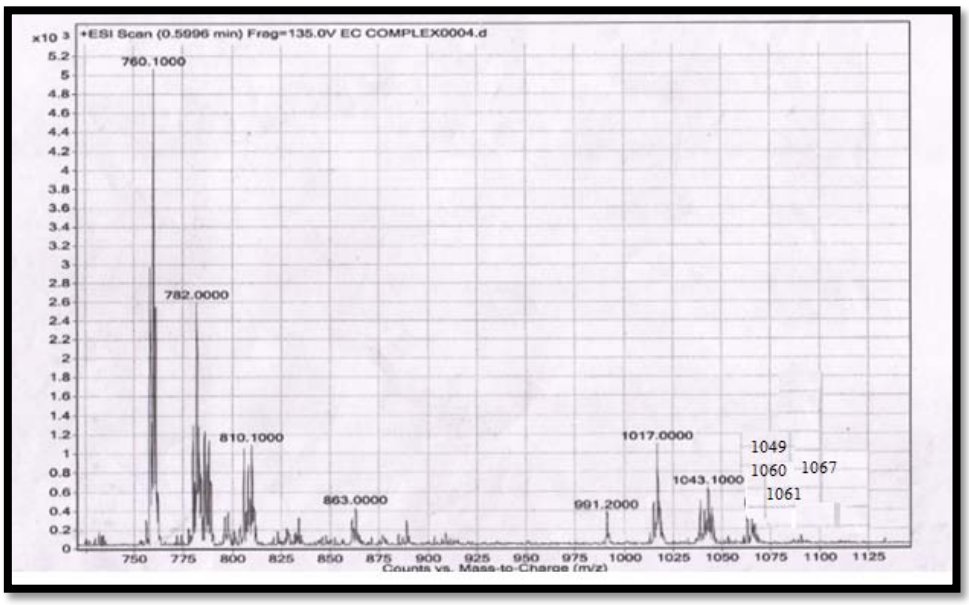

Fig. 6: MS/MS fragments of phospholipids complexes of HYPE of *Punica granatum*

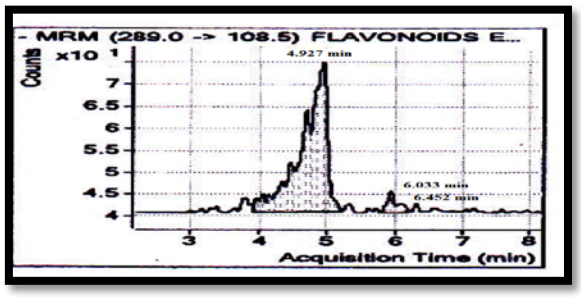

Fig. 7: LC-ESI-MS-MS chromatogram for *Punica granatum* peel extract

***In vivo* pharmacokinetics study of polyphenol metabolite**

Concentrations of glucuronides 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 mins and then added 2 ml acetone and cold centrifuged

for 10 mins at 12, 000 G. An aliquot of 10 µL of the supernatant was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in the mobile phase and injected into LC/MS/MS system. The plasma concentration of all the biomarkers at different time points was expressed as mean ±SD, and the mean concentration –time curve was plotted. The maximum plasma concentration and time to reach peak concentration was obtained directly from the observed value. The apparent elimination rate constant was calculated using fitting mean data at four terminal points of the plasma concentration profile. $T_{1/2}$ was calculated as $0.693/K_e$. the area under the plasma concentration- time curve from zero to the time of the final measurable sample ($AUC_{0\text{-}t}$) was calculated using the linear trapezoidal up to the last sampling point with detectable level (C). the area under plasma concentration time curve from zero to infinity ($AUC_{0\text{-}\infty}$) was calculated using trapezoidal rule with extrapolation to infinity with K_e. Volume of distribution (V_d) was calculated from $Dose/C_o$ and CLt was calculated from $K_e \times V_d$ formula. Bioavailability was calculated by using $F=(CLt/Dose) * AUC0-t$.

RESULTS AND DISCUSSION

Phospholipids complex of biomarkers and polyphenols from *Punica granatum* was supported by ^{1}H-NMR, FT-IR and Mass spectroscopy. Under the LC-ESI-MS/MS conditions, good separation of biomarkers was obtained within 7.5 mins which were analysed by electrospray ionization (ESI) techniques with the gradient mobile phase mentioned. Complexation of bio-markers with phospholipid was confirmed by NMR spectroscopy depicting disappearance of peaks between 6-9 ppm. The bio-markers measured by LC-ESI-MS/MS as catechin 125 µg, quercetin 18.3 µg & myricetin 0.6 µg for the amount present in 1000 µg of extract. (fig. 1-7).

Anti lipid peroxidation activity

Pomegranate peel extract and biomarkers mixture showed a significant reduction in lipid peroxidation induced by $FeSO_4$ and ascorbic acid in a dose dependent manner. Under similar experimental conditions result was faced with well-known antioxidants curcumin. Peroxide and hydroxyl radicals are important agents that mediate lipid peroxidation therefore damaging cell membranes. IC_{50} values of diverse groups like biomarker mixture, phospholipids complex of biomarkers mixture and extract of *P. granatum* indicated higher activity for lipid complexes table 1. The phospholipid complex of biomarker mixture inhibited the lipid peroxides more efficiently than its spontaneous form suggesting the fact of improvement in the lipid penetration during oxidative stress. Literature reported quercetin and catechin being more effective anti-lipid per-oxidant than myricetin perhaps due to poor lipid solubility of myricetin [9]. The inhibition ratio was calculated using the equation:

$$\% \text{ lipid peroxidation inhibition } = \left[\frac{(\text{absorbance of control} - \text{absorbance of test})}{\text{absorbance of control}}\right] * 100$$

HYPE of *Punica granatum* exhibited a greater free radical scavenging activity than the mixtures of biomarkers suggesting the presence of other polyphenols which contributed to the antioxidant activities. But the lower activity of lipid complexes of HYPE than lipid complexes of biomarkers mixture suggested limitation of the polyphenols present in the extract to form lipid complexes and/or presence of less hypothetical anti-oxidants in complex form. (fig. 8).

Table 1: Anti-lipid peroxidation activity presented as IC_{50} value

Group	IC_{50} (µg/ml)
Biomarker mixture	63.52
Biomarker mixture complex	17.41
Extract of *P. granatum*	50.74
Extract Complex of *P. granatum*	23.84
Curcumin	48.28

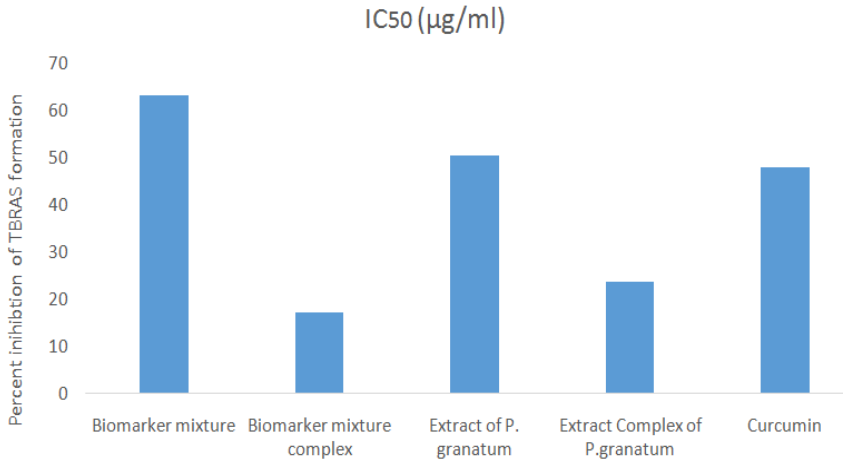

Fig. 8: IC_{50} value comparison of Iso mix (biomarker mixture), IC (phospholipids complex of Biomarker mixture), Extract (peel of *P. granatum*), EC (phospholipid complex of extract) and curcumin

Anti-inflammatory activity

The carrageenan-induced rat paw edema is a biphasic process. The release of histamine or serotonin occurs in the first phase and the second phase is linked to the production of bradykinin, protease, prostaglandin, and lysosome.

Both phospholipids complexes of biomarkers and HYPE of *Punica granatum* exhibited greater activity in a dose dependent study (50 mg/kg and 100 mg/kg) at 3rd hour with significant results (*P<0.05 and **P<0.001). The anti-inflammatory activity was determined by the (fig. 9). At third hour the extract complexes (HYPE complex) exhibited the highest activity (21.95%) than their isolated form, it may be due to the presence of parent catechin, quercetin, myricetin and their metabolites along with other polyphenols like allergic acid present in the extracts which reaches to the systemic circulation in greater amount due to lipid carrier phospholipids complexes. Anti-

inflammatory activity of HYPE complexes was more than HYPE in 3rd hour may be due to the greater bioavailability of ellagic acid or other polyphenols along with long circulating metabolite of quercetin (glucuronide and sulphated). These complexes of the HYPE exhibited the greater anti-inflammatory activity not only due to the faster absorption and increase bioavailability of parent biomarkers and their metabolites, but also demonstrated the contribution of synergistic effect due to the presence of elastic acid and other unknown polyphenols in HYPE of *Punica granatum*. Peel extracts exhibited anti-inflammatory activity of 3rd and 4th hour suggesting inhibition of the enzyme cyclooxygenase and subsequent inhibition of prostaglandin synthesis [10]. (fig. 9).

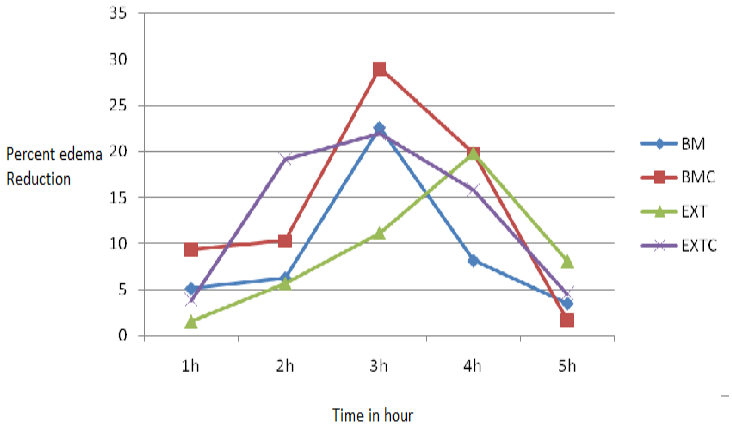

Fig. 9: Anti-inflammatory activity presented for BM= biomarkers, BMC= Biomarker complex, EXT = extract, EXTC= Extract complex

***In vivo* pharmacokinetics study of polyphenols and their metabolites**

LC-ESI-MS/MS method was used to determine pharmacokinetics parameters of quercetin, catechin, myricetin and their phase-II metabolites after per oral administration of their mixture, HYPE of an agro waste of *Punica granatum* and their phospholipids complexes in rat serum at a dose of 50 mg/kg and 100mg/kg. Mean serum concentration–time profile of quercetin, catechin, myricetin and its conjugates after per oral administration of their mixture in 2:2:0.5 ratios, (HYPE) of an agro waste of *Punica granatum* and their phospholipids complexes is shown in tables 2, 3. Detection level of these biomarkers as their parent form is much lower than their glucuronide conjugates (phase II metabolite) during the experiment. The half-life of these biomarkers and their conjugates was reported in table 4, 5. $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ of all these biomarkers was compared both with the equal dose and the equivalent dose.

The glucuronide metabolite canbe established due to lack of authentic standard. Therefore, concentrations of quercetin in serum sample were determined before and after respectively treatment with β-glucuronidase in order to calculate the concentration of quercetin glucuronides. Ascorbic acid was added to the serum as an antioxidant to prevent the decomposition of these biomarkers liberated from its conjugates. Half-life of conjugated form of biomarkers was longer than their parent form possibly due to their enter hepatic circulation [11].

As per the reported literature quercetin aglycone is more absorbable than its glycoside from intestine [11]. But as per the earlier literature in the presence of catechin, quercetin absorption was low and is erratic because it was observed the presence of quercetin enhances the absorption of catechin [12]. Greater water solubility of myricetin may be responsible for more absorption than quercetin due to its carrier dependent absorption process. After per oral administration of these biomarkers, the glucuronide metabolites appeared before 0.25 hr and were exclusively circulating in the bloodstream. Gallocatechin 3-gallate was reported to have almost zero metabolisms in the body where the parent catechin was reported to have a slow metabolism [12]. During the experiment catechin as the parent form was detected at longer time than quercetin parent form indicating a slow metabolism of the flavan-3-ol residue over flavonol moiety. A previous pharmacokinetics study of quercetin suggested enterohepatic circulation of quercetin glucuronide metabolites [3, 11].

During the course of pharmacokinetics study prolong detection of quercetin glucuronide metabolite was observed than catechin and myricetin metabolite after per-oral administration of admixtures of these biomarkers, suggesting that quercetin glucuronide served as a long circulating metabolite than the glucuronides of catechin and myricetin. After per oral administration of these biomarkers as a mixture and their phospholipids complexes, the $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ values of glucuronides metabolites of these biomarkers were higher in phospholipids complexes with higher Cmax, $t_{1/2}$ and shorter t_{max} indicating the greater bioavailability of these biomarkers in phospholipids complexes during an equivalent dose study Table- 6-11 (Fig. 10-12). Phospholipids complexes of these biomarkers not only enhanced their absorption (indicated a shorter t_{max}) by an equal dose study but also exhibited slow hepatic metabolism which leads to detection of quercetin glucuronide conjugate in a higher level than it's free form of 3rd and 4th hours. At equal dose study, concentration of myricetin and catechin glucuronide metabolites was found to be lesser than quercetin metabolite supporting the fact that the long circulating nature of quercetin glucuronide metabolites is due to enteroheptatic circulation than myricetin and catechin.

Table 2: Quantity of biomarkers in serum from the biomarkers mixtures and its phospholipid complexes

Time in hour	Catechin (ng/mL) from biomarkers mixtures	Myricetin (ng/mL) from biomarkers mixtures	Quercetin (ng/mL) from biomarkers mixtures	Catechin (ng/mL) from lipid complex of biomarkers mixtures	Myricetin (ng/mL) from lipid complex of biomarkers mixtures	Quercetin (ng/mL) from lipid complex of biomarkers mixtures
0hr	0	0	0	0	0	0
0.75hr	2.43	11.7	8.3	27.95	1.01	19.8
1.25hr	3.8	2.24	1.8	4.36	0	3.831
1.75hr	5.6	0.412	1.1	0	0	1.1
2.25hr	2	0	0.102	0	0	0

Table 3: Quantity of biomarkers in serum from the HYPE and its phospholipid complexes

Time in hour	Catechin (ng/mL) from HYPE	Myricetin (ng/mL) from HYPE	Quercetin (ng/mL) from HYPE	Catechin (ng/mL) from lipid complex of HYPE	Myricetin (ng/mL) from lipid complex of HYPE	Quercetin (ng/mL) from lipid complex of HYPE
0hr	0	0	0	0	0	0
0.75hr	3.44 µg	1.0681	6.22	1.1	0.204	1.274
1.25hr	0.5	0.35028	1.1	0.802	0	0.759
1.75hr	0.1483	0	0.7	0.355	0	0.525
2.25hr	0	0	0	0	0	0

Table 4: Pharmacokinetic parameters of parent biomarkers

Material administered (Per oral route)	Name of the phytoconstituents	Concentration of phytoconstituents detected at T_{max} in micrograms C_{max}	
Biomarkers mixture 100mg/kg	Quercetin	0.8h	8.3 µg
	Catechin	1.75h	5.6 µg
	Myricetin	0.85h	11.7 µg
Biomarkers mixture 50mg/kg	Quercetin	Not detected	Not detected
	Catechin	Not detected	Not detected
	myricetin	Not detected	Not detected
Phospholipids complex of biomarkers mixtures 100mg/kg	Quercetin	0.75h	6.22 µg
	Catechin	0.75h	3.44 µg
	Myricetin	0.758h	1.0681 µg

Table 5: Pharmacokinetic parameters of biomarkers and their Phospholipid complexes of *Punica granatum*

Material administered (Per oral route)	Name of the phyto-constituents	Concentration of phytoconstituents detected at T_{max} in micrograms C_{max}	
HYPE 100mg/kg	Quercetin	0.8h	9.8 µg
	Catechin	1.25h	15.55 µg
HYPE complex 100mg/kg	Quercetin	0.7h	5.5 µg
	Catechin	0.7h	2.55 µg

Table 6: Serum estimation of metabolites of quercetin

Time	Quercetin (from 50mg of biomarkers mixture)	Quercetin (from 100mg of biomarkers mixture)	Quercetin (from 100 mg of phospholipids complex of biomarkers)
hour	Log nM	Log nM	Log nM
0.25	3.59914043	4.371829	4.23638
0.75	4.06402723	4.177971	3.998526
1.25	3.56135187	3.903775	3.823587
2.25	3.17317169	3.45294	3.431117
3.25	2.91789919	2.917899	3.337525

Table 7: Serum estimation of metabolites of catechin

Time	Catechin (from 50mg of Biomarkers mixture)	Catechin (from 100mg of Biomarkers mixture)	Catechin (from 100 mg of Phospholipids complex of Biomarkers)
hour	Log nM	Log nM	Log nM
0.25	3.16595619	3.397905	3.333447
0.75	3.32431868	3.087796	3.448725
1.25	3.19077977	3.031722	3.19078
2.25	2.57895994	2.634477	2.57896
3.25	2.0816353	2.350481	2.081635

Table 8: Serum estimation of metabolites of Myricetin

Time	Myricetin (from 50mg of Biomarkers mixture)	Myricetin (from 100mg of Biomarkers mixture)	Myricetin (from 100 mg of Phospholipids complex of Biomarkers)
hour	Log nM	Log nM	Log nM
0.25	3.21764184	3.439491	3.801111
0.75	3.58098516	3.646702	3.501804
1.25	3.2067525	3.416561	3.284234
2.25	2.47520614	2.898883	2.594393
3.25	2.04155058	2.041551	2.310396

Table 9: Pharmacokinetic parameters of glucuronide metabolites of biomarkers from 50mg/kg dose of biomarkers mixture

Pharmacokinetic parameters	Catechin (from 50mg/kg of biomarkers mixture)	Quercetin (from 50mg/kg of biomarkers mixture)	Myricetin (from 50mg/kg of biomarkers mixture)
T_{max} (hr)	0.75	0.75	0.75
C_{max} (µg/ml)	612	3500	1212
AUC_{0-t} (µg. min/ml)	877.25	4600	1233.75
$AUC_{0-\infty}$ (µg. min/ml)	1008.08	4848.75	1280.54
elimination $t_{1/2}$ (hr)	2.595	.690	.926
K_e (hr^{-1})	0.267	1.0035	0.748
V_d (Lt)	0.117	0.041	0.095
CL_T (hr^{-1}Lt)	0.0312	0.0411	0.0710
F=$(CLt/Dose) * AUC0-t$	0.547	0.378	0.1751

Table 10: Pharmacokinetic parameters of glucuronide metabolites of biomarkers from 100mg/kg dose of biomarkers mixture

Pharmacokinetic parameters	Catechin (from 100mg/kg of biomarkers mixture)	Quercetin (from 100mg/kg of biomarkers mixture)	Myricetin (from 100mg/kg of biomarkers mixture)
T_{max} (h)	0.25	0.25	0.75
C_{max} (µg/ml)	725	5205	1410
AUC_{0-t} (µg. min/ml)	750.25	6849.5	1624
$AUC_{0-\infty}$ (µg. min/ml)	1330.60	7305.70	2051.52
Elimination $t_{1/2}$ (hr)	6.187	1.264	1.506
K_e (hr^{-1})	0.112	0.548	0.460
V_d (Lt)	0.137	0.0140	0.114
CL_T (hr^{-1}Lt)	0.0153	0.0076	0.0524
F=$(CLt/Dose) * AUC0-t$	0.1147	0.5205	0.850

Table 11: Pharmacokinetic parameters of glucuronide metabolites of biomarkers from 100mg/kg dose of phospholipids complex of biomarkers mixture

Pharmacokinetic parameters	Catechin (from 100 mg of phospholipids complex of biomarkers)	Quercetin (from 100mg/kg mg/kg of phospholipids complex of biomarkers)	Myricetin (from 100 mg/kg of phospholipids complex of biomarkers)
T_{max} (h)	0.75	0.25	0.25
C_{max} (µg/ml)	815	5205	2012
AUC_{0-t} (µg. min/ml)	1136.25	4706.7	1624
$AUC_{0-\infty}$ (µg. min/ml)	1307.46	6615.88	1773.42
elimination $t_{1/2}$ (hr.)	1.205	2.0145	1.593
K_e (hr^{-1})	0.575	0.344	0.435
V_d (Lt)	0.16	0.019	0.0571
CL_T (hr^{-1}Lt)	0.092	0.00653	0.0248
F= $(CLt/Dose) * AUC0-t$	0.1136	0.3059	0.4027

Table 12: Paw volume reading after per oral administration

Treatment	Dose (mg/kg)	Paw volume reading (ml) (Percent inhibition in rat paw edema ±SD) (n=6)				
		1h	2h	3h	4h	5h
Blank		1.004	1.104	1.15	1.01	0.655
Standard	40	0.965 (3.8±0.044)	0.994 (10.65±0.234)	0.926 (19.47±0.1406)	0.76 (24.75±0.124)	0.616 (1.37±0.068)
Biomarkers	100	0.9525 (5.12±0.5072)*	0.99 (6.25±0.1067)*	0.89 (22.60±0.071)*	0.9275 (8.16±0.117)*	0.632 (3.51±0.074)*
Biomarkers complex	100	0.91 (9.36±0.1660)**	1.104 (10.32±0.2611)**	0.816 (29.04±0.114)**	0.81 (19.80±0.125)**	0.644 (1.67±0.043)**
Extract	100	0.988 (1.59±0.2133)*	1.04 (5.7±0.1555)*	1.022 (11.13±0.084)*	0.81 (19.80±0.089)*	0.602 (8.09±0.173)*
Extract Complex	100	0.965 (3.88±0.123)*	0.8925 (19.15±0.115)*	0.8975 (21.95±0.071)*	0.85 (15.84±0.042)*	0.625 (4.58±0.079)*

The values in parenthesis indicate percent inhibition in rat paw edema (±S. D.). Level of significance for all groups is*$p<0.05$ and for biomarker complex is **$p<0.001$

***In vivo* (anti-inflammatory) potential of circulating metabolites**

Pharmacokinetics study revealed the fact that at 3rd hour no parent form of quercetin, catechin and myricetin was detected in blood. The glucuronide metabolites detection of 3rd hour confirmed that the anti-inflammatory properties of these biomarkers in 3rd hour were only due to their metabolites. From the above pharmacokinetics study and previous literature, it can be concluded that the inhibition of paw edema by these biomarkers in 3rd hour was governed by a long circulating phase II metabolites. The above study recorded high amount of quercetin glucuronide metabolite than catechin and myricetin at 3rd hr. The mixture also showed an inhibition of paw edema at 2nd hr. was due to the presence of parent form of catechin and the phase II metabolites of catechin, quercetin and myricetin. The glucuronide metabolites of these biomarkers were previously reported producing the anti-inflammatory activity by reaching the target site and getting cleaved by β-glucuronidase enzyme to release free biomarkers to enter at the site of inflammation [4]. The anti-inflammatory activity is presented as inhibition in rat paw edema in table 12. At fourth hour the extract complexes (HYPE complex) exhibited the highest activity than complexes of biomarkers mixture and their isolated form, which may be due to the presence of parent catechin, quercetin, myricetin and their metabolites along with other polyphenols like allergic acid [13, 14] etc. The anti-inflammatory activity of HYPE complexes was more than HYPE and mixture of the biomarkers at 3rd and 4thhr which may be due to the greater

bioavailability of ellagic acid or other polyphenols along with long circulating metabolite of quercetin. These complexes of the HYPE exhibited greater anti-inflammatory activity not only due to the faster absorption, increased bioavailability of parent biomarkers and their metabolites, but also demonstrated the contribution of synergistic effect due to the presence of elastic acid and other unknown polyphenols in HYPE of Punica granatum. No significant data obtained from dose dependent study (50 mg/kg), hence data not shown.

CONCLUSION

Detection of free quercetin at a dose of 50 mg/kg and 100 mg/kg was a challenge for any analyst by conventional HPLC or even LC-MS methods. But with the help of LC-ESI-MS/MS, a successful attempt was made to quantify these biomarkers like catechin, myricetin and quercetin at this low dose of rat serum and even successfully applied to estimate the metabolites after bio enhancement through phospholipid complex. The most important attribute demonstrated by phospholipids complex form was alteration of the pharmacokinetics behaviour of polyphenols in completed form (shorter t_{max} and higher C_{max}) leading to the faster metabolism.

The AUC_{0-t} and $AUC_{0-\infty}$ values of glucuronide metabolites of these biomarkers were higher in phospholipids complexes with higher C_{max}, $t_{1/2}$ and shorter t_{max} indicating the greater bioavailability of these biomarkers in phospholipids complexes. *In vitro* and *in* vivo activity profile also confirmed the bio enhancement of polyphenols from natural sources after forming a supramolecular complex with phospholipids. Anti lipid peroxidation inhibitory potential was found greater in the case of phospholipid complexes with IC_{50} value 17.41 µg for biomarker complex as compared to biomarker mixture (63.52 µg). For plant extract complex the potential was found to be higher with IC_{50} value 23.84 µg as compared to extract alone (50.74 µg). Anti-inflammatory activity of 100 mg/kg of agro waste extracts complex of *P. granatum* was found very significant at 3rd hour with (21.95±0.071 %) inhibition in rat paw edema which was greater than the extract alone (11.13±0.084 %). Similar results were observed for biomarker complex with (29.04±0.114%) inhibition and found better than biomarker mixture alone, (22.60±0.071%). Anti-inflammatory activity after 3rd hour demonstrated that not only free forms of biomarkers are active; but the metabolites detected after the second hour also are responsible for contributing to the bioactivity. Thus to conclude it can be stated that bio enhancement of polyphenols was observed and achieved through phospholipids complexes and was demonstrated by *in vitro* and *in vivo* activity testing.

CONFLICT OF INTEREST

The authors confirm that this article has no conflict of interest.

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