ejpmr, 2016,3(4), 521-533

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

DIVERSE BIOMOLECULES PRESENT IN ETHANOLIC EXTRACT OF FRUITS OF *PUNICA GRANATUM* L(EEF-PG) ACT AS POTENTIAL HEPATOCYTES REGENERATOR

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Article Received on 23/02/2016 **Article Revised on 14/03/2016** **Article Accepted on 04/04/2016**

ABSTRACT

Punica granatum is grown as a fruit crop plant, and as ornamental trees and shrubs in parks and gardens. The edible fruit of *Punica granatum* is a berry and is between a lemon and a grapefruit in size, 5–12 cm in diameter with a rounded hexagonal shape and has thick reddish skin. The main aim and objectives of the present work was to isolate the bioactive molecules from the ethanolic extract of fruits *Punica granatum* (EEF-PG) and evaluate the *in vivo* hepatoprotective activity. The isolationation and characterization of biomolecules was carried out by ESI-MS-MS. *In vivo* hepatoprotective activity was performed against CCl_4 induced rat hepatocytes. The Results displayed that the elevated levels of SGOT, SGPT, ALP and Serum bilirubin due to CCl_4 intoxication were reduced significantly (*P<0.05) in rats, after treatment with EEF-PG. Treatment with EEF-PG at a both doses of 200 and 400 mg/kg b.w. significantly decreased the SGOT, SGPT, ALP, Serum Bilirubin levels by 10.76%, 23.30%, 10.18% and 36.98% (at low dose) and 25.33%, 42.26%, 22.66% and 54.79% (at high dose) respectively. Silymarin used as standard drug showed a reduction of 74.19%, 76.91%, 86.7% and 78.08% receiving CCl_4 alone. So depending upon the experimental evidence, it was confirmed that the biochemical parameters of the group treated with EEF-PG was significantly lower than the CCl_4-treated group. Histopathological examination of liver section of the rat treated with both 200 mg/kg, 400 mg/kg body weight of EEF-PG intoxicated with carbon tetrachloride had shown minimal inflammation with moderate portal triditis, moderate necrotic debris, centrilobular regeneration with restoration of central vein, sinusoids and hepatocytes with mild necrosis and their lobular architecture was normal.

KEYWORDS: Bioactive molecules, Hepatoprotective activity, ESI-MS-MS, Portal triditis, centrilobular regeneration etc.

INTRODUTION

The *Punica granatum* leaves are opposite or sub-opposite, glossy, narrow oblong, entire, 3–7 cm long and 2 cm broad. The flowers are bright red, 3 cm in diameter, with four to five petals (often more on cultivated plants). Some fruitless varieties are grown for the flowers alone. The edible fruit is a berry and is between a lemon and a grapefruit in size, 5–12 cm in diameter with a rounded hexagonal shape, and has thick reddish skin. The exact number of seeds in a pomegranate can vary from 200 to about 1400 seeds, contrary to some beliefs that all pomegranates have exactly the same number of seeds.[1] Each seed has a surrounding water-laden pulp—the edible sarcotesta that forms from the seed coat—ranging in color from white to deep red or purple. The seeds are embedded in a white, spongy, astringent membrane.[2] The preliminary phytochemical screening displayed the present of following phytoconstituents in *Punica granatum*: Energy -346 kJ (83 kcal), Carbohydrates (18.7 g), Sugars (13.67 g) , Dietary fiber (4 g) Fat (1.17 g), Protein (1.67 g), Thiamine (vit. B1) - 0.067 mg (6%), Riboflavin (vit. B2) 0.053 mg (4%), Niacin (vit. B3) - 0.293 mg (2%), Pantothenic acid (B5) - 0.377 mg (8%), Vitamin B6 - 0.075 mg (6%), Folate (vit. B9) - 38 µg (10%), Choline - 7.6 mg (2%), Vitamin C - 10.2 mg (12%), Vitamin E - 0.6 mg (4%), Vitamin K - 16.4 µg (16%), Calcium - 10 mg (1%), Iron - 0.3 mg (2%), Magnesium - 12 mg (3%), Manganese - 0.119 mg (6%), Phosphorus - 36 mg (5%), Potassium - 236 mg (5%), Sodium - 3 mg (0%), Zinc - 0.35 mg (4%). Pomegranate seeds provide 12% of the Daily Value (DV) for vitamin C and 16% DV for vitamin K per 100 g serving, and

contain polyphenols, such as ellagitannins and flavonoids. Pomegranate seeds are excellent sources of dietary fiber which is entirely contained in the edible seeds. People who choose to discard the seeds forfeit nutritional benefits conveyed by the seed fiber and micronutrients.[3,4]

In preliminary laboratory research and clinical trials, juice of the pomegranate may be effective in reducing heart disease risk factors, including LDL oxidation, macrophage oxidative status, and foam cell formation**.**[5,6,7] In mice, "oxidation of LDL by peritoneal macrophages was reduced by up to 90% after pomegranate juice consumption.[8] In a limited study of hypertensive patients, consumption of pomegranate juice for two weeks was shown to reduce systolic blood pressure by inhibiting serum angiotensin-converting enzyme.[9] Juice consumption may also inhibit viral infections[10], while pomegranate extracts have antibacterial effects against dental plaque.[11] Metabolites of pomegranate juice ellagitannins localize specifically in the prostate gland, colon, and intestinal tissues of mice[12], leading to clinical studies of pomegranate juice or fruit extracts for efficacy against several diseases. In 2013, 44 clinical trials were registered with the National Institutes of Health to examine effects of pomegranate extracts or juice consumption on a variety of human disorders, including[13]: prostate cancer, prostatic hyperplasia, diabetes, lymphoma, rhinovirus infection, common cold, oxidative stress in diabetic hemodialysis, atherosclerosis, coronary artery disease, infant brain injury, hemodialysis for kidney disease, male infertility, aging, memory, pregnancy complications, osteoporosis and erectile dysfunction. One pilot study in adult subjects found that daily consumption of pomegranate juice over two weeks increased salivary testosterone levels by 24% and had other effects on blood pressure, mood, anxiety or emotions.[14]

MATERIALS AND METHOD

Drugs and chemicals

The all chemicals used for the extraction and phytochemical screening were of LR and AR grade. Standard drugs silymarin was purchased from Local Retail Pharmacy Shop and solvents and other chemicals were used from Institutional Store and were of AR grade.

Instrumentation

The Polyphenols present in EEF-PG were determined by ESI-MS/MS**.** The pomegranate fruits extract was analyzed using a Waters Quattro Premier XE tandemquadrupole mass spectrometer equipped with electro-spray ionization (ESI) source.

Experimental animals

White male albino Wister rats weighing about 200-250 g was used. They were obtained from the animal house of C.L. Baid Metha College of Pharmacy, Chennai. They were kept under observation for about 7 days before the onset of the experiment to exclude any intercurrent infection, had free access to normal diet and water. The animals were housed in plastic well aerated cages at normal atmospheric temperature (25±5°C) and normal 12- hour light/dark cycle under hygienic conditions. The experimental protocol was approved by Institutional Animal Ethics Committee **(IAEC) of CPCSEA: IAEC/XXIX/12/2015**.

CHEMISTRY

Methodology for extraction

Weigh 20 g of fruits of *Punica granatum* paste (ripen can be mashed to prepare a paste) into a 250 ml round-bottomed flask. Add 50 ml of ethanol and 60 ml of dichloromethane. Heat the mixture under reflux for 5 min on steam-bath with frequent shaking. Filter the mixture under suction and transfer the filtrate to a separatory funnel. Wash this mixture containing bioactive compounds with three portions of 150 ml each with sodium chloride solution. Dry the organic layer over anhydrous magnesium sulfate.[15] Filter and evaporate most of the solvent in vacuum without heating and obtained ethanolic extract of fruits of *Punica granatum* (EEF-PG).

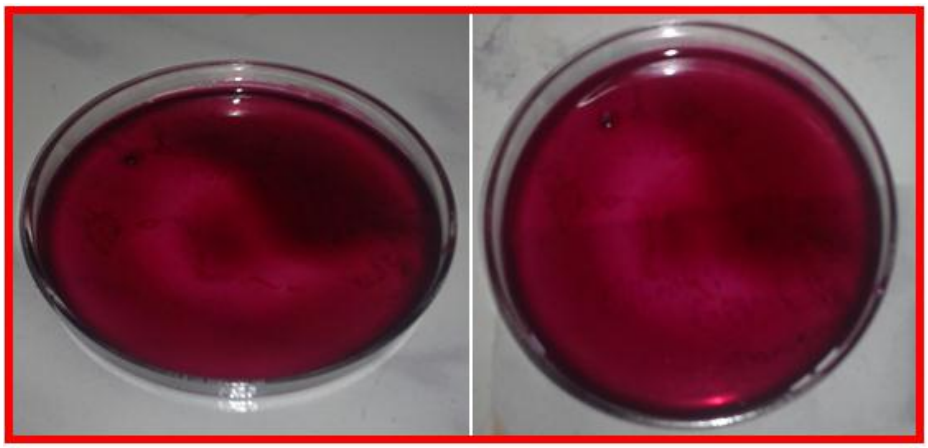


Fig 1: Ethanolic Extract of fruits of *Punica granatum* (EEF-PG).

Phytochemical screening[16,17,18]
Preliminary Phytochemical screening of ethanolic extract of fruits of Punica granatum L (**EEF-PG**) had shown the presence of various bioactive compounds such as carbohydrates, aminoacids and peptides, phytosterols, carotenoids, and polyphenols etc.

Characterization of biomolecules present in EEF-PG
The Polyphenols present in EEF-PG were determined by ESI-MS/MS**.** The pomegranate fruits extract was analyzed using a Waters Quattro Premier XE tandemquadrupole mass spectrometer equipped with electro-spray ionization (ESI) source. Instrument control and data acquisition were performed using Mass Lynx ver. 4.1 software. The instrument was calibrated for nominal resolution for MS1 and MS2 up to 1200 m/z using the sodium cesium iodide standard calibration solution. The sample was introduced by direct infusion at a flow rate of 40 μL/min and was run in both positive and negative ionization mode for the preliminary screening (full scan mode) from 50 m/z to 1200 m/z. The compounds of interest were tuned by optimizing the sample cone voltage (20-50 V) and a capillary voltage to 3.5 KV in order to observe the bioactive substances most amenable to electro spray ionization. The parent ions of the compounds of interest were then subjected to tandem MS analysis using the daughter scan function using the following tuning parameters: (ESI), capillary voltage set between 3.3 to 3.6 KV, cone voltage (30 V), extractor (3 V), RF Lens (0.2 V), source temperature (100°C), dissolvation temperature (150°C), multiplier (650 V), the collision energy was optimized in the range from 20 to 45 eV. Most of the daughter peaks were identified by matching the fragmentation patterns obtained from the daughter scan to those theoretically and experimentally documented in literature.[19-31]

PHARMACOLOGY

Protocol for the study of acute oral toxicity of EEF-PG
In the present study the acute oral toxicity of the ethanolic extract of fruits of Punica granatum was performed by acute toxic class method. In this method the toxicity of the extract was planned to test using step wise procedure, each step using three Wister rats. The rats were fasted prior to dosing (food but not water should be withheld) for three to four hrs. Following the period of fasting the animals were weighed and the extract was administered orally at a dose of 2000 mg/Kg b.w. Animals were observed individually after dosing at least once during the first 30 min; periodically the surveillance was carried out for the first 24 hrs with special attention given during the first 4 hrs and daily thereafter, for a total of 14 days.[32]

Experimental protocol for the evaluation of *in vivo* hepatoprotetive activity[33]
A total of 30 rats were taken and divided into 5 groups of 6 rats each
(A) Group I: Normal Control Group [**NCG -** (only the vehicle (1 mL/kg/day of 1% CMC; p.o.)].
(B) Group II: Negative Control Group **[Neg.CG** – (CCl4 1 mL/kg (1:1 of CCl_4 in olive oil) i. p].
(C) Group III: Positive Control/Standard Group **[SG -** CCl_4 1 mL/kg (1:1 of CCl_4 in olive oil) i.p.+ Standard Silymarin 100 mg/kg orally (p.o.) for 7 days]

Treatment Groups
(D) Group V: Low Dose Group [**LDG** - CCl_4 1 mL/kg (1:1 of CCl_4 in olive oil) i. p + **EEF-PG** (200 mg/ kg b. w., p.o.)]. Treatment was given daily for seven days orally.
(E) Group IV: High Dose Group [**HDG** - CCl_4 1 mL/kg (1:1 of CCl_4 in olive oil) i.p + **EEF-PG** (400 mg/ kg b. w., p.o.)].

Collection of blood
On the 8^{th} day, blood was collected by retro orbital puncture, under mild ether anesthesia after 8 hr fasting. Blood samples were centrifuged at 3000 rpm for 20 mins. Serum was separated and stored at – 200^0C until biochemical estimations.

Biochemical Analysis
The Serum sample was taken for the determination of SGOT, SGPT, **S**ALP and Serum bilirubin etc.

RESULTS AND DISCUSSION

Phytochemical screening
Preliminary Phytochemical screening of ethanolic extract of fruits of Punica granatum L (**EEF-PG**) had shown the presence of various bioactive compounds such as carbohydrates, aminoacids and peptides, phytosterols, carotenoids, and polyphenols which are ascertained by their qualitative confirmatory tests.

Characterization of biomolecules by ESI-MS/MS
The present study demonstrated the fractionation of pomegranate fruit in ethyl acetate as a solvent which was very efficient in exhibiting various compounds of polyphenols when subjected to the ESI-MS/MS analysis. ESI-MS/MS spectra of the direct infusion of Punica ethyl acetate fraction showed the presence of wide array of phenolic compounds. The main constituents of ethyl acetate fraction were monitored by diode-array and mass spectrometry. Typical direct infusion mass spectra of phenolic compounds of interest from the pomegranate fruit extract are shown in **Fig 1-5**. **Table: 1** outlines the peak list for the full scan (precursor ions) and daughter scan (product ions) mass spectra for the major compounds of interest. The profile of the extract showed 16 major phenolic compounds and several derivatives. The phenolic compounds were classified into three categories: hydroxycinnamic acids, hydrolysable tannins and hydroxybenzoic acids. Peak identities were obtained by matching their pseudo molecular ions [M–H] - obtained by ES/MS and tandem MS with the expected theoretical molecular weights from literature. It is evident that various antioxidant substances are present in pomegranate fruits. In addition, the fruits contain

significant amounts of ellagitannins, such as punicalin (Compound 22) and punicalagin (Compound 26) together with hexahydroxydiphenic acid (HHDP) derivatives (Compounds 23 & 24) and flavonoids such as quercetin (Compound 17) as well as the ellagic acid (Compounds 14,19) and gallic acid derivatives (Compounds 27-35).

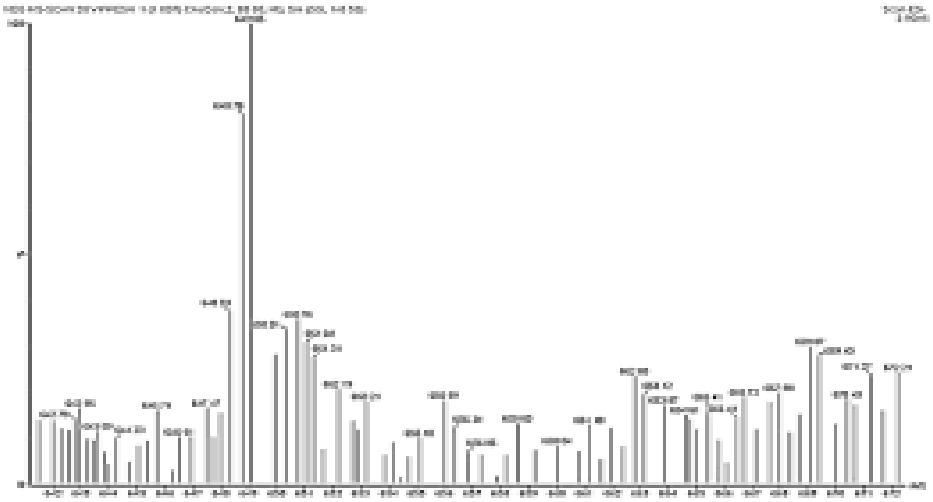


Fig 1

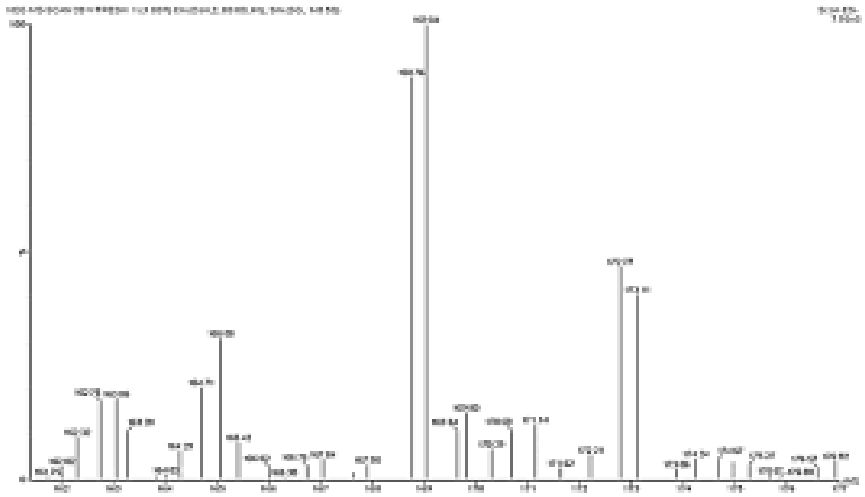


Fig 2

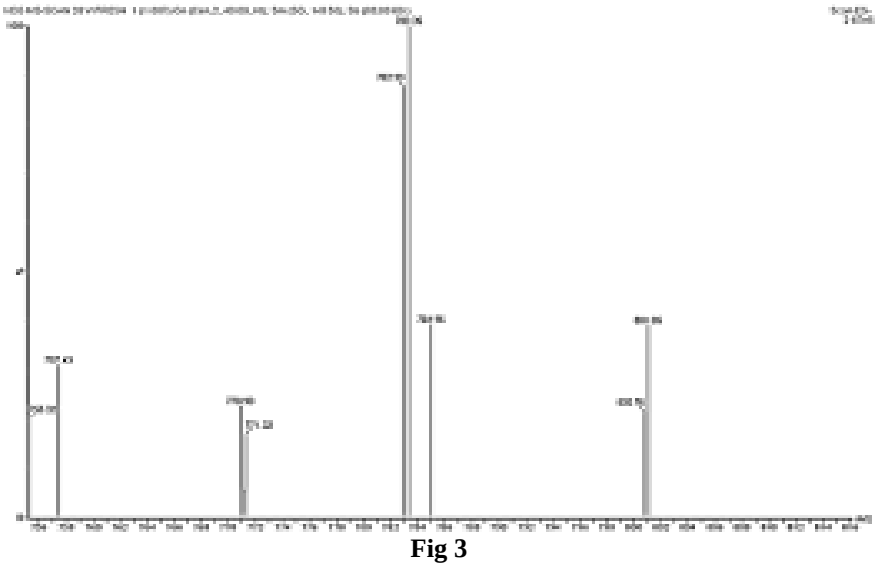


Fig 3

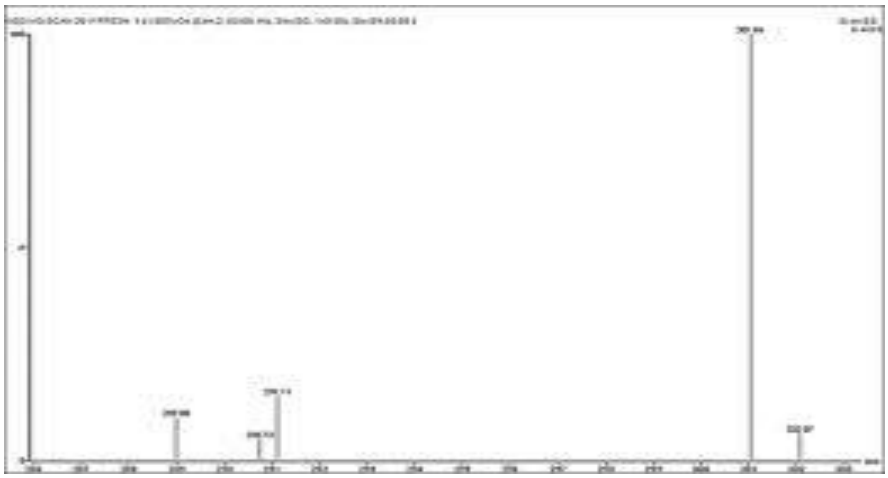


Fig 4

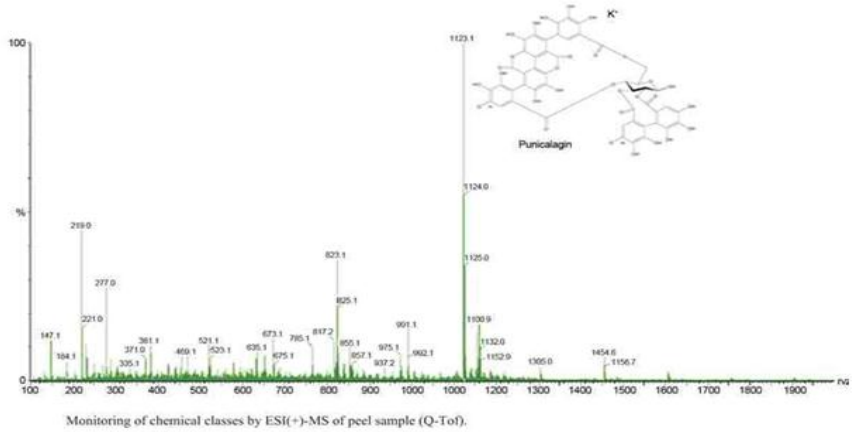


Fig 5

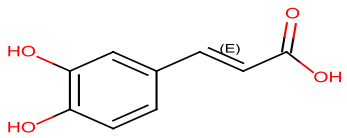
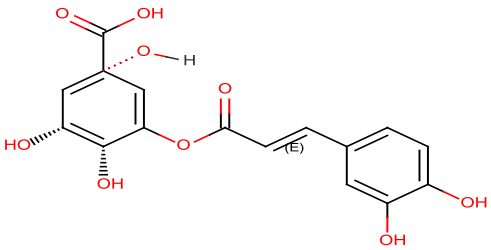
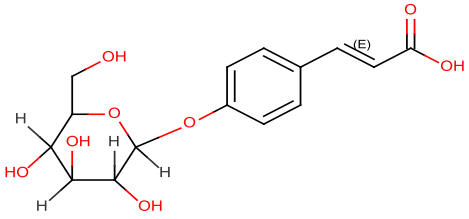
Table 1: for Characteristic ions of phenolic compounds of pomegranate fruit extract

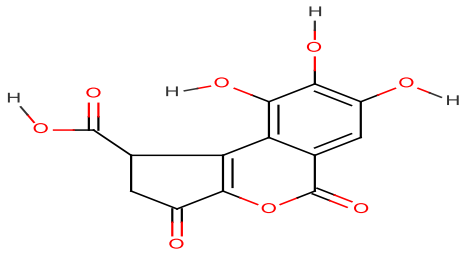
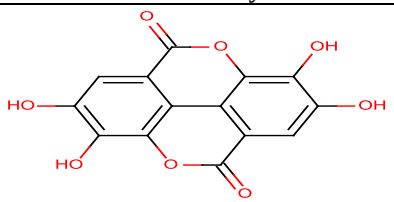
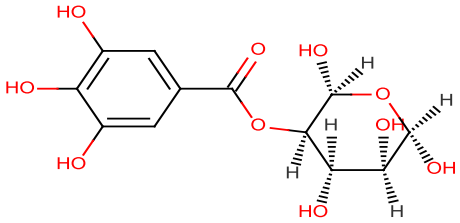
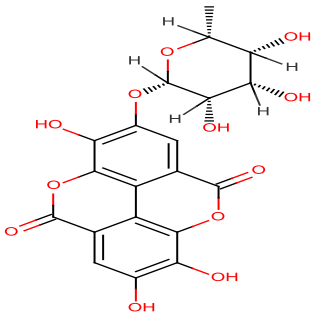
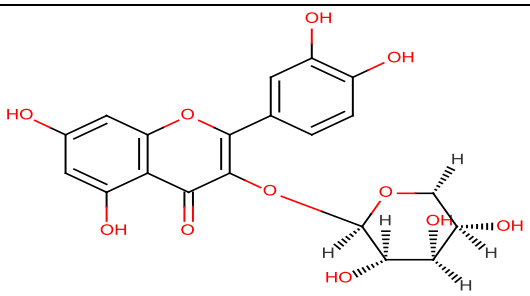
Compound	Assignment	Chemical formula	[M-H]-m/z	MS-MSm/z
Hydroxycinnamic acid derivatives				
C1	Caffeic acid	$C_9H_8O_4$	179	59, 71, 75,89, 135
C2	Fragment of caffeic acid	$C_8H_8O_2$	135	71, 75
C3	Fragment of caffeic acid	$C_8H_6O_4$	165	59,71, 75
C4	Caffeic acid derivative	$C_9H_{10}O_4$	181	59, 71, 89
C5	Caffiec acid derivative	-	215	59, 71, 89, 179, 225
C6	Caffiec acid derivative	-	217	59, 71, 89, 179, 181
C7	Caffiec acid derivative	-	219	179
C8	Caffeic acid phenethylester	$C_{17}H_{16}O_4$	283	59, 179
C9	Methyl gallate derivative	-	325	169, 183
C10	Caffeoylquinic acid	$C_{16}H_{18}O_9$	353	111, 129
C11	Caffeic acid derivative	-	457	179, 215
C12	P-Comaric acid glucuronide	$C_{15}H_{16}O_9$	339	163
Hydrolysable tannins				
C13	Brevifolin carboxylic acid	$C_{13}H_8O_8$	291	80, 247
C14	Ellagic acid	$C_{14}H_6O_8$	301.13	185, 229, 257
C15	Galloyl-hex(glucogallin)	$C_{13}H_{16}O_{10}$	331	125, 169
C16	Eschweilenol C	$C_{20}H_{16}O_{12}$	447.17	432, 389, 77
C17	Quercetin-3-O-glucoside	$C_{15}H_{10}O_7$	463	89, 265, 301
C18	HHDP-hex	$C_{13}H_{18}O_{14}$	481. 2	57, 275, 301, 180
C19	Ellagic acid der	-	515	229, 275, 301

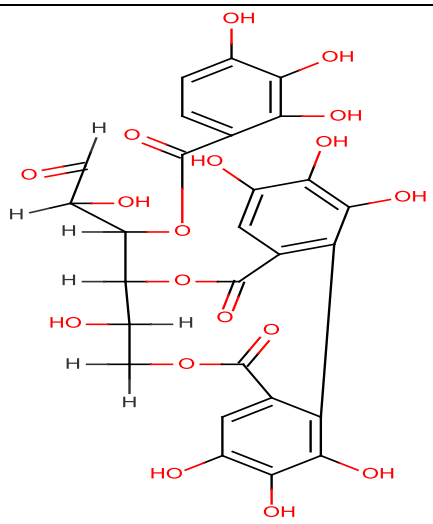
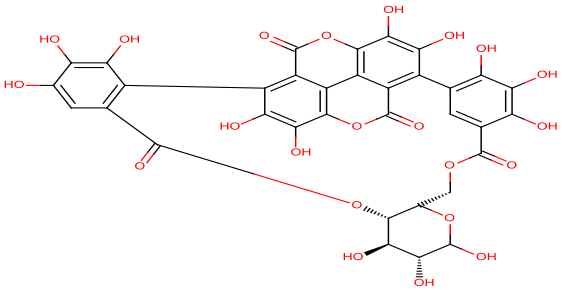
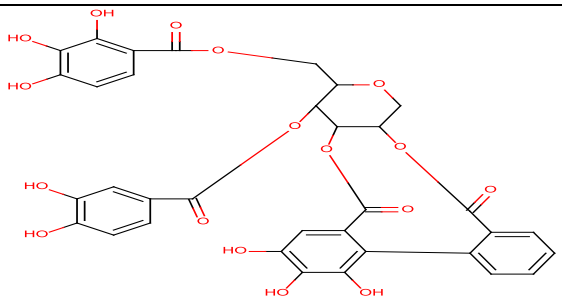
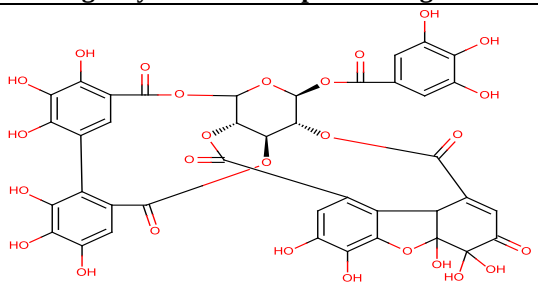
C20	Galloyl-HDDP-hex	$C_{27}H_{22}O_{18}$	633	275, 301
C21	Galloyl-HDDP-gluconide Lagerstannin C	$C_{27}H_{22}O_{19}$	649	301
C22	Punicalin	$C_{34}H_{22}O_{22}$	781	601, 299, 300
C23	Bis-HHDP-hex Pedunculagin 1	$C_{34}H_{24}O_{22}$	783	301, 602, 481
C24	Digalloyl-HHDP-hex Pedunculagin II	$C_{34}H_{26}O_{22}$	785	633
C25	Galloyl-HHDP- DHHDP-Glucoside GranatinB	$C_{34}H_{28}O_{27}$	951	481
C26	HHDP- gallagylglucoside Punicalagin	$C_{48}H_{28}O_{30}$	1083	781, 601, 602
Hydroxy benzoic acid				
C27	Gallic acid	$C_7H_6O_5$	169	69, 79, 83, 97,110,
C28	Fragment of gallic acid	$C_6H_6O_3$	125	69, 79
C29	Gallic acid der	-	277	79, 97
C30	Gallic acid der	-	279	79, 97
C31	Gallic acid der	-	395	125, 169, 215
C32	Gallic acid der	-	397	127, 171, 181, 217
C33	Vanilic acid der	-	255	128, 167
C34	Protocatechuic acid der	-	435	79, 89, 153,171
C35	Methyl gallate der	-	311	169, 183

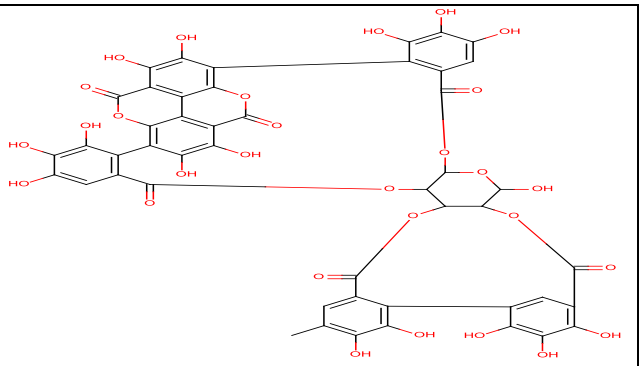
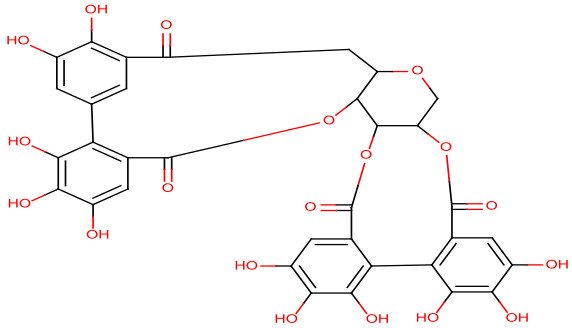
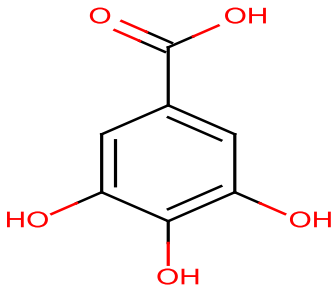
The chemical structures of major identified compounds are represented in **Table: 2**. Several [M-H] - ions were detected in the extract of freeze-dried pomegranate fruit using a tandem mass spectrometry (MS/MS) equipped with electro-spray ionization (ESI) interface. This study represents the first phenolic analysis for pomegranate fruit and as far as we are aware also reports the identification of some molecular ions that have not been reported before such as caffeic acid derivatives m/z 215, 217, 283, methyl gallat ester, quinic acid methyl ester and hexahydroxydiphenic acid (HHDP)-acetyl glucoside derivatives. Due to the large number of detected ions and unavailability of authentic standards, identifications of all molecular ions were carried out by direct infusion analysis using the negative full scan mode and their structural information elucidated by performing a daughter scan on the molecular ion of interest.

Table: 2 for the chemical structures of major identified compounds

Sl. No.	Compounds
1.	 **Caffic acid**
2.	 **Caffeoylquinic acid**
3.	 **p-comaric acid glucoronide.**

4.	 **Brevifolin carboxylic acid**
5.	 **Ellagic acid**
6.	 **Glucogallin**
7.	 **Eschweilenol-C**
8.	 **Quercetin-3-O-glucoside**

9.	 **Galloyl-HHDP-glucoronide-Lagerstanin C**
10.	 **Punicalin**
11.	 **Digalloyl-HHDP-hex-pedunculagin II**
12.	 **Galloyl- HHDP-DHHDP-glucoside gratin B**

13.	 **HHDP-gallagyl glucoside punicalagin**
14.	 **Bis-HHDP-hex-pedunculagin I**
15.	 **Gallic acid**

Chemical structure of major compounds found in the fruits extract of Punica granatum using ESI-MS/MS analysis

Hydroxycinamic acids

Compound 1 had [M-H] - at m/z 179 which fragmented into MS 2 ion at m/z 135 (loss 44 u [COO]) and it was identified as caffeic acid. Compounds 2 -7 in **Table 1** were considered caffeic acid fragments and derivatives because they showed similar product ion spectra. The MS/MS fragmentation pathway of compound 8 at m/z 283 showed two fragments m/z 179 (loss of phenethyl alcohol group with m/z and with m/z 104 water 18 u) and m/z 59, so the compound was assigned as caffeic acid phenethyl ester. Compound 12 at m/z 339 was identified as p-comaric acid glucuronide because its further fragmentation lost glucuronic acid moiety (176 u) yielding the fragment m/z 163.

Hydrolysable tannins

A number of 14 metabolites were identified in this group. The predominant compound was ellagic acid at m/z 301 with fragments m/z 257, 229, 185. The efficacy of this important compound has been considered atreatment for some low to mild and chronic disorders. Compound 13 was identified as brevifolin carboxylic acid at m/z 291 as its MS/MS fragmentation showed a [M-H-COO] - fragment at m/z 247. The loss of 162 u and 18 u from m/z 481 to m/z 301 was most likely due to the breakdown of glucose and water, so this compound was identified as hexahydroxydiphenoyl - glucoside (HHDP - glucoside). Compounds 21- 26 exhibited [M-H] - ions with m/z 633, 649,781, 783, 785, 951 and 1083. Their MS 2 fragmentations nearly led to the same basic structure and were all identified as Galloyl-HHDP-hex, Galloyl-HHDP-gluconidelagerstannin C, punicalin, bis-HHDP-hex pedunculagin 1, Digalloyl-HHDP-hexpedunculagin II, Galloyl- HHDP-DHHDP-glucosidegranatin B, and HHDP-gallagyl glucoside punicalagin.

Hydroxybenzoic acids

Four gallic acid derivatives were detected in the extract from the negative ionization mode at m/z 277, 279, 395 and 397 as their MS/MS fragments matched well with basic fragmentationof gallic acid. Beside gallic acid derivatives, vanilic acid and protocatechuic acid

derivatives were also detected with molecular ions m/z 255 and 435. A compound m/z 311 was characterized as methyl gallate derivative; this was because it showed two fragments m/z 183 and 169. The 14 u difference between the two fragments was due to the loss of CH_2.

Acute oral toxicity study

(i) Acute oral toxicity studies were performed according to the OECD guideline 423 method.

(ii) This method has been designed to evaluate the substance at the fixed doses and provide information both for hazard assessment and substance to be ranked for hazard classification purposes.

(iii) The **EEF-PG** was administered initially at a dose of 2000 mg/kg b.w and 1% CMC (p.o) and observed 14 days mortality due to acute toxicity.

(iv) Careful observation were made at least thrice a day for the effect on CNS, ANS, motor activity, salivation and other general signs of toxicity were also observed and recorded.

(v) Since no sign of toxicity observed at 2000 mg/kg b.w. to the group of animals, the **LD_{50}** value of the **EEF-PG** expected to exceed 2000 mg/kg b. w. and represented as class 5 (2000 mg/kg < LD50 < 2500 mg/kg).

(vi) From the toxicity studies the data revealed that all the synthesized compounds proved to be non toxic at tested dose levels and well tolerated by the experimental animals as there **LD_{50}** cut of values > 2000 mg/kg b. w.

Table 3: for the dose selection by acute toxicity class method (OECD) guide lines 423 of EECS

Sl. No.	Treatment group	Dose mg/kg	Sign of toxicity	Onset of toxicity	Duration
1	EE	200	No	No	14 days
2	EE	400	No	No	14 days

Evaluation of *In vivo* hepatoprotective activity

Statistical analysis: The data were expressed as mean ± SD. Statistical differences at *P < 0.05 between the groups were analyzed by one-way ANOVA followed by Dunnett's Multiple Comparison Test using Graph Pad Prism 5.04 Instate software package. The data's were compared with group-II i. e. Negative Control group.

Biochemical analysis

The effects of **EEF-PG** on liver marker enzymes and serum bilirubin content are displayed in **Table 4.** The data exhibited that Normal Control Group demonstrated a normal range of AST, ALT and bilirubin levels while the CCl_4-treated group showed elevated levels of AST, ALT and bilirubin, thus confirming that CCl_4 causes hepatocellular degeneration at higher doses. The elevation of cytoplasmic AST and ALT is considered an indicator for the release of enzymes from disrupted liver cells. Bilirubin concentration has been used to evaluate chemically induced hepatic injury. The Results displayed in **Table 2 and Fig: 1-2** indicated that the elevated levels of SGOT, SGPT, ALP and Serum bilirubin due to CCl_4 intoxication were reduced significantly (*P<0.05) in rats, after treatment with **EEF-PG**. Treatment with **EEF-PG** at a both doses of **200 and 400 mg/kg b.w.** significantly decreased the SGOT, SGPT, ALP, Serum Bilirubin levels by **10.76%, 23.30%, 10.18%, and 36.98% (at low dose) and 25.33%, 42.26%, 22.66% and 54.79% (at high dose)** respectively. Silymarin used as standard drug showed a reduction of **74.19%, 76.91%, 86.7% and 78.08%** receiving CCl_4 alone. So depending upon the data of **Table 1** it was confirmed that the biochemical parameters of the group treated with **EEF-PG** was significantly lower than the CCl_4-treated group. Moreover the treatment with the **EEF-PG** significantly reduced the previously raised levels of AST, ALT, ALP and bilirubin in hepatotoxic rats.

Table: 4 for the assessment of Biochemical parameters

Sl. No	Treatment	AST(SGOT) IU/L	ALT(SGPT) IU/L	ALP(SALP) IU/L	Sr. bilirubin mg/dL
1.	NCG-I	52.25±0.3566	48.11±0.1533	51.95±0.1531	0.7268±0.0069
2.	Neg. CG-II	202.5±0.3038***	208.4±0.1961***	391.9±1.411***	7.383±0.1014***
3.	LDG-III	180.7±0.2176***	160.6±0.08021***	351.1±2.629***	4.665± 0.039***
4.	HDG-IV	151.2±0.1461***	120.9±0.6005***	302.8±0.1769***	3.397±0.3531*
5.	SG-V	91.60±1.795***	109.8±6.818***	155.7±6.240***	0.9000 ± 0.0577^{ns}

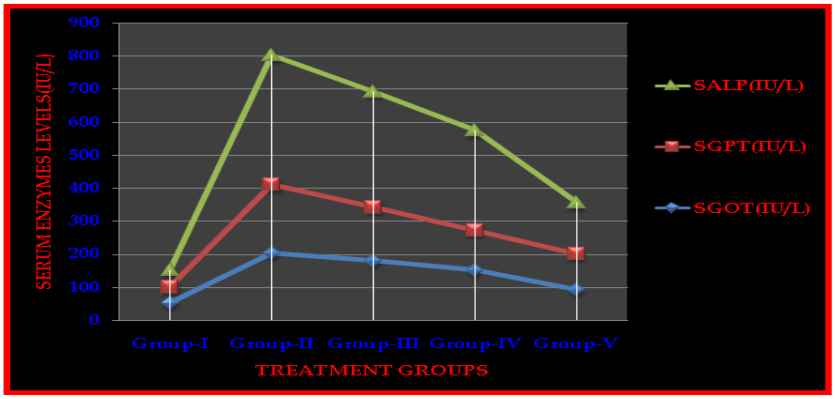


Fig 1: Comparison of sr. enzymes levels in different groups.

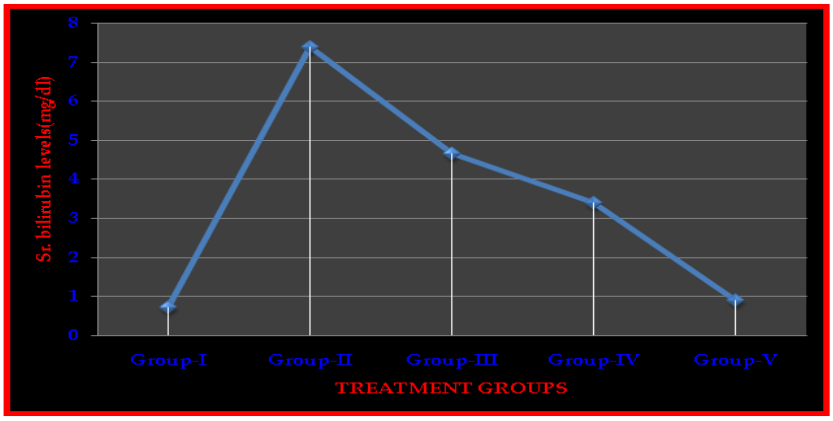


Fig 2: Comparison of sr. bilirubin level in different groups.

Histopathological Analysis

The results of light microscopy examination of the transverse section of control, CCl_4-treated and treated with **EEF-PG** rat livers were represented **in Fig 3 and 4.** It was revealed that the liver section of animals treated with CCl_4 showed a high degree of damage characterized by cell vacuolation, pyknotic and degenerated nuclei and wall of bile capillaries. The normal architecture of the liver was lost. The intralobular vein was badly damaged with wide spaces at some sinusoids. Liver sections of these rats indicated necrosis, ballooning and degeneration in hepatic plates and loss of cellular boundaries. There was also a heavy accumulation of neutrophils surrounding the portal vein. These neutrophils act as an indicator of the occurrence of cell damage as they are absent in normal healthy tissues. The hepatocytes are disrupted and sinusoids are damaged as well.

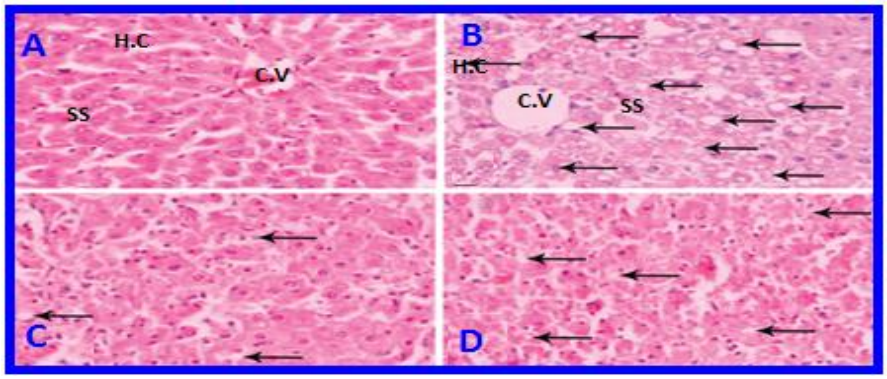


Figure 3: Hematoxylin and eosin staining, X400: (A) Normal control rat liver section (Group-I): Normal histological findings in the liver parenchyma; (B) Liver section of the rat intoxicated with Carbon tetrachloride (Group-II): Indicated variable degree of bridging necrosis of hepatocytes, most marked in centrilobular and mononuclear cellular infiltrate in the lobule. Mild degree of liver cell necrosis is seen as ballooning degeneration; (C) Liver section of the rat treated with silymarin and intoxicated with carbon tetrachloride (Group-V) and (D) Lliver section of the rat treated with 400 mg/kg of EEF-PG intoxicated with carbon tetrachloride (Group-IV): Showing centrilobular regeneration with restoration of c.v, ss and hepatocytes with mild necrosis. C.V: Central vein, V.C: Vacuole, S.S: Sinusoidal spaces, H.C: Hepatocytes.

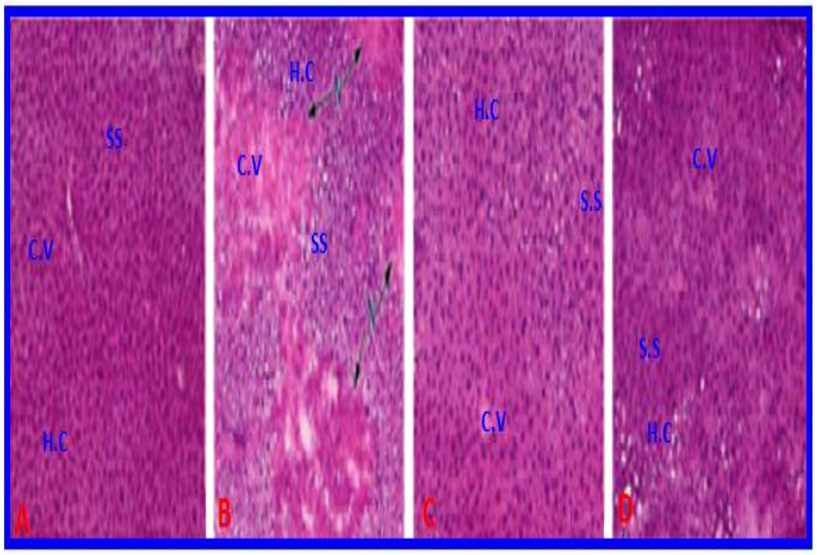


Figure 4: Histopathology report. (A) Normal control rat liver section (Group-I): Showed a normal structure of the liver; (B) Liver section of the rat intoxicated with Carbon tetrachloride (Group-II): Showed portal inflammation and panacinar necrosis necrosis; (C) Liver section of the rat treated with silymarin and intoxicated with carbon tetrachloride (Group-V): Showed minimal portal inflammation and their lobular architecture was normal; (D) Lliver section of the rat treated with 200 mg/kg of EEF-PG and intoxicated with carbon tetrachloride (Group-III): Showed minimal inflammation with moderate portal triditis, moderate necrotic debris and their lobular architecture was normal.

CONCLUSION

From the above exprimental data, here I concluded that the EEF-PG contained various bioactive molecules which were isolated by ESI-MS-MS and executed moderate to good hepatoprotective activity. When compared with standard drug silymarin it displayed that EEF-PG had the ability to restore and regenerate the CCl_4 induced hepatocytes due to the presence of diverse biomolecules.

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