Comprehensive *In Vivo* and *In Silico* Approaches to Explore the Hepatoprotective Activity of Poncirin Against the Paracetamol Toxicity

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Abstract

Purpose: In the present study, the Poncirin was evaluated against the Paracetamol-induced liver injury using *in vivo* and computational approaches.

Methods: The Paracetamol was administered intraperitoneally to establish the liver injury in mice and subsequently, to investigate the hepatoprotective effect of the Poncirin on the liver injury. The effect of the Poncirin was evaluated against the liver injury markers and inflammatory cytokines. Similarly, in the present study the antioxidants and oxidative stress parameters were also assessed following paracetamol-induced liver injury. The histological studies following liver injury was also assessed using H and E staining, Masson's trichrome staining, and periodic acid Schiff staining.

Results: The Poncirin markedly improved the antioxidant enzymes, while attenuated the oxidative stress markers and inflammatory cytokines. The Poncirin also markedly improved hematological parameters and electrolytes profile. Furthermore, the Poncirin treatment significantly improved the histological parameters using H and E staining, Masson's trichrome and PAS staining compared to the control. The Poncirin treatment also improved the liver function tests and liver synthetic activity compared to Paracetamol treated group. The immunohistochemistry analysis revealed significant decrease in the inflammatory signaling protein such as NF- κ B (nuclear factor kappa light chain enhancer of activated B cells), JNK (Jun N-terminal Kinase) and COX-2 (Cyclooxygenase-2) expression level compared to the Paracetamol treated group. The *in silico* approach was also used to analyze the potential activity and explore the underlying molecular mechanism of Poncirin.

Conclusion: The Poncirin improved the sign and symptoms associated with the liver injury using both *in vivo* and computational approaches.

Introduction

The Paracetamol is widely used antipyretic and considered usually safe at therapeutic doses i.e. the dose up to 4 gram per day is well tolerated, however, greater than recommended dose can trigger hepatotoxicity (Pradhan and Girish 2006). The Paracetamol is metabolized primarily conjugation reaction such as glucuronidation and sulphonation, while 30–55 % of the Paracetamol is excreted in urine as conjugated form (Jóźwiak-Bebenista and Nowak 2014). The small amount of Paracetamol is metabolized microsomal cytochrome p450 enzymes system and result into the formation of the N-acetyl-p-benzoquinone (NAPQI) electrophilic intermediate which is inactivated by the glutathione (Jóźwiak-Bebenista and Nowak 2014). However, at toxic doses the cytochrome p450 system and glutathione is saturated with resultant accumulation of the NAPQI and subsequent binding with the cellular protein as well as necrosis. These pathological changes results into jaundice, decreased synthetic capacity, bilirubin accumulation and fulminant hepatic dysfunction (Ullah Khan et al. 2018; Khan et al. 2020). These pathological changes might also involve the generation of reactive oxygen and nitrogen species (RONS)

which further results into the damage of the cellular component and alteration of cellular hemostasis (Ullah et al. 2018).

The ROS are generated as byproducts, which at physiological concentrations are required for regular cellular functions like transcriptional activation (Rasheed et al. 2018). However, excessive generation or inadequate clearance leads to imbalance between production and neutralization of ROS causing oxidative damage of biomolecules i.e. lipids, DNA and cellular proteins (Phull et al. 2017; Khan et al. 2020). Cumulative effects of excessive ROS production can be manifested by variety of injurious events like hepatotoxicity (Lukaszewicz-Hussain and Moniuszko-Jakoniuk 2004; Khan et al. 2020). The reactive free radical induce the production of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α , which further exacerbate the underlying condition (Rømsing et al. 2002; Ali et al. 2019). These pro-inflammatory cytokines are induced by the activation of upstream signaling mechanism such as MAPKs and NF- κ B, which interact with the intermediate signaling protein and transcriptional factors such as AP-1 (Rasheed et al. 2018; Khan et al. 2020). The body is armed with the endogenous antioxidant defense mechanism such as Nrf2 signaling, which are induced during the oxidative stress and induce the expression of the related antioxidants such as HO-1, SOD, Catalase, GSH and GST (Ali et al. 2019). Plant derived natural antioxidants like polyphenols and flavonoids have been widely reported for their free radical scavenging activity (Khalid et al. 2018). Natural products are of prime importance which are used for the treatment of many disease including inflammation, cancer, arthritis etc (Khalid et al. 2018; Sana et al. 2021). Similarly, the Poncirin is natural compound obtained from the immature fruit of *Poncirus trifoliata* and has been reported for various biological activities (Khalid et al. 2018). Based on the previously reported biological activities, the present study was aimed to investigate the hepatoprotective activity of the Poncirin against the Paracetamol-induced liver injury using *in vivo* and *in silico* analysis (Khalid et al. 2018).

Materials And Methods

Chemicals and Reagents

The various chemical and reagents used in the present study includes Paracetamol (Sigma–Aldrich, Germany), ketamine (Sigma–Aldrich, Germany), formalin (Sigma–Aldrich, Germany), Xylazine (Sigma–Aldrich, Germany), (Sigma–Aldrich, Germany) and Poncirin (Sigma–Aldrich, Germany). ELISA kits for the determination of TNF- α , IL-1 β and IL-6 were purchased from the Thermo Fisher Scientific (Thermo Fisher Scientific, USA). All the chemical used in the present study were of analytical grades and diluted with normal saline 0.9 % (2 % DMSO). Neubauer hemocytometer (Feinoptik, Germany) was used for hematological analysis, while Hemoglobin (Hb) content was determined by Sahli's haemoglobin meter. Liver function tests (LFTs) were analyzed by AMP diagnostic kits (Graz, Austria) according to manufacturer's instructions. The primary and secondary antibodies were obtained from the Santa Cruz (Santa Cruz Biotechnology, Inc).

Animals

The animals (Male Albino BALB/c, age 8-9 weeks and weighing 28-31 gm) were obtained from the National Institute of Health (NIH, Islamabad). All the animal activities were performed in the department of pharmacy (pathogen free zone), Quaid-i-Azam University, Islamabad (Pakistan) and the study was approved by the animal ethical committee of Quaid-i-Azam University, Islamabad (Pakistan) (No. #BEC-FBS-QAU2018-90). The experimental animals were housed under standard conditions such as temperature 23 ± 0.5 °C and humidity of 50 ± 5 % with 12 h light-dark cycles. "Principles of Lab Animals Care" from NIH publication were followed in all laboratory procedures (Zimmermann 1983). The behavioral activities were performed from 7.30 a.m. to 7.30 p.m. and animals were used once during the experiment. The number of animals were used less to avoid unnecessary harm and discomfort to the animals.

Grouping of animals

Animals were classified into six groups (n=8) such as normal control (normal saline with 2% DMSO), Paracetamol treated 640 mg/kg, Silymarin 50 mg/kg (dissolved in normal saline and 2 % DMSO) and three groups of Poncirin (5, 15, 30 mg/kg (dissolved in normal saline and 2 % DMSO)). The dose of the Poncirin was selected based on the previously reported study (Afridi et al. 2019; Khan et al. 2020; Ullah et al. 2020). All the drugs (Paracetamol, Silymarin and Poncirin) were administered via intraperitoneal (i.p.) route. The animals were pre-treated with the Poncirin and Silymarin before the induction of the Paracetamol-induced hepatic injury.

Body weight and Liver weight variation

The changes in the body weight of all the treated groups were assessed before (day 0) and after (day 8) the disease induction. The animals were weighted using digital electronic weighing balance and their respective weights were noted, while the changes in the body weights were during the course of the study were analyzed as reported previously (Afridi; et al. 2019). Similarly, the liver weight variation was performed to determine the in the changes in the liver weight variation following Paracetamol-induced liver injury and to observe the effect of the Poncirin treatment on the hepatic changes. The liver weight variations were determined as reported previously (Afridi et al. 2019; Ullah et al. 2020).

Food assessment

The food intake was assessed in all the treated groups before the commencement of disease induction and every day after the diseases induction (Afridi et al. 2019). The food was weighted, placed for each group and 24 h latter the amount consumed as well as the quantity of the feed used was calculated as described previously. The amount of the feed consumed in all the treated groups were compared (Afridi et al. 2019, Ullah et al. 2020).

Survival Rate

The Kaplan-Maier analysis was used for the survival in all the treated groups following Paracetamol-induced liver injury. The effect Poncirin on the survival rate was evaluated daily for 8 days as reported

previously (Afridi et al. 2019; Ullah et al. 2020).

Collection of Blood Samples and Organ

At the end of experiment the animals were euthanized with cervical dislocation as reported previously (Khan et al. 2019). Following cervical dislocation, the organs were removed carefully, rinsed, and washed with distilled water and placed in ice cold solution. For further analysis such as histological and biochemical analysis the tissues were processed according to the previously described methods (Khan et al. 2019; Ullah et al. 2020).

Hematological Studies

The hematological analysis was performed to assess the changes in the blood composition following induction of the Paracetamol-induced liver injury in all the treated groups. The blood was withdrawn directly from the cardiac puncture carefully, placed in EDTA tubes and analyzed as reported (Khan et al. 2019).

Serum Analysis

In order to separate the serum from the cellular components, the blood was centrifuged at 6000 rpm at 4°C for 10 min. The serum analysis such as alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate transaminase (AST) and bilirubin were analyzed in triplicate as reported previously (Ullah et al. 2020).

NO (Nitric Oxide) determination

The NO assay was performed using Griess reagent method and the effect of the Poncirin (5, 15, 30 mg/kg, i.p) was evaluated (Atiq, Shal et al. 2019). The NO concentration was determined in both the liver tissue and plasma in all the treated groups. The blood was collected directly from the cardiac puncture and centrifuged at (5000 rpm for 10 min at 4 °C) to separate the plasma from the cellular contents as reported previously (Khan et al. 2019).

Histopathological study of liver tissues

The histological changes in the liver tissue following induction of injury with the Paracetamol was evaluated using H and E staining (Atiq et al. 2019). The H and E staining was performed via previously reported method. The histological changes were quantified as normal, moderate and severe based on the hepatic cytoplasm inflammation, centrilobular necrosis, cellular hypertrophy, vacuolization and steatosis (Keppler et al. 2007). The histopathological changes were quantified by two independent histopathologist and scored double blindly (Kazmi et al. 2018). The Masson's trichrome staining was performed to assess the changes in the liver parenchyma following Paracetamol-induced liver injury (Khan et al. 2020). The left lateral lobe was selected and sliced. The tissue were fixed in formalin and subjected to the Masson's trichrome staining. The histological changes were quantified double blindly by the histopathologist as

reported previously (Khan et al. 2020). The fibrosis score was calculated by selected minimum of 10 fields per slice and their mean was calculated. The extent of the fibrosis was graded from 0-4, while the 0 means no fibrosis and 4 indicates maximum fibrosis. The Periodic acid-Schiff (PAS) staining was performed to determine the glycogen accumulation in the liver tissue following Paracetamol-induced liver injury (Khan et al. 2020). The PAS staining was performed according to the previously reported method. The changes in the PAS staining were quantified ranging from normal to inflammation and even cirrhosis (Khan et al. 2020).

Effect of Poncirin on antioxidant and oxidative stress markers

The antioxidants and oxidative stress balance is significantly altered following Paracetamol-induced liver injury (Atiq et al. 2019). The various antioxidants such as GST, GSH, Catalase and SOD, while the oxidative stress marker such as MDA were analyzed and the effect of the Poncirin was evaluated as reported previously (Khalid et al. 2018).

Myeloperoxidase (MPO) activity

The MPO serves as a marker of the neutrophilic infiltration and neutrophils are the first cells to respond against the inflammatory insult (Shal et al. 2019). The neutrophilic infiltration into the liver following Paracetamol-induced liver injury was evaluated and the effect of the Poncirin was investigated. The MPO activity was determined in all the treated groups and the results were compared (Atiq et al. 2019).

Measurement of IL-1 β , IL-6 and TNF- α production

The cytokines production in liver tissue following Paracetamol-induced liver injury were determined according to the previously described method (Shal et al. 2019; Zeeshan et al. 2019). The various cytokines that are commonly implicated in the numerous inflammatory disorders such as IL-1 β , IL-6 and TNF- α were studied and the effect of the Poncirin was determined. At Day 8 of Paracetamol administration (i.p.), the liver tissue was removed, and their proteins were extracted from 100 mg tissue/ml PBS to which 0.4 M NaCl, 0.05% Tween 20, and protease inhibitors were added. The liver tissues were homogenized and centrifuged for 10 min at 3,000 g, and the supernatant was frozen at -80 °C for later quantification. The TNF- α , IL-1 β , and IL-6 production was calculated using a commercially available TNF- α , IL-1 β and IL-6 ELISA kit (eBioscience, Inc., San Diego, CA) (Rasheed et al. 2018).

Immunohistochemistry analysis

The immunohistochemistry analysis was performed to assess the changes in the expression level of the various inflammatory signaling proteins such as NF- κ B, JNK and COX-2 following Paracetamol-induced liver injury (Rasheed et al. 2018). The paraffin embedded tissues were treated with xylene, alcohol (graded wise), normal goat serum (NGS), primary antibodies and placed overnight. The next day primary antibodies were washed, while treated with secondary antibodies, avidin-biotin complex and stained with the DAB reagent as reported (Rasheed et al. 2018).

Prediction of target genes associated with Paracetamol-induced liver injury and genes target for Poncirin

The genes associated with the Paracetamol-induced liver injury from the PUBMED database (National center for biotechnology information (<https://www.ncbi.nlm.nih.gov/>)) with search query of “Paracetamol-induced liver injury” and humans (Xinqiang et al. 2020). A total of 434 genes were retrieved from the GeneBank search engine which were implicated in the pathogenesis of the Paracetamol-induced liver injury and filtered for Homo sapiens. Similarly, to retrieve the genes list related with the Poncirin various search engines were utilized such as Comparative toxicogenomics database (<http://ctdbase.org/>), Therapeutic targets database (<http://bidd.nus.edu.sg/group/cjttd/>) and traditional chines medicines system pharmacology database and an-alysis platform (<http://lsp.nwu.edu.cn/index.php>) as reported previously (Xinqiang et al. 2020). The Comparative toxicogenomics database was used to assess the gene interacting with the Poncirin. These interacting genes were plotted in the form of the cluster using String online software (<https://string-db.org/>). The Comparative toxicogenomics database using disease query to assess the interaction of the genes associated with the Poncirin (Xinqiang et al. 2020). Similarly, the genes that are related with the Poncirin were also assessed and the implication in other disease based on the similarity were analyzed (Shal et al. 2020; Xinqiang et al. 2020). Similarly, the Funrich analysis was performed for the functional enrichment and protein-protein interactions. Furthermore, the cellular component, transcription factors and molecular function based on the P value using Funrich enrichment analysis on the genes retrieved from Comparative toxicogenomics database (Xinqiang et al. 2020; Naveed et al. 2021).

Construction of the target gene network

The PUBMED database was used to retrieve the genes associated with the Paracetamol-induced liver injury and imported to the Cytoscape 3.7.1 software to create a network based on their interaction (Xinqiang et al. 2020). Furthermore, gene ontology enrichment analysis was performed to identify the interaction and to assess the systemic involvement of the concern genes. The 0.90 score was set as confidence level for minimal interaction (Ali et al. 2020; Xinqiang et al. 2020).

Molecular docking and active site prediction

The molecular docking analysis was performed using Autoock vina 4.2 software. The crystal structure of respective proteins were downloaded from the Protein Data Bank (<http://www.pdb.org/>) and saved as PDB file (Naveed et al. 2019; Khan et al. 2021). The protein were prepared i.e. energy was minimized, water molecules were removed, co-crystalized ligand were removed and hydrogens were added using Swiss PDB viewer and discovery studio visualizer_16 (Khan et al. 2020). The active site of the proteins were analyzed using CASTp online software and the amino acids within the active pockets were assessed within the chain of interest (Khan et al. 2020). The ligand (Poncirin) was prepared using ChemBioDraw_14, minimized and saved as SDF file. The ligand was then converted to PDB file using discovery studio visualizer_16 (Khan et al. 2020; Shal et al. 2021). The Autodock vina was used to assess the binding interaction of ligand with the proteins. The ligand and protein were converted to PDBQT, Grid parameter files and configuration file was generated (Khan et al. 2020; Xinqiang et al. 2020). Finally, the

vina was used to assess the binding energy of the ligand with the protein targets. The results were visualized using discovery studio visualizer_16, while the ligand-protein complex 3D and 2D images were saved (Khan et al. 2020). The binding energies of the ligand-protein complex and mode of the interaction i.e. hydrogen bonding, van der Waals, salt bridges, π - π interaction, π -sigma bond, and many other hydrophobic interactions were shown. The number of hydrogen bonds and the maximum negative energies of the ligand-protein complex indicate stable complex (Naveed et al. 2019). Similarly, the ligand interaction was evaluated against the protein binding site of the DNA i.e. c-fos binding site of the DNA.

Validation of Molecular docking

The Molecular docking analysis was validated by re-docking the ligand following extraction from the protein target and RMSD value was calculated (Khan et al. 2020). The model is considered valid if the root mean square deviation is less than 2 Å. The ligRMSD software was used to determine the RMSD value of the docked ligand from the re-docked ligand and superimposed ligand were shown using discovery studio visualizer_16 (Khan et al. 2020).

Molecular Dynamics Simulation and Analysis

The AMBER20 simulation package was utilized to accomplish molecular dynamics simulation of Poncirin-1vkx complex. The protein parameters were generated using ff14SB whereas general Amber force field was applied assign Poncirin parameters (Khan et al. 2020). The complex was solvated explicitly in TIP3P water box where counter ions were added to achieve charge neutralization. The complex was forwarded to energy minimization phase and subjected to 2500 rounds of steepest descent and conjugate gradients algorithms. Afterward, the complex was equilibrated via 1000 ps of heating and density equilibration in the presence of weak restraints. Pressure equilibration was achieved for 2 ns at temperature of 300 K. The production run was performed for 100 ns using NPT ensemble under periodic boundary conditions. Particle mesh Ewald method was considered to explain electrostatic interaction setting the cut-off value of 10 Å. The hydrogen bonds were constrained using SHAKE algorithm to keep the bond length at equilibrium. To evaluate complex structural stability, CPPTRAJ module was used (Khan et al. 2020).

Estimating MM-GBSA Binding Free Energies

Molecular mechanics energies combined with the generalized Born surface area (MM-GBSA) was run further to estimate binding free energies of the complex. Five hundred snapshots at regular intervals were selected and MM-GBSA analysis was performed as performed previously (Khan et al. 2020).

In silico pharmacokinetic analysis and toxicokinetic analysis

The pharmacokinetic analysis of the Poncirin was performed using Swiss ADME (absorption, distribution, metabolism, and elimination) software. The various parameters that were studied includes physicochemical properties, lipophilicity, water solubility, pharmacokinetics, drug likeness and medicinal chemistry aspects (Atiq et al. 2019, Xinqiang et al. 2020). Furthermore, the preADMET software utilized

to assess the potential metabolites and the metabolites were predicted based on their probability such as Rank1, Rank2, Rank3 etc (Atiq et al. 2019). The aim of the *in silico* toxicity assessment was to determine the untoward effect of the Poncirin exposure against an organism using known route of administration such as oral, subcutaneous, intravenous, inhalation and inhalation (Atiq et al. 2019). The LD50 for the IP, oral, sub-cutaneous and IV route were computationally assessed, while the training set of the previously compounds were screened against the Poncirin based on the toxicity (Xinqiang et al. 2020). Furthermore, based on the GUSAR online software prediction, the Poncirin toxicity against the rodents was calculated. Furthermore, the Poncirin was assessed for further classification according to the Organization for Economic Co-operation and Development (OECD) chemical classification (Xinqiang et al. 2020). Based on the search query the software predicts the nature of the compound and gives the class of the compounds. Similarly, the PASS online software based approach was used to assess the potential activity of Poncirin and the activity was based on the probability of being active (pa) to the probability of being in active (pi) as reported (Xinqiang et al. 2020).

Statistical analysis

The data was analyzed as Mean $\pm$ S.D using SPSS version _18. The difference between different groups in the present study was determined using one way analysis (ANOVA) followed by Dunnett's t test. P value less than 0.05 was chosen as statistically significant. The Graphpad Prism version_5 was used for the plotting of data.

Results

Body weight variation, liver weight variation, survival rate and food intake

The Poncirin effect was evaluated on the various parameters such as body weight variation, liver weight variation, survival rate and food intake following Paracetamol-induced liver injury. The Poncirin administration at the dose of the 30 mg/kg showed improvement in the food intake compared to the only Paracetamol treated group Fig. 1. Similarly, the effect of the Poncirin was analyzed on the liver weight variation following disease induction. The Poncirin administration dose dependently reduced the liver weight variation and the dose of the 30 mg/kg showed maximum response Fig. 1. The Kaplan-Maier survival analysis showed no significant statistical changes in the mortality rate as shown in the Fig. 1. Additionally, the body weight changes were analyzed in all the treated groups following disease induction and the changes were analyzed during the course of the disease. The body weights before the disease induction and at the end of the disease were assessed. There were no significant changes in the body weight of all the treated groups as shown in the Fig. 1.

Effect of the Poncirin on liver function markers and hematological parameters

The changes in the liver function markers following Paracetamol-induced liver injury were studied and the effect of the Poncirin was assessed. The Paracetamol markedly increased the liver function tests markers such as ALT, AST, ALP, Serum bilirubin, Serum albumin and total protein. However, the Poncirin administration markedly attenuated the serum markers compared to the only Paracetamol treated groups as shown in the Fig. 2. Similarly, the Poncirin treatment markedly improved the hematological parameters compared to Paracetamol treated group as shown in Table 1.

Table 1

Effect of the Poncirin treatment on the hematological changes following Paracetamol-induced liver injury.

Sample	WBC (10 ⁹ /L)	NEU (10 ⁹ /L)	MON (10 ⁹ /L)	RBC (10 ¹² /L)	HB (g/dL)
Normal control	2.23 ± 0.42	3.19 ± 0.10	0.5 ± 0.10	13.1 ± 3.45	12.89 ± 0.34
Paracetamol	10.1 ± 0.48 ^{###}	9.89 ± 0.51 ^{###}	1.1 ± 0.3 ^{###}	8.5 ± 2.12 ^{###}	10.0 ± 0.04 ^{###}
Silymarin 50 mg/kg	3.27 ± 0.21 ^{***}	3.91 ± 0.06 ^{***}	0.6 ± 0.09 ^{**}	12.56 ± 5.64 ^{**}	12.38 ± 0.01 ^{**}
Poncirin 5 mg/kg	8.12 ± 0.01	6.12 ± 0.21 [*]	0.89 ± 0.07	11.43 ± 3.18	10.5 ± 0.03
Poncirin 15 mg/kg	6.51 ± 0.01 [*]	4.71 ± 0.03 ^{**}	0.70 ± 0.12	11.9 ± 7.81 [*]	11.53 ± 0.03 [*]
Poncirin 30 mg/kg	5.12 ± 0.38 ^{**}	3.5 ± 0.04 ^{***}	0.58 ± 0.01 ^{**}	12.89 ± 4.91 ^{**}	12.4 ± 0.01 ^{**}
All values are expressed as mean ± S.D (n = 8). ###P < 0.05 compared with vehicle control group *P < 0.05, **P < 0.01 and ***P < 0.001 compared with negative control group.					

Effect Of Poncirin On The Histopathological Parameters

The effect of the Poncirin on the Paracetamol-induced histological changes on the liver tissue were assessed using H and E staining. The Paracetamol administration markedly altered the liver architecture and shown significant pathological changes in the liver parenchyma. However, the Poncirin treatment markedly improved the histological parameters dose dependently compared to the only Paracetamol treated groups as shown in Fig. 3.

Effect of the Poncirin treatment on the Massons's trichrome and PAS staining

The Paracetamol treatment showed marked increase in the fibrosis of the liver. However, the Poncirin (5 mg/kg, 15 mg/kg, 30 mg/kg) administration dose dependently protected the liver from the fibrosis compared to the Paracetamol treated group as shown in the Fig. 4. Similarly, the PAS staining showed

marked elevation in the glycogen content of the Paracetamol treated group, however, the Poncirin treated group showed significant improvement in the glycogen content as evident from the Fig. 5.

Effect Of The Poncirin On The Antioxidants

The antioxidants such as GST, GSH, Catalase and SOD were significantly compromised following Paracetamol-induced liver injury. The Poncirin treatment markedly enhanced the antioxidants compared to the Paracetamol treated groups. Similarly, the Silymarin treated groups also enhanced the antioxidants level and showed promising protection as shown in Fig. 6.

Effect of the Poncirin on the NO, LPO and MPO

The Poncirin was evaluated against the NO production in both plasma and liver homogenate following Paracetamol-induced liver injury. The NO production was significantly attenuated in Poncirin treated groups and the response was dose dependently Fig. 6. The LPO assay was performed to assess the effect of the Poncirin on the Malonaldehyde (MDA) production. The Poncirin treatment significantly reduced the level of MDA in dose dependent fashion and the dose of the 30 mg/kg showed maximum response as shown in the Fig. 5. Similarly, the MPO activity was performed to assess the effect of the Poncirin on the neutrophilic infiltration marker. The Paracetamol treated group showed sharp increase in the MPO activity, however, the Poncirin treatment markedly neutralized the MPO activity compared to control as shown in the Fig. 6.

Effect Of The Poncirin Treatment On The Inflammatory Cytokines

The effect of the Poncirin treatment on the pro-inflammatory cytokines following establishing Paracetamol-induced liver injury. The Paracetamol administration markedly enhanced production of the inflammatory mediators such as IL-1 β , IL-6 and TNF- α using ELISA assay. The Poncirin treatment significantly attenuated the production of the cytokines compared to the Paracetamol-induced group as shown in Fig. 7.

Immunohistochemistry Analysis

The Poncirin treatment showed marked reduction in the expression level of the NF- κ B, JNK, and COX-2 protein using immunohistochemistry analysis compared to the Paracetamol treated group. Similarly, the Silymarin treated group also showed significant reduction in the NF- κ B, JNK, and COX-2 level as shown in the Fig. 8.

Pathways and genes network related with the Paracetamol-induced liver injury

The total of 434 genes related with the Paracetamol-induced liver injury were retrieved using Cytoscape software from the PUBMED using query “Paracetamol-induced liver injury” and import to the Cytoscape. The PUBMED gives reported 434 studied genes related with the Paracetamol-induced liver injury and the results were retrieved from 1971 to 2020. The proteins retrieved from the PUBMED have been implicated in many other diseases such as inflammatory, cancer, arthritis, renal injuries, and lung injuries. These proteins after important were formatted and visualized as shown in the Fig. 9. Similarly, the Comparative Toxicogenomics database was exploited, and the genes related with the Poncirin were retrieved. The Poncirin related genes were visualized using STRING software as shown in the Fig. 8. Similarly, another gene enrichment analysis was performed using STRING software and the enrichment analysis was performed with the query molecules in the study as shown in Fig. 9. Similarly, the common genes related with the Poncirin and Paracetamol-induced liver injury were functionally enriched using STRING software as shown in the Fig. 9. Furthermore, Comparative Toxicogenomics Database and Funrich analysis showed interaction of Poncirin related genes with the different diseases and drugs, while the bar graphs were shown according to the P value in different roles such as cellular component, transcriptional factors, and molecular functions. Furthermore, the genes related with the Poncirin also overlap with the other drugs and were classified based on the similarity with other drugs as shown in Fig. 10.

Molecular Docking Analysis

The molecular docking analysis was used to assess the binding interaction with the inflammatory cytokines such as IL-1 β , IL-6 and TNF- α . The Poncirin showed significant affinity with the cytokines via multiple hydrogen and hydrophobic bonds as shown in the Fig. 7. Similarly, the computational tool was also used to assess the binding affinity of the Poncirin with the signaling protein and assess the mode of the interaction. The Poncirin showed variable interaction with the signaling proteins via both hydrophilic and hydrophobic bonds as shown in the Fig. 11. Similarly, the interaction of the Poncirin was evaluated against the c-fos and DNA complex and the site of interaction. The Poncirin anchored within the active pocket of the protein-DNA complex and interacted within the active pocket via multiple hydrogens and hydrophobic bonds as shown in the Fig. 10. Additionally, the docking validation was commenced to assess the molecular docking process and the RMSD value were calculated. The RMSD values of the re-docked ligand showed RMSD values less than 2 Å and proved that validity of the docking process as shown in Fig. 12. The interaction of the ligand with various targets and their respective binding energies are shown in the Table 2.

Table 2
Molecular docking analysis of Poncirin

Sample	Number of H-bonds	Hydrophobic bonds	H-bond Amino acid	Energy (kcal/mol)
p65 (1vkx)	3	1	GLY361, VAL358, GLY413	-10.5
JNK (1uki)	1	6	ASN114	-8.5
IL-1 β (1itb)	3	5	GLU11, SER21, LYS27	-8.2
IL-6 (1p9m)	6	2	ASP160, ARG113, LYS46, ARG104, GLN156, GLN152	-8.0
TNF- α (2az5)	4	2	SER60, LYS98, LEU120, TYR151	-8.3
c-fos and DNA complex (2wt7)	3	4	ARG157, GLU267, DT4	-6.8

Table 3
MM-GBSA binding free energies estimated for Poncirin-1vkx complex. All energy values can be read in kcal/mol.

Energy Component	Average	Std. Dev.	Std. Err. of Mean
VDWAALS	-64.94	2.45	0.24
EEL	-33.03	2.73	0.27
EGB	42.58	2.59	0.25
ESURF	-7.70	0.14	0.01
DELTA G gas	-97.97	3.41	0.34
DELTA G solv	34.88	2.52	0.25
DELTA TOTAL	-63.09	2.24	0.22
VDWAALS (van der Waals energy), EEL (electrostatic energy), EGB (polar solvation energy), ESURF (non-polar solvation energy), DELTA G gas (net gas phase energy), DELTA G solv (net solvation energy), DELTA TOTAL (net complex energy).			

All Atoms Molecular Dynamics Simulation

The Poncirin-1vkx complex was used in all atoms molecular dynamics simulation to unveil dynamics stability of the complex and to decipher the binding mode and interactions stability of the Poncirin at the docked site of 1vkx protein. It was important to describe that the compound remain intact with the protein in dynamic environment in order to perform its biological action in living cells. In this regard, 100 ns of molecular dynamics simulation of the complex was performed, and the generated trajectories were analyzed first through carbon alpha RMSD. All the frames of the complex produced during simulation time were overlapped over the initial docked structure, which was taken as a reference, and plotted in the

form of graph to easily understand time dependent evolution of the Poncirin-1vkx complex (Fig. 13A). As can be noticed, that structurally the complex remained in high equilibrium for the first 12 ns touching RMSD of 1 Å. Afterward, the complex experienced a sudden minor surge and RMSD reached to RMSD of ~ 2 Å (which is still depicting very stable nature of the complex). The consistent behavior of complex is reported till the end of simulation time. These low structural deviations and smooth RMSD trend is demonstration of the fact the Poncirin-1vkx complex enjoys intermolecular affinity and the shows good docked conformation with respect to each other. Next, root mean square fluctuation (RMSF) was determined for all residues of the protein in the presence of the Poncirin Fig. 13B. This parameter measures individual residue fluctuations from its mean as a function of time. The N- and C-terminal of the protein is showing higher flexibility compared to the rest of protein structure. However, these fluctuations are not driving any major conformational changes in the complex structure neither affect the compound binding. Last, radius of gyration (Rg) was performed to get insight about complex structure compactness as function of time Fig. 13. It was revealed that the protein remained in highly compact nature and does not acquire any relaxation in the presence of Poncirin. All these analyses unveiled the high intermolecular affinity of Poncirin and 1vkx and their stable nature in dynamics.

Calculating MM-GBSA Binding Free Energies

The MM-GBSA binding free energy method was run for Poncirin-1vkx complex simulation trajectories to determine the different atomic level binding free energies the govern the intermolecular interactions and responsible for overall complex stability. The MM-GBSA method is more reliable in predicting complex stability compared to traditional scoring functions and is less computational expensive therefore providing a user friendly validation method in re-evaluating compounds binding affinity. The complex interactions are mainly dominated by van der Waals energy as well by electrostatic energy both of which contribute to high gas phase energy compared to the solvation energy. The net van der Waals energy is -64.94 kcal/mol which is double of the electrostatic energy (-33.03 kcal/mol). The polar solvation energy is illustrated to play less role in complex formation while the non-polar energy is favorable complex stabilization. The net binding energy of the complex is -63.09 kcal/mol that is reflection of highly stable nature of the complex. Details about the contribution of each energy component to the overall complex intermolecular interactions is provided in Table 2.

In Silico Pharmacokinetic Analysis

The Poncirin pharmacokinetic profile was analyzed using Swiss ADME and the studied parameters includes the physico-chemicals properties such as number of heavy atoms, hydrogen bond donors and acceptor, number of rotatable bonds and number of aromatic heavy atoms. The Software also calculated the lipophilicity of the Poncirin and showed logP value of 3.02. Similarly, it was also predicted the water solubility characters such as GIT absorption, BBB permeability, substrate for cytochrome, P-glycoprotein, and skin permeability. The drug likeness and medicinal aspect were also considered by the software as

shown in the Fig. 14. Similarly, the preADMET predicted the possible metabolites of the Poncirin and were classified according to Ranks as shown in Fig. 15.

Discussion

The liver is an important and vital organ of the GIT regulating the various physiological processes of the body such as synthetics, metabolism (endogenous and exogenous substances) and detoxification (Burrell et al. 2003). The liver facilitates the process of metabolism either by phase I or phase II reactions and armed with the microsomal mixed function oxidases system. The phase I metabolism includes the hydroxylation, oxidation and reduction, while the phase II reaction comprises of conjugation reaction such as glucuronidation and sulphonation (Clark et al. 2002; Shal et al. 2018). Similarly, the liver is also very critical site for the metabolism of the xenobiotic, where liver make the drugs either active or in active to perform the intended therapeutic effect or increase the clearance from the body (Clark et al. 2002; Hernandez-Gea and Friedman 2011). The Paracetamol is commonly prescribed analgesic and anti-pyretic and considered very safe at therapeutic doses. However, at very high doses it can compromise the liver hemostasis and generation of reactive intermediate which interact with the cellular components such as lipids, proteins and DNA (Hernandez-Gea and Friedman 2011). This interaction of the reaction intermediate with cellular component trigger the generation of RONS and other inflammatory mediators, which results into hepatic inflammation and finally necrosis (Hernandez-Gea and Friedman 2011). Pre-clinically the Paracetamol-induced liver injury model is commonly used to assess the protective effect of the new chemical against this insult and explore the underlying protective mechanism (Hernandez-Gea and Friedman 2011).

In the present study the effect of the Poncirin was evaluated against the Paracetamol-induced liver injury in rodents. The effect of the Poncirin was evaluated against the survival rate, food intake, body weight variation and liver weight variations. The Poncirin treatment significantly improved the liver weight variations and food intake, however, no marked changes were noticed in survival rate and body weight variations. The liver functions tests (LFTs) such as ALT, AST, total protein and bilirubin serves as indicator of the liver function (Hernandez-Gea and Friedman 2011). During oxidative stress and inflammatory conditions, the liver synthetic function is compromised and results in marked elevation of these markers. It is well established that Paracetamol administration markedly enhanced the level of liver functions tests and compromised the synthetic activity of the liver (Kumar et al. 2011). During present study, the Paracetamol administration markedly increase the LFTs, however, the Poncirin treatment significantly normalized the LFTs compared to the control. Similarly, the Paracetamol administration markedly alter the hematological parameters, however, Poncirin treatment significantly improved the hematology compared to the control. The histological changes can be used to assess the effect of any toxin on the histological architecture of any organ and assess the degree of damage. The H and E staining is commonly used to assess the histological changes and can be used to analyze the course of the disease (Mohite 2010). The Paracetamol administration markedly altered the histopathology of the liver tissue, however, the Poncirin treatment dose dependently improved the histology compared to the control. The Paracetamol is well known for increasing the reactive oxygen and nitrogen species (RONS) and oxidative

stress (Mohite 2010). These reactive substances interact covalently with the cellular structures and alter their physiology. The effect of the Poncirin treatment was evaluated against the Paracetamol-induced antioxidants and oxidative stress such as GSH, GST, Catalase, SOD, and MDA. The Poncirin treatment markedly alleviated the oxidative stress induced by the Paracetamol administration via enhancing the antioxidants. Similarly, the Paracetamol administration markedly enhanced the production of the NO production in both plasma and liver tissue, however, the Poncirin intervention markedly attenuated the NO production compared to the control. The MPO serves as indirect marker of the neutrophilic infiltration and its concentration is enhanced significantly during inflammatory conditions (Mohite 2010; Khan et al. 2020). The Paracetamol administration markedly elevated the MPO activity in the liver tissue and hence, indicates marked infiltration of the neutrophils into the liver tissue. However, the Poncirin treated groups showed marked reduction in the MPO activity compared to the Paracetamol treated groups and the response was dose dependent. The pro-inflammatory cytokines are critically involved in the Paracetamol-induced hepatic injury and inflammation. The cytokines are induced by the oxidative stress and regulate the inflammatory process within the liver tissue via positive feedback mechanism (Ilavarasan, Malika et al. 2005, Khan, Shal et al. 2020). The Paracetamol administration markedly enhanced the pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α . However, the Poncirin treatment markedly attenuated the production of Poncirin treatment compared to the control.

The network based pharmacology approaches were used to assess the diseases and Poncirin related genes and developed a network based on their interaction as reported previously (Ilavarasan et al. 2005; Khan, Shal et al. 2019). The Poncirin and diseases-based query showed fruitful interaction of the common genes and these genes were implicated in various diseases. Furthermore, the computational approach was used to determine the binding affinity of the Poncirin with the various protein targets and analyzed the mode of interaction. The various protein targets that were studied computationally includes p65, JNK, p38, AP-1 and inflammatory cytokines (IL-1 β , IL-6 and TNF- α). The Poncirin showed variable interaction with the protein targets and binding energies varied significantly. Similarly, the Poncirin also showed variable mode of interaction with the protein targets i.e. the number of hydrogen bonds, hydrophobic interaction and the amino acid involve. The docking results were further validated through more sophisticated approaches of molecular dynamics simulation and MM-GBSA binding free energies. Both of these techniques translate the good binding affinity of Poncirin for 1vkx protein and good equilibrium of the complex. Similarly, the pharmacokinetic analysis was performed to assess the various properties of the Poncirin using computational approaches. The physico-chemical properties, lipophilicity, water solubility, metabolites, medicinal chemistry aspects and drug likeness behavior were analyzed. Similarly, the Potential activities of the Poncirin were analyzed using *in silico* approaches and the best activities against the various diseases were reported.

Conclusion

In the present study, the Poncirin was evaluated against the Paracetamol-induced liver injury using *in vivo* and *in silico* approaches. The Poncirin showed significant improvement in the liver functions tests and inflammatory cytokines. Similarly, the Poncirin treatment improved the histological parameters,

antioxidants status and attenuated the oxidative stress. The Poncirin treatment also attenuated the expression level of various signaling protein and inflammatory cytokines. Furthermore, the Poncirin showed several drug likeness properties, promising interactions with the protein targets and various important genes using *in silico* and molecular dynamic simulation. From the results of the present study, we can speculate that poncirin could be promising agent/ligand attenuating liver injuries induced by many factors mainly drugs including paracetamol.

Declarations

Ethical approval

The study was approved by the animal ethical committee of Quaid-i-Azam University, Islamabad (No. #BEC-FBS-QAU2018-90). All the animal experiments were regulated according to the guidelines of the institutional bioethical committee.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Author contribution

HA and AUK performed the *in vivo* activities. The SA, AUK performed the computational studies. AAA, FAA, YSA, SDA and SK designed the project. The HA and AUK performed the biochemical assays. AAA, FAA, YSA, SDA and SK analyzed the results and drafted the manuscript. SK supervised the project. All authors read and approved the final manuscript. The authors declare that all the data were generated in-house and that no paper mill was used.

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Competing interest

The author declares no conflict of interest.

Availability of data and materials

Not applicable.

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Figures

Figure 1

The effect of the Poncirin treatment (5 mg/kg, 15 mg/kg, 30 mg/kg) on the food intake (A), liver weight variation (B), survival rate (C) and body weight changes (D) were assessed following Paracetamol-induced liver injury. The Poncirin treatment at the dose of the 30 mg/kg improved the food intake compared to the control. Similarly, the liver weight variation was dose dependently improved by the Poncirin administration compared to the Paracetamol-induced liver injury. However, the Poncirin did not showed any effect on the body weight variation and survival rate. All values are expressed as mean $\pm$ S.D (n=8). ###P < 0.05 compared with vehicle control group; *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the Paracetamol treated group.

Figure 2

The Poncirin (5 mg/kg, 15 mg/kg, 30 mg/kg) treatment was evaluated against the ALT (A), AST (B), ALP (C), total protein (D), total bilirubin (E) and serum bilirubin (F). The Paracetamol treated group showed marked elevation in the ALT, AST, bilirubin and reduction in the total protein. However, the Poncirin treatment dose dependently improved the liver functions tests and the dose of the 30 mg/kg showed maximum response. All values are expressed as mean $\pm$ S.D (n=8). ###P < 0.05 compared with vehicle control group; *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the Paracetamol treated group.

Figure 3

The influence of the Poncirin treatment (5 mg/kg, 15 mg/kg, 30 mg/kg) was investigated on the histopathology following Paracetamol-induced liver injury. The histological changes in all the treated groups such as Normal control, Paracetamol treated, Silymarin treated and Poncirin treated (5 mg/kg, 15 mg/kg, 30 mg/kg) were studied and the histopathological changes were quantified as described in the “Materials and methods” section. The Poncirin treatment dose dependently improved the histological changes compared to the control. The images were taken at 40X and quantified using Image_j software. All values are expressed as mean $\pm$ S.D (n=8). ###P < 0.05 compared with vehicle control group; *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the Paracetamol treated group.

Figure 4

The effect of the Poncirin treatment on the Masson's trichrome staining following disease induction with the Paracetamol-induced liver injury. The Poncirin treatment dose dependently protected the liver tissue from the fibrosis compared to the control. The fibrosis score ranges from the 0-4, while 0 represent no fibrosis and 4 indicates maximum fibrosis as described in the “Materials and methods” section. The images were taken at 40X using simple microscope attached with the camera. All values are expressed as mean $\pm$ S.D (n=8). ###P < 0.05 compared with vehicle control group; *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the Paracetamol treated group.

Figure 5

The influence of the Poncirin treatment on the PAS staining following Paracetamol-induced liver injury. The glycogen content was assessed in all the treated groups and compared. The Poncirin treatment showed significant improvement in the glycogen content compared to the Paracetamol-induced group. The PAS staining was quantified from 0-4 as described in the "Material and methods" section. The images were taken at 40X using simple microscope attached with the camera. All values are expressed as mean $\pm$ S.D (n=8). ###P < 0.05 compared with vehicle control group; *P < 0.05, **P < 0.01 and ***P < 0.001 compared with Paracetamol treated group.

Figure 6

The effect of the Poncirin (5 mg/kg, 15 mg/kg, 30 mg/kg) treatment on Paracetamol-induced changes in antioxidants and the oxidative stress markers such as GSH (A), GST (B), Catalase (C), SOD (D), MPO (E), MDA (F) and NO (G, H). The Poncirin treatment dose dependently restored the antioxidants, however, the dose of the 30 mg/kg showed maximum increase in the antioxidants compared to the control. Similarly, the Silymarin treated group also improved the antioxidants level. Furthermore, the Paracetamol administration significantly increase the NO, MPO and MDA level, however, the Poncirin administration showed significant amelioration in the NO production, MPO activity and MDA production compared to the Paracetamol treated group. All values are expressed as mean $\pm$ S.D (n=8). ###P < 0.05 compared with vehicle control group; *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the Paracetamol treated group.

Figure 7

The impact of the Paracetamol administration on the production of pro-inflammatory cytokines and subsequently, the investigation of the Poncirin treatment on these inflammatory mediators. The

Paracetamol administration markedly enhanced the level of the IL-1 β (A), IL-6 (B) and TNF- α (C). However, the Poncirin treatment statistically attenuated the production of the pro-inflammatory mediators compared to the control. The Silymarin treatment also showed promising inhibition of the pro-inflammatory mediators. All values are expressed as mean $\pm$ S.D (n=8). ###P < 0.05 compared with vehicle control group; *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the Paracetamol treated group. The computational approach was used to assess the binding interaction of the Poncirin with the inflammatory cytokines such as IL-1 β , IL-6 and TNF- α . The Poncirin formed complex with these mediators via hydrogen bonds, hydrophobic and pi bonds. Once the ligand-protein complexes were formed, they were visualized using discovery studio visualizer to obtain 3 dimensional and 2 dimensional images.

Figure 8

The immunohistochemistry analysis following Paracetamol-induced liver injury. The Poncirin treatment showed marked reduction in the level of the NF-κB, JNK, and COX-2 protein expression level compared to the Paracetamol treated group. Similarly, the Silymarin treated group also showed marked reduction in the inflammatory proteins. ###P < 0.05 compared with vehicle control group; *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the Paracetamol treated group.

Figure 9

The network pharmacology based approach was exploited to using diseases based query such as Paracetamol-induced liver injury (A) and STRING (B, C, D) software. The Paracetamol-induced liver injury based query was used in PUBMED (from 1971 to 2020) and imported into the Cytoscape and topological based network was generated. Similarly, the STRING software was used and network was established involving Poncirin and Paracetamol-induced liver injury query as described in “Materials and methods” section.

Figure 10

The Comparative Toxicogenomics Database (A, B) using diseases based and Poncirin based queries to develop the disease link with the Poncirin and genes involved. Similarly, the Funrich enrichment analysis (C, D, E) was used and P value was generated for the genes involved in the diseases and Poncirin studies as described in the “Materials and methods” section.

Figure 11

The computational approach was used to assess the binding interaction of the Poncirin with the molecular signaling proteins such as p65, JNK and p38. The Poncirin showed binding interaction via several hydrogen bonds, hydrophobic and pi bonds. The ligand-protein complexes were analyzed using discovery studio visualizer and 3 dimensional and 2 dimensional images were obtained. The computational analysis of the ligand with the DNA and protein complex was performed using autodock tools. The Poncirin was docked against the transcriptional factor and DNA complex. The Poncirin interacted within the active pocket of the protein-DNA complex via multiple hydrogen hydrophobic bonds. The 3D and 2D interaction of the ligand and protein-DNA complex are shown.

Figure 12

The molecular docking process was validated by redocking the ligand with the same protein and determining the root mean square deviation (RMSD) of the docked ligand with redocked ligand. The RMSD value less than the 3 Å was considered valid docking. The ligRMSD showed that RMSD value of all the ligand-protein complex less than 2 Å. The heatmap was generated based on the binding energy of the ligand and protein targets.

Figure 13

Different post molecular dynamics simulation analysis to evaluate dynamics stability of Poncirin-1vkx complex. (A) RMSD, (B) RMSF, and (C) Rg.

Molecule 1

SMILES OCC1OC(Oc2cc3OC(CC(=O)c3c(c2)O)c2ccc(cc2)OC)C(C(C1O)O)OC1OC(C)C(C(C1O)O)O

Physicochemical Properties

Formula	C ₂₈ H ₃₄ O ₁₄
Molecular weight	594.56 g/mol
Num. heavy atoms	42
Num. arom. heavy atoms	12
Fraction Csp ³	0.54
Num. rotatable bonds	7
Num. H-bond acceptors	14
Num. H-bond donors	7
Molar Refractivity	139.38
TPSA	214.06 Å ²

Lipophilicity

Log <i>P</i> _{o/w} (iLOGP)	3.02
Log <i>P</i> _{o/w} (XLOGP3)	-0.11
Log <i>P</i> _{o/w} (WLOGP)	-1.19
Log <i>P</i> _{o/w} (MLOGP)	-2.57
Log <i>P</i> _{o/w} (SILICOS-IT)	-1.08
Consensus Log <i>P</i> _{o/w}	-0.39

Water Solubility

Log S (ESOL)	-3.21
Solubility	3.70e-01 mg/ml ; 6.22e-04 mol/l
Class	Soluble
Log S (Ali)	-3.93
Solubility	6.95e-02 mg/ml ; 1.17e-04 mol/l
Class	Soluble
Log S (SILICOS-IT)	-1.18
Solubility	3.96e+01 mg/ml ; 6.65e-02 mol/l
Class	Soluble

Pharmacokinetics

GI absorption	Low
BBB permeant	No
P-gp substrate	Yes
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log <i>K</i> _p (skin permeation)	-10.00 cm/s

Druglikeness

Lipinski	No; 3 violations: MW>500, NorO>10, NHorOH>5
Ghose	No; 4 violations: MW>480, WLOGP<-0.4, MR>130, #atoms>70
Veber	No; 1 violation: TPSA>140
Egan	No; 1 violation: TPSA>131.6
Muegge	No; 3 violations: TPSA>150, H-acc>10, H-don>5
Bioavailability Score	0.17

Medicinal Chemistry

PAINS	0 alert
Brenk	0 alert
Leadlikeness	No; 1 violation: MW>350
Synthetic accessibility	6.28

Figure 14

The pharmacokinetic analysis using SwissADME tool was used to assess the ADME, physico-chemical properties, drug likeness properties, water, and lipid solubility. Similarly, the various drugs obeying rules such as Lipinski, Ghose, Veber, Egan and Muegge were also considered by the software.

Figure 15

The preADMET software was used to assess the potential metabolites of the Poncirin. The software predicted 5 metabolites and ranked them according to the probability of their production.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryParacetamoldata.xlsx](#)