Small molecule inhibitors of HCV replication from Pomegranate

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SUPPLEMENTARY INFORMATION

Characterization of purified compounds by spectral analyses.

Compound-1**:** The sub-fraction-IB was eluted in water obtained as a yellowish green crystalline powder. It is also soluble in water and methanol, but insoluble in n-hexane and chloroform. It has given a single prominent peak with retention time of 3.81 minutes respectively in the LC profile and provided its unique mass signals at m/z 1083.13 $[M-H]^-$, 541.27 $(M-2H)^-$ and 301.33 $(M-3H)^-$, in the negative ion modes corresponding to its absolute mass of 1084.7 g/mol, and is a characteristic feature of punicalagin ($C_{48}H_{28}O_{30}$) (Supplementary Figure -3).

Compound-2**:** is a yellowish green crystalline powder, eluted in the sub-fraction-IA (Supplementary Fig. 2). It is soluble in water and methanol, but insoluble in n-hexane. It has given a single prominent peak along with small peak with retention time of 3.75 and 4.94 minutes respectively in the LC profile and provided their identical mass signal as m/z 781.20 $[M-H]^-$ in the negative ion mode corresponding to the absolute mass of 782.5 g/mol, and is a characteristic feature of punicalin ($C_{34}H_{22}O_{22}$) (Supplementary Figure-4). Resulted, this compound is existed as isomeric condition in the fruit peel.

Compound-3**:** The sub-fraction-2A was eluted in ethanol obtained as a creamish light brown amorphous powder. It is sparingly soluble in water, alcohol and acetone. It has given a single prominent peak with retention time of 5.14 minutes respectively in the LC profile and provided its unique mass signal at m/z 301.20 $[M-H]^-$, in the negative ion mode corresponding to its absolute mass of 302.5g/mol, and is a characteristic feature of ellagic acid ($C_{14}H_6O_8$) (Supplementary Figure-5).

Assessment of accuracy of NS3-telaprevir modeled complex.

Docking of NS3 protease structure with the structure of telaprevir resulted in a streochemically sound model and it was compared with the crystal structure of the complex. Comparative analysis revealed that several ligand binding residues in NS3 protease, protein binding chemical groups in the telaprevir and protein-ligand interactions are correctly predicted (Supplementary Figure 7a and 7b) (Detailed list of correctly predicted residues and interactions are listed in supplementary table S1a). This shows that the knowledge based docking analysis carried out in this analysis can provide reliable clues about the general region of binding of the inhibitor and mode of inhibition.

Another interesting observation is the presence of good overlap of ligand interacting residues of NS3 protease bound to telaprevir and NS3 protease bound to ligands (Punicalin, Punicalagin and Ellagic acid) from Punica granatum. This shows that the mode of interaction and inhibition of the ligands namely, Telaprevir, Punicalin, Punicalagin and Ellagic acid are similar.

Supplementary table S1

Correctly predicted interacting residues and interactions in the modelled complex of NS3 bound to Telaprevir

Correctly predicted ligand interacting residues from enzyme, enzyme interacting ligand residues and interactions in the docked NS3-telapravir complex when compared to the crystal structure of NS3-telaprevir complex (PDB id : 3sv6) are listed. The amino acid numbering and the ligand atom names are according to the PDB structure : 3sv6.

1. Ligand interacting residues in enzyme	**Catalytic residues**: H1057, S1139 **Substrate interacting residues**: I1132, L1135, K1136, F1154, R1155, A1156, A1157, V1158, S1159 **Other ligand interacting residues**: Q1041, T1042, F1043, V1055, G1058, G1137
2. Enzyme interacting ligand atoms (Telaprevir)	CA, CAH, CAI, CAK, CAM, CAN, CAO, CAP, CAY, CB, CBA, CBB, CBC, CBD, CBE, CBF, CBI, CBJ, CBK, CBL, CBM, CBO, CD2, CG, NAA, NAE, NAF, O, OBR, OBS, OBT, OBW
3. Interactions	CAN - G1137, CAO - Q1041, CAO - F1043, CAO - T1042, CAP - Q1041, CAP - K1136, CBA - I1132, CBB - V1158, CBC - I 1132, CBC - K1136, CBD - S1159, CBK - R1155, CBL - R1155, NAA - Q1041, OBS - S1139, OBW - S1159, O - H1057 (Interactions shown in figure S1)

Supplementary tables S2, S3, S4 and S5

Unfavourable interactions between porcine pancreatic trypsin and ligand molecules

Ligand interacting residues which are also substrate interacting residues in NS3 protease is listed. The structurally equivalent residue of the substrate interacting residues in NS3 protease in trypsin is also listed. Unfavourable structural and physicochemical features in the amino acids of trypsin as listed in the tables could be a reason for inability of the ligands to inhibit trypsin.

Table S2:

Punicalin interacting residues	**NS3 protease**	**Trypsin**	**Possibility of interaction**	**Remarks**
Substrate binding residues	V132	K188	No	
	L135	C191	No	Disulphide bonded with other cysteine
	F154	V213	No	
	A156	W215	No	Short contact with ligand
	A157	G216	No	Short contact with ligand
	V158	Y217	No	
	C159	G218	No	

Table S3:

Punicalagin interacting residues	**NS3 protease**	**Trypsin**	**Possibility of interaction**
Substrate binding residues	V132	D189	No
	K136	Q192	No
	F154	V213	No
	A156	W215	No
	A157	G216	No
	C159	G218	No

Table S4:

Ellagic acid interacting residues	NS3 protease	Trypsin	Possibility of interaction	Remarks
Substrate binding residues	V132	K188	No	
	L135	C191	No	Short contact with ligand
	K136	Q192	No	
	F154	V213	No	
	A157	G216	No	
	C159	G218	No	

Supplementary tables S5 and S6

S5: Acute toxicity studies: organ weights of freshly dissected liver and spleen from control and treated groups of mice were recorded. **S6**: **Subacute toxicity studies**: organ weights of freshly dissected liver and spleen from control and treated groups of mice were recorded. Here A1, A2, and A3 represent three different mice of same group. SDW (sterile distilled water),

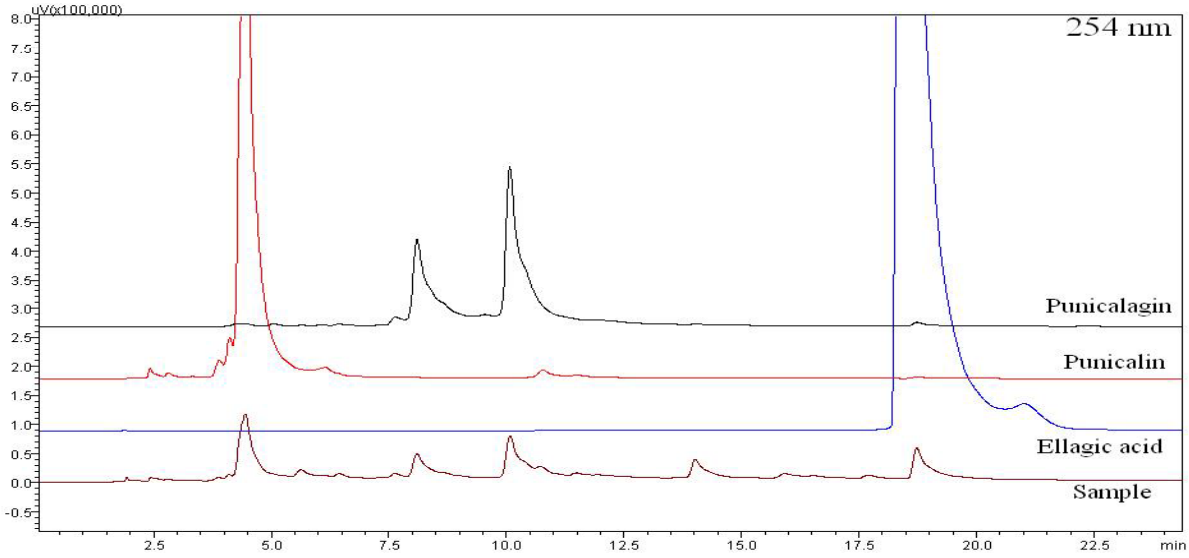
Table S5:

Groups	Sl no. of mice/group	Drugs	Liver (g)	Spleen (g)
Group 1	A1	SDW	1.27	0.07
	A2	SDW	1.32	0.08
	A3	SDW	1.38	0.07
Group 2	A1	CE	1.33	0.081
	A2	CE	1.30	0.101
	A3	CE	(died)	(died)
Group 3	A1	PLN	1.20	0.07
	A2	PLN	1.35	0.08
	A3	PLN	1.22	0.09
Group 4	A1	PGN	1.41	0.07
	A2	PGN	1.36	0.09
	A3	PGN	1.29	0.09
Group 5	A1	EA	1.38	0.08
	A2	EA	1.41	0.09
	A3	EA	1.28	0.12

Table S6:

Groups	Sl no of mice/ group	Drugs	Liver (g)	Spleen (g)
Group 1	A1	SDW	1.37	0.081
	A2	SDW	1.35	0.078
	A3	SDW	1.33	0.079
Group 2	A1	PLN	1.30	0.080
	A2	PIN	1.31	0.078
	A3	PLN	1.32	0.091
Group 3	A1	PGN	1.40	0.079
	A2	PGN	1.37	0.089
	A3	PGN	1.34	0.089
Group 4	A1	EA	1.33	0.085
	A2	EA	1.40	0.092
	A3	EA	1.38	0.121

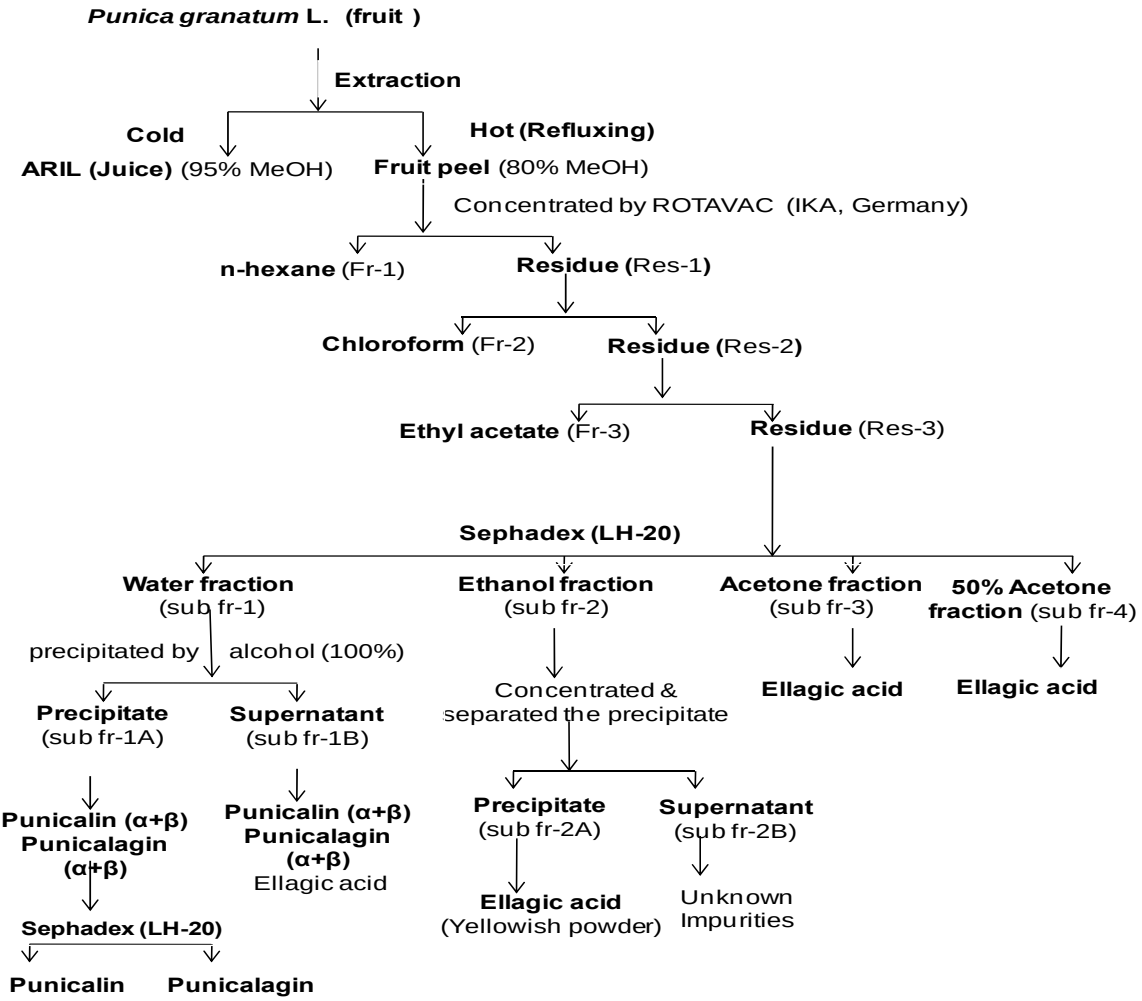
SUPPLEMENTARY FIGURE: S1



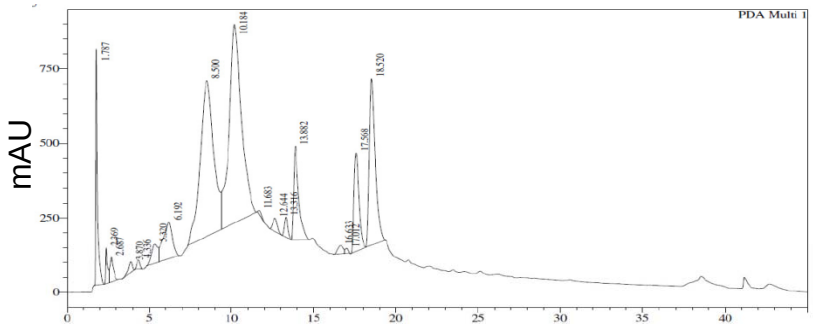
HPLC finger print of *Punica granatum* fruit peel. The analytical RP-HPLC of *P. granatum* fruit peel methanolic extract (sample) was run along with standard reference compounds punicalin, punicalagin (α+β) and ellagic acid. These compounds were identified as major constituents in extract

SUPPLEMENTARY FIGURE: S2

A

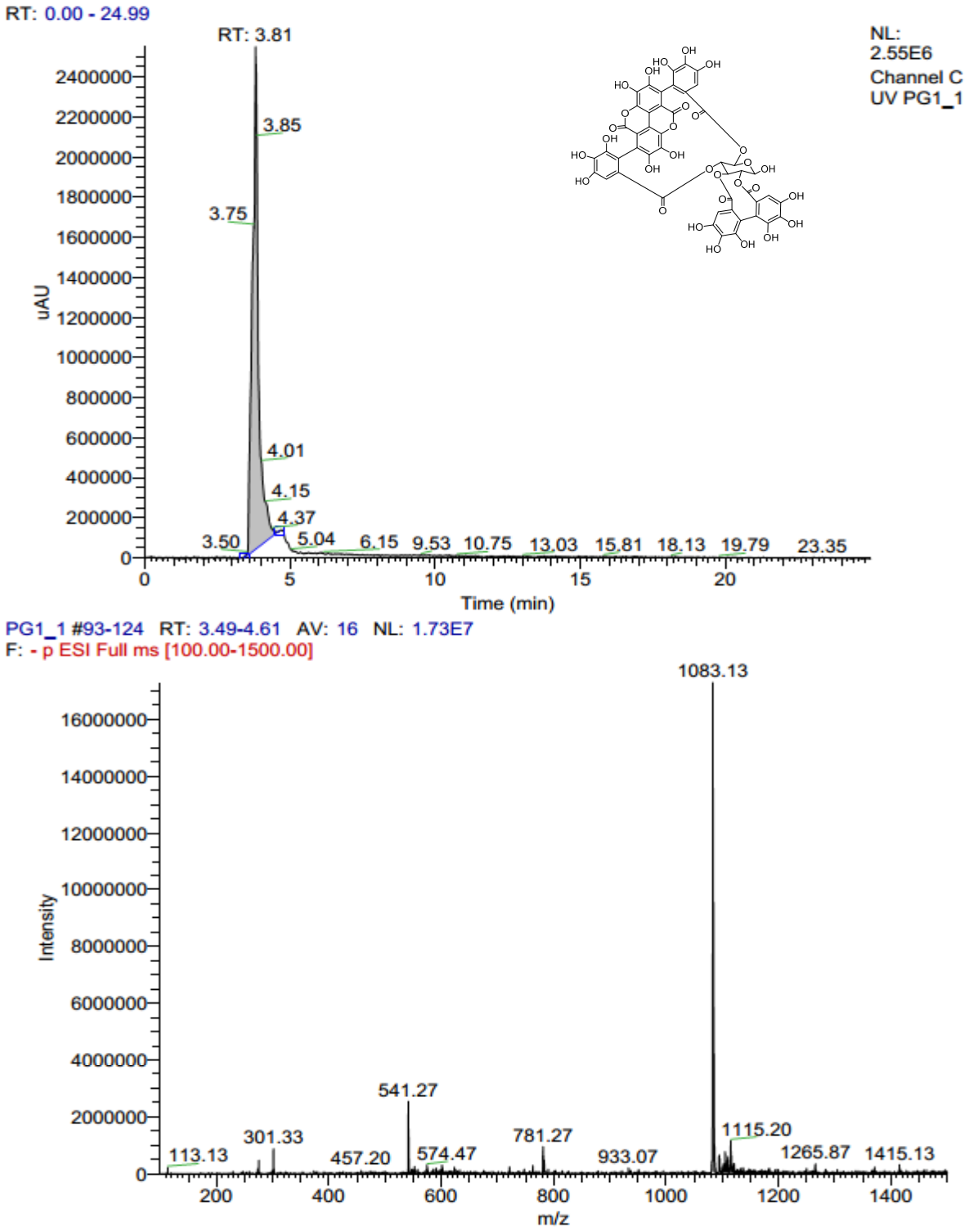


B



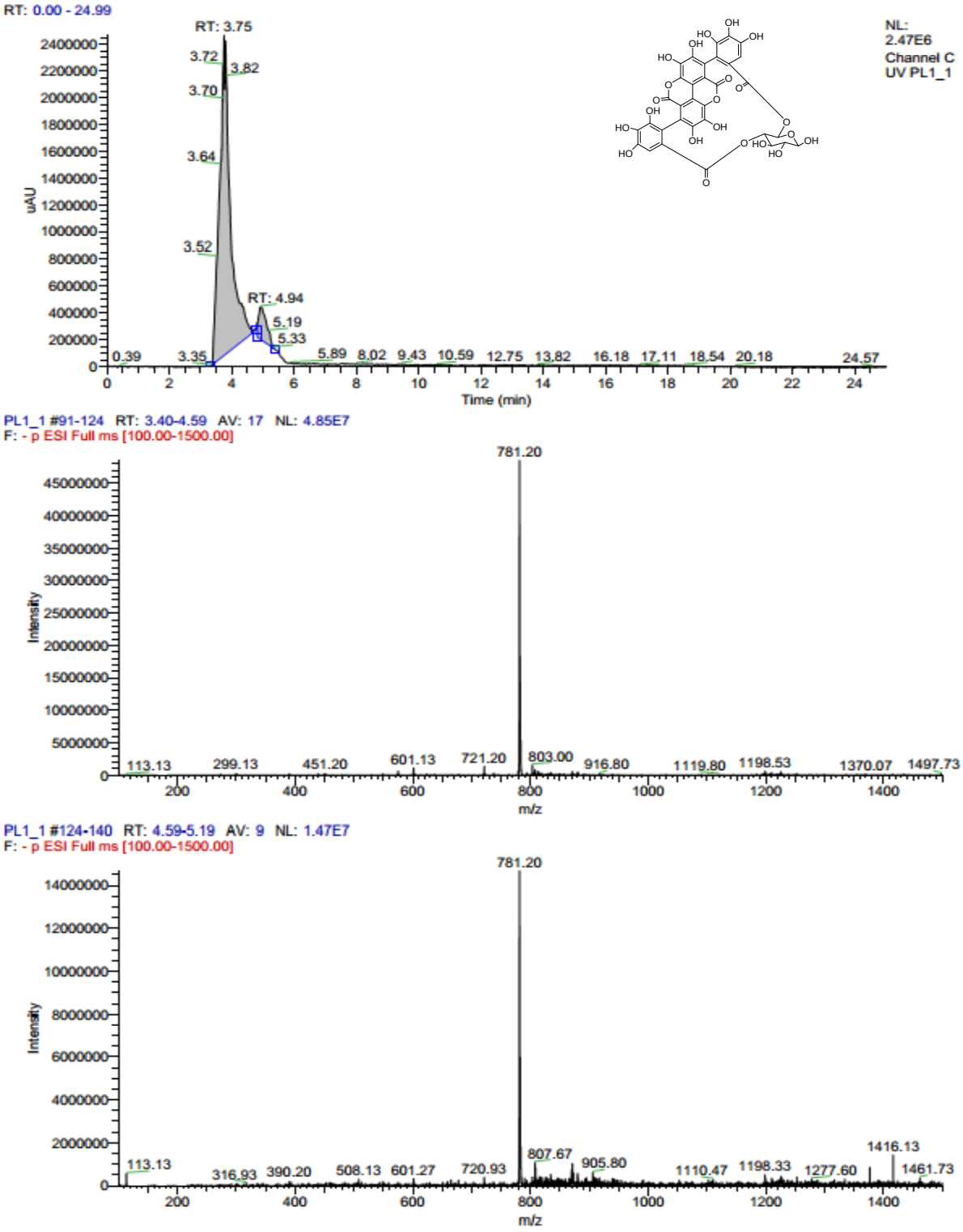
(A) Schematic of the work flow. Bioassay guided fractionation and purification of bioactive components from *P. granatum* fruit. **(B) RP-HPLC finger print of the residue -3 of *P. granatum* peel extract.** The major peaks detected in polar region (in chromatogram) of the residual fraction were identified as punicalin (20.8%, retention time 1.7 min), punicalagin α+β (13.7% & 17.4%, retention time 8.5 and 10.18 minutes) and ellagic acid (14.64%, retention time). The percentage of these compounds present in the crude fraction was calculated based on area of normalization. The identities are confirmed by the retention times of their corresponding reference standards.

SUPPLEMENTARY FIGURE: S3



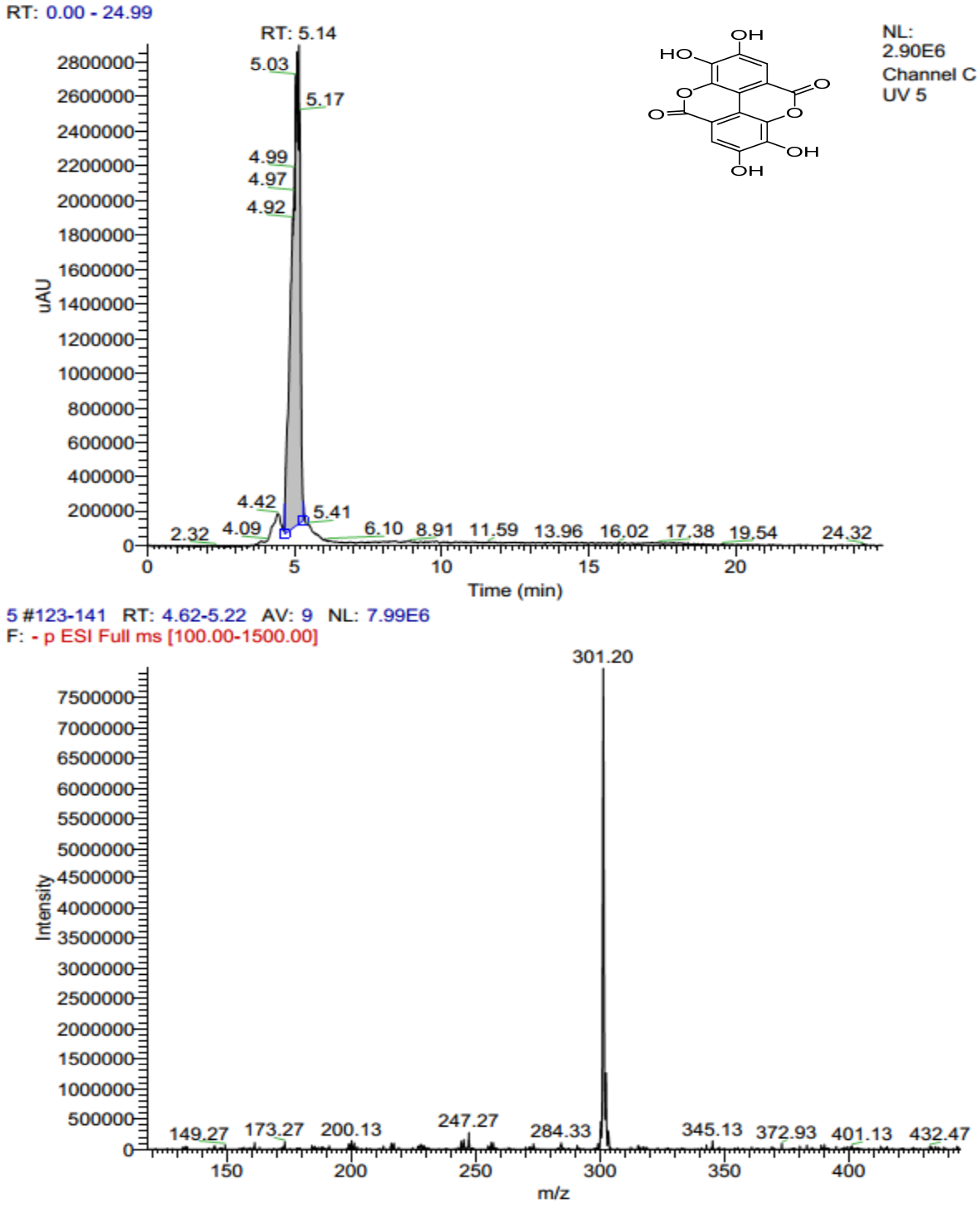
LC ESI - MS of purified punicalagin. Liquid chromatography profile of punicalagin (PGN) showing a prominent major peak at retention time 3.81 minutes. PGN signal was obtained in negative ion mode of ESI **-** MS at m/z 1083.13 $[M-H]^-$ and 541.27 $[M-2H]^-$ corresponding to its absolute mass m/z 1084.7 Daltons. Chemical structure of PGN was drawn (adopted from Cerda *et al.,* 2003) by using Chem Draw software.

SUPPLEMENTARY FIGURE: S4



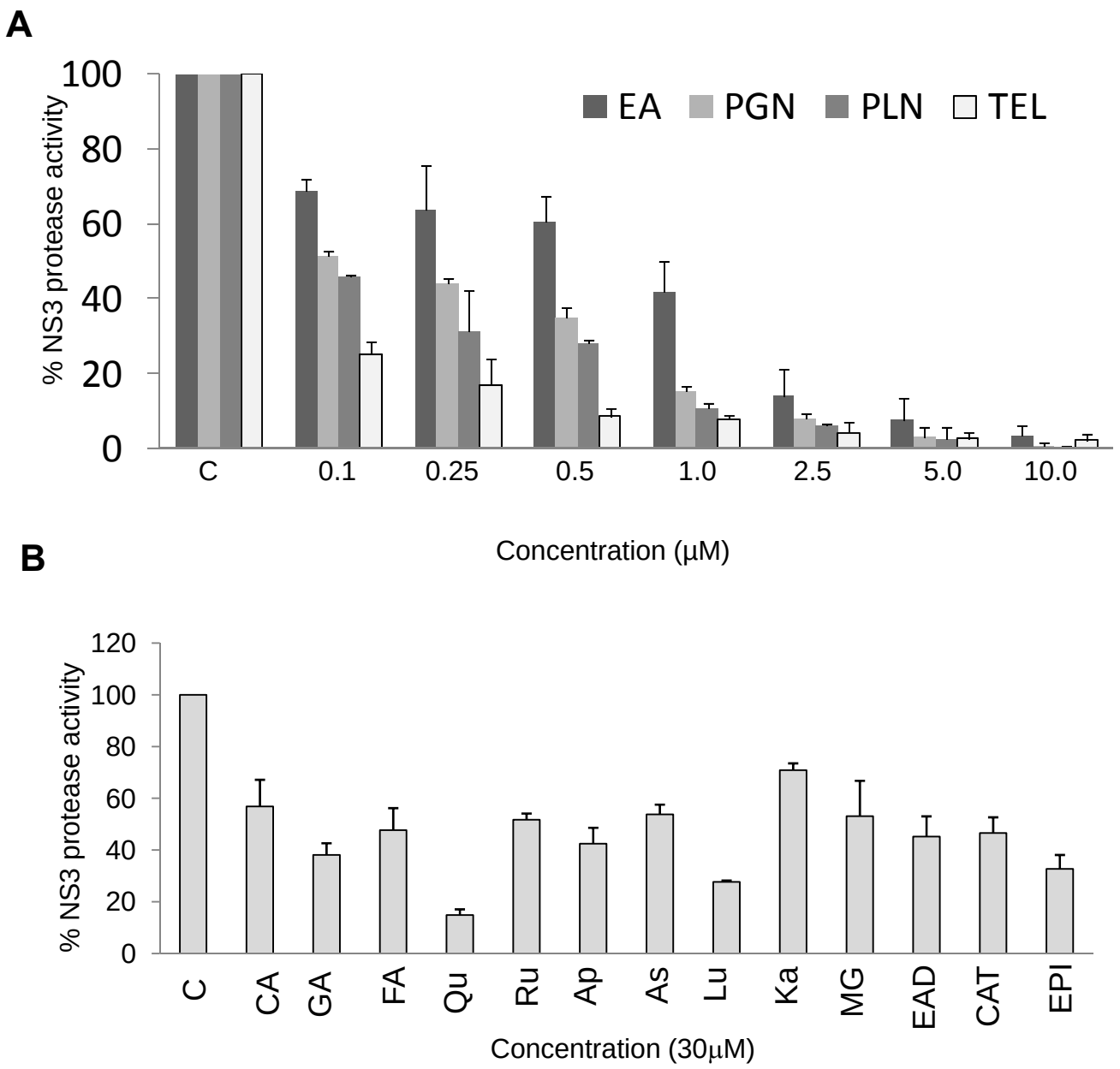
LC ESI - MS of purified punicalin Liquid chromatography profile of punicalin (α+β) showing prominent major peaks at retention time 3.75 and 4.94 minutes. Punicalin (PLN) signal was obtained in negative ion mode of ESI **-** MS at m/z 781.20 $[M-H]^-$ corresponding to its absolute mass m/z 782.5 Daltons. Chemical structure of PLN was drawn (adopted from Cerda *et al.,* 2003) by using Chem Draw software.

SUPPLEMENTARY FIGURE: S5



LC ESI - MS of purified ellagic acid. Liquid chromatography profile of ellagic acid (EA) showing a single prominent peak at retention time 5.14 minutes. EA signal obtained in negative ion mode of ESI **-** MS at m/z 301.20 $[M-H]^-$ corresponding to its absolute mass 302.197 Daltons. Chemical structure of EA was drawn (adopted from Cerda *et al.,* 2003) by using Chem Draw software.

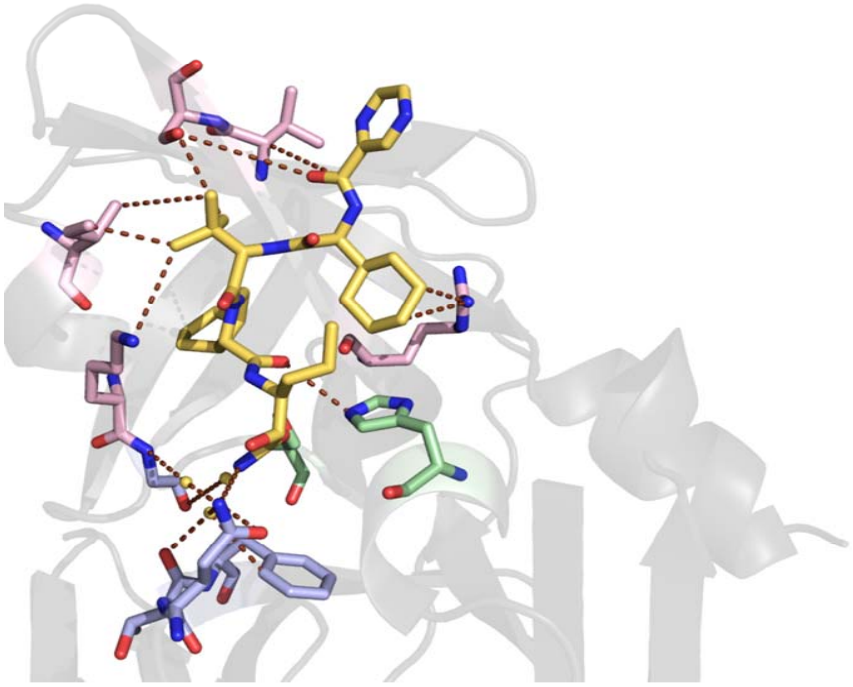
SUPPLEMENTARY FIGURE: S6



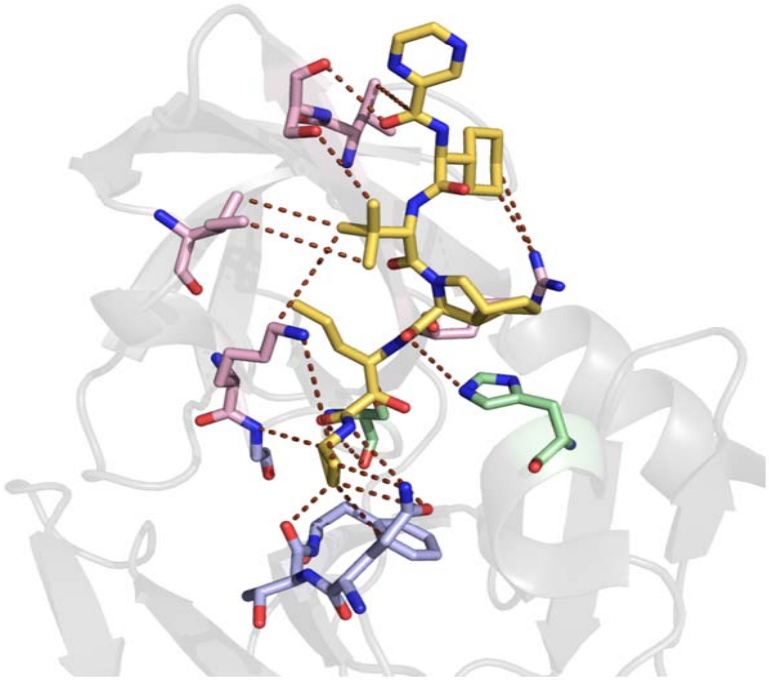
Effect of different bioactive compounds from *P. granatum* (commercially procured) against HCV NS3/4A protease activity. (A) HCV-NS3/4A protease was pre-incubated with increasing concentrations (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10.0μM) of commercially obtained EA, PGN, PLN followed by addition of EGFP-NS5A/B site-CBD fusion substrate protein. The potential of these compounds to inhibit substrate cleavage efficiency of enzyme was quantified by fluorometry. Telaprevir served as positive control. **(B)** Experiment similar to panel A, was performed with 13 more pure compounds (present in *P. granatum)* using 30μM concentration each. Vehicle control (C), caffeic acid (CA), gallic acid (GA), ferulic acid (FA), quercetin (Qu), rutin (Ru), apigenin (Ap), asiatic acid (As), luteolin (Lu), kaempferol (Ka), methyl gallate (MG), 3,3′-O-methyl ellagic acid-4′-O-β-D-xylopyranoside (EAD), catechin (CAT) and epicatechin (EPI).

SUPPLEMENTARY FIGURE: S7

A

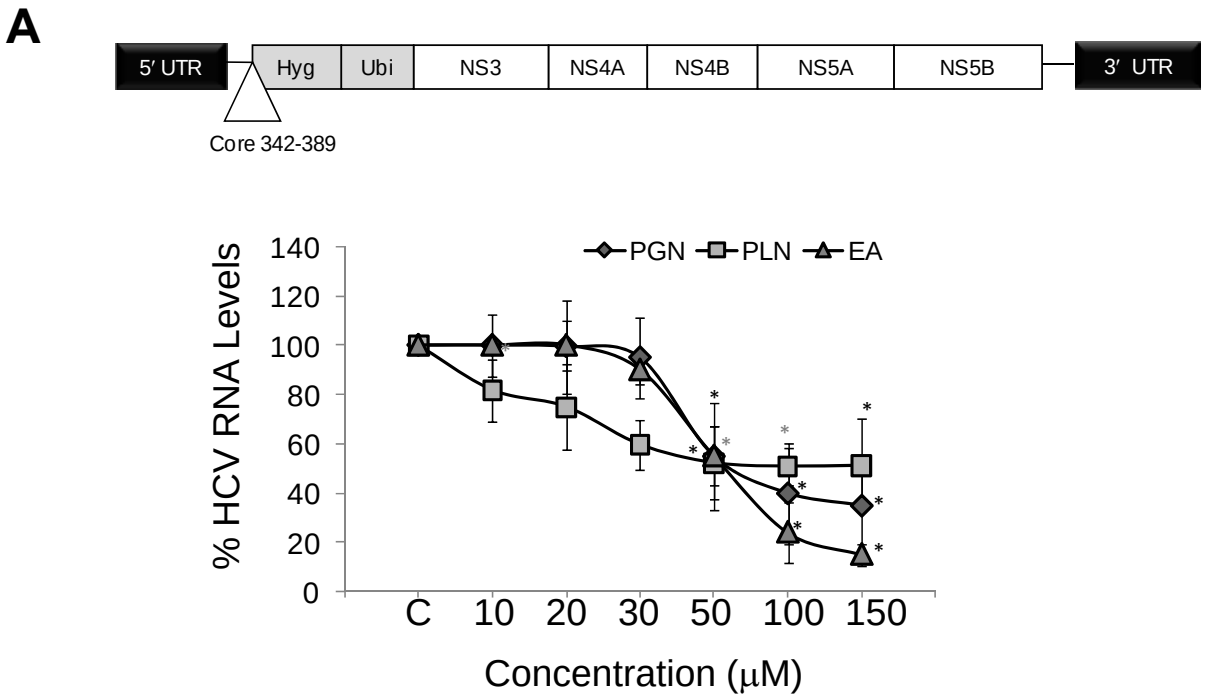


B



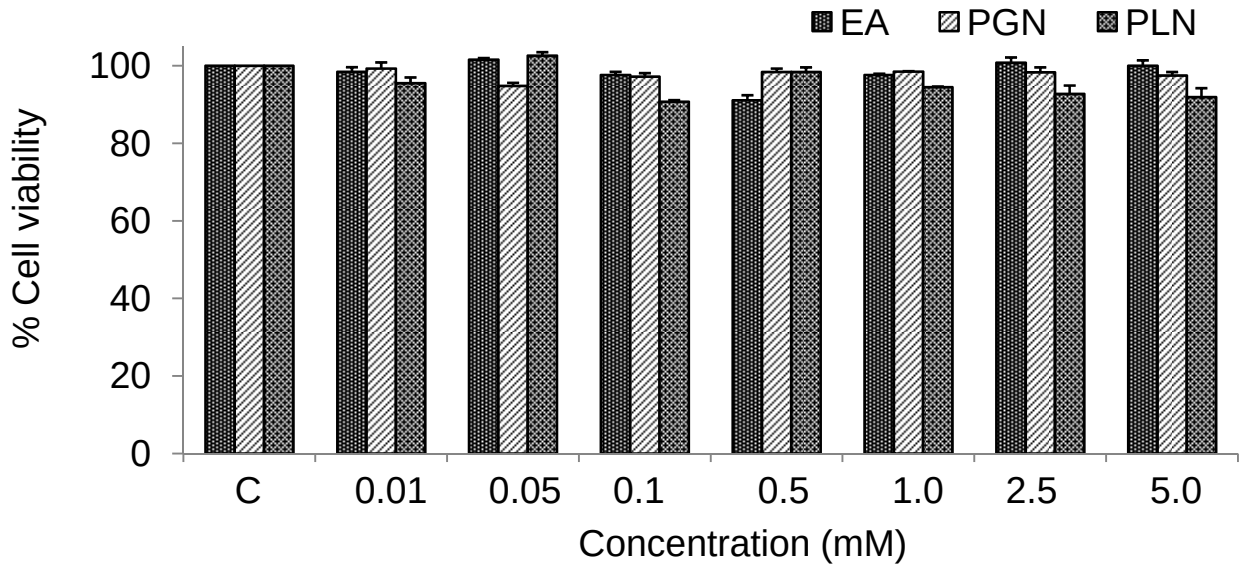
(A) and (B) Correctly predicted interactions in the modeled complex of NS3 protease bound to Telaprevir. The overlap of sixteen correctly predicted interactions in the docked NS3-Telaprevir and crystal structure of NS3-Telaprevir complex is shown in panels A and B respectively. The ligand interacting residues in NS3 protease which are catalytic residues, substrate binding residues and other ligand interacting residues are coloured in green, pink and blue respectively. Telaprevir is coloured in yellow and the protein-ligand interactions are coloured as brown dotted lines.

SUPPLEMENTARY FIGURE: S8



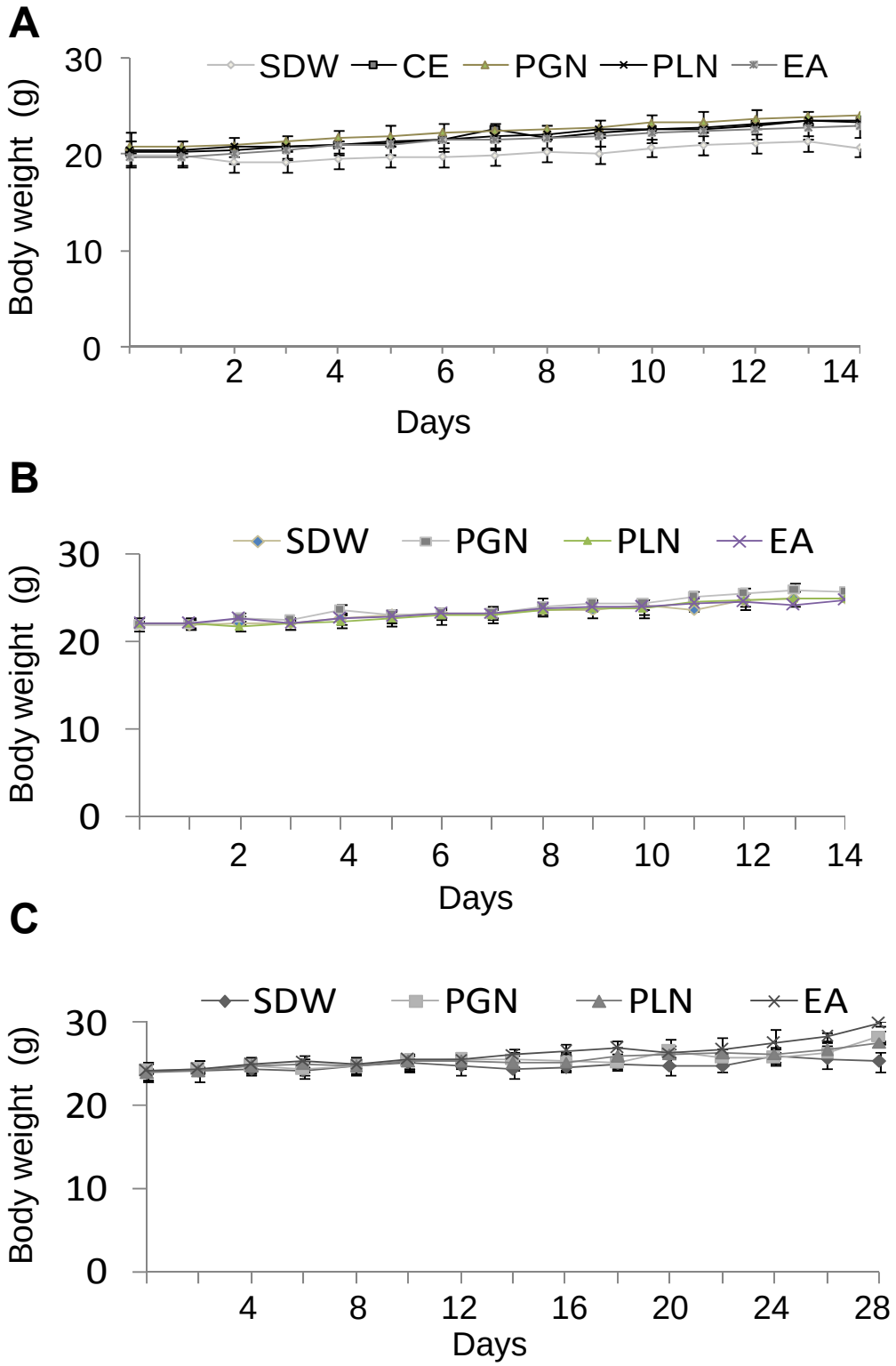
(A) Huh7 cells harboring subgenomic replicon 2a were treated with increasing concentrations (as indicated) of PLN, PGN and EA. At 48 hr post-treatment, RT-qPCR was performed to detect HCV negative strand RNA levels. Schematic above the panel represents the HCV subgenomic replicon. * denotes significant values ($P < 0.05$) **(B) Strand specific tagged RT-PCR showed decreased negative strand synthesis in presence of PGN, PLN and EA.** For experiment performed in panel A, strand specific tagged cDNA RT-PCR was also performed to specifically detect HCV negative strand. **(C) Western blot analysis** showing levels of phosphorylated STAT 1 (P-STAT 1) in JFH1 infected cells in the presence of 150 μM of PGN or PLN. IFN α (100U/ml) was used as positive control. 'C' represents negative control (no treatment) .

SUPPLEMENTARY FIGURE: S9



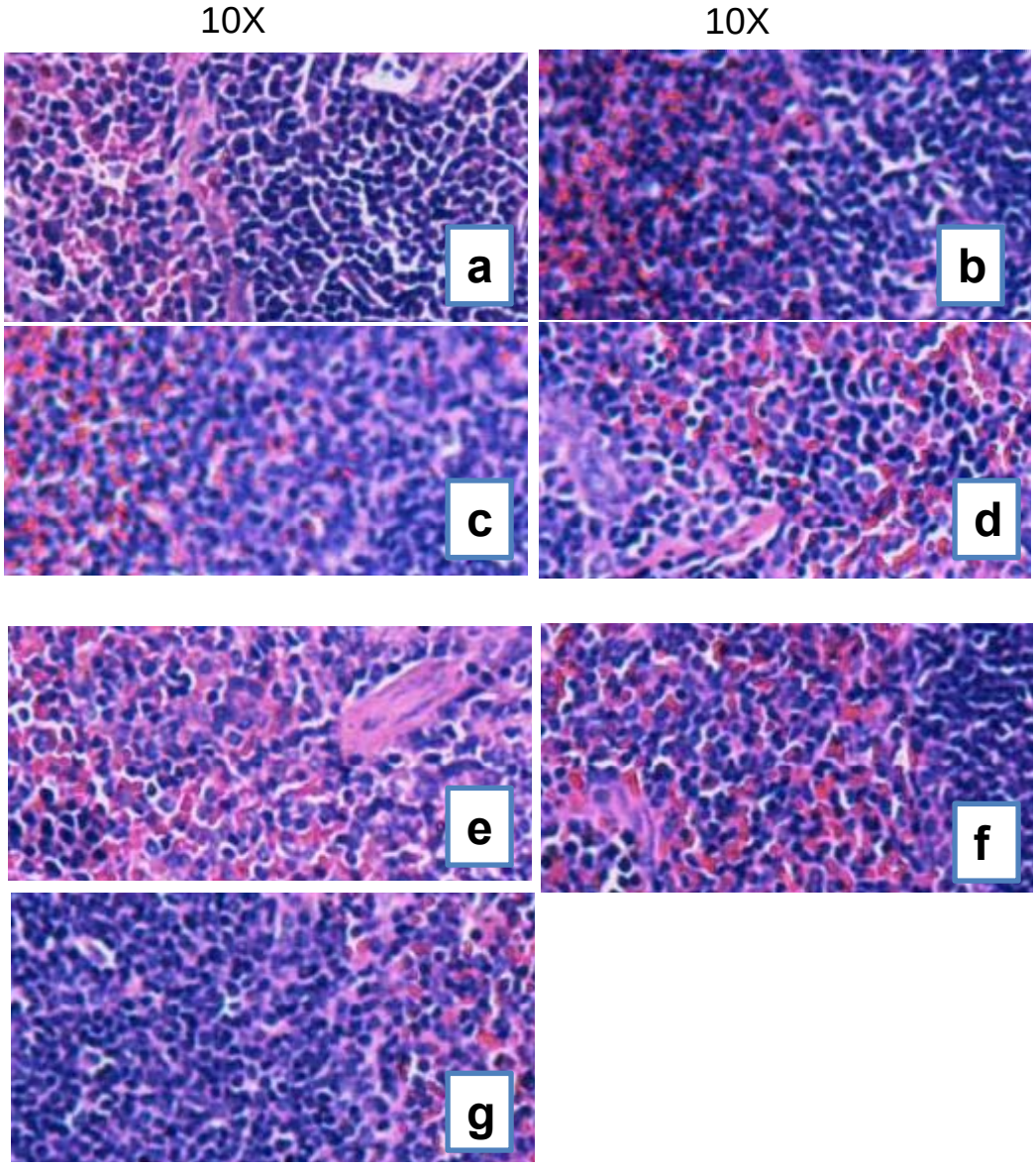
MTT assay showing the effect of increasing concentrations (0.01, 0.05, 0.1, 0.5, 1.0, 2.5, and 5.0 mM) of EA, PGN, and PLN on viability of Huh7 cells harboring HCV (2a) subgenomic replicon

SUPPLEMENTARY FIGURE: S10



Determination of body weight gain for toxicity studies: (A) An acute dose (2000 mg/kg b.wt.) of crude extract, PGN, PLN, and EA was orally administered to BALB/c mice and body weight was measured for 14 days. **(B)** Experiment similar to panel A was performed with an acute dose (5000mg/kg b.wt.) of PGN, PLN, and EA. **(C)** BALB/c mice were orally administered everyday with a dose (1000 mg/kg/day) of PGN, PLN and EA for 28 days. Body weights were taken during this period and plotted graphically. (SDW = sterile distilled water as vehicle control).

SUPPLEMENATARY FIGURE: S11



Histopathological examination of mice spleen to determine the toxicity of test compounds. BALB/c mice were orally administered with an acute dose (5000 mg/kg b.wt.) of **(b)** PGN, **(c)** PLN, and **(d)** EA. On 14 th day post treatment, spleen was taken out for histopathological analysis. 'a' denotes untreated healthy control mouse. BALB/c mice were orally administered everyday with a dose (1000 mg/kg b.wt./day) of PGN **(e)**, PLN **(f)**, and EA **(g)** for 28 days. On 28th day post treatment, spleen was studied for histopathological analysis. **(a)** represents vehicle control.