Comprehensive in vivo and in silico approaches to explore the hepatoprotective activity of poncirin against paracetamol toxicity

Hadayat Ullah1,2, Ashrafullah Khan1,2,3, Tehmina Bibi1,2, Sajjad Ahmad2,3, Omer Shehzad2,4, Hussain Ali1,2, Eun Kyoung Seo2,5, Salman Khan1,2

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Abstract

In the present study, poncirin was evaluated against paracetamol-induced liver injury using in vivo and computational approaches. Paracetamol was administered intraperitoneally (i.p.) to establish liver injury in mice and, subsequently, to investigate the hepatoprotective effect of poncirin (administered intraperitoneally) on liver injury. The effect of 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 were also assessed using H and E staining, Masson’s trichrome staining, and periodic acid-Schiff staining. Similarly, the computational approach was used to assess the pharmacokinetic parameters of poncirin and its interaction with various protein targets. Poncirin markedly improved the antioxidant enzymes while attenuated the oxidative stress markers and inflammatory cytokines. Poncirin also markedly improved hematological parameters. Furthermore, poncirin treatment significantly improved the histological parameters using H and E staining, Masson’s trichrome, and PAS staining compared to the control. 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 nuclear factor kappa light chain enhancer of activated B cells (NF-κB), Jun N-terminal kinase (JNK), and cyclooxygenase-2 (COX-2) expression level compared to the paracetamol treated group. Computational analysis (molecular docking and molecular dynamic simulation) showed significant binding affinity of poncirin with the NF-κB, JNK, COX-2, IL-1β, IL-6, and TNF-α via multiple hydrophilic and hydrophobic binds. Similarly, the SwissADME software revealed that poncirin follows various drug-likeness rules and exhibited better pharmacokinetic parameters. Poncirin improved the sign and symptoms associated with liver injury using both in vivo and computational approaches.

Keywords Poncirin · Oxidative stress · Antioxidant enzymes · Cytokines · Hepatoprotection

Hadayat Ullah and Ashrafullah Khan contributed equally to this work.

Salman Khan
skhan@qau.edu.pk; udrsalm@gmail.com
Hadayat Ullah
hadayatwaxir@gmail.com
Ashrafullah Khan
ashrafwazir6@gmail.com
Tehmina Bibi
tehmeenagul42@gmail.com
Sajjad Ahmad
sajjademaan@gmail.com
Omer Shehzad
omersnu@yahoo.com
Hussain Ali
h.ali@qau.edu.pk

Eun Kyoung Seo
yuny@ewha.ac.kr

1 Pharmacological Sciences Research Lab, Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad 45320, Pakistan
2 Faculty of Pharmacy, Abasyn University, Peshawar 25000, Pakistan
3 Department of Health and Biological Sciences, Abasyn University, Peshawar 25000, Pakistan
4 Department of Pharmacy, Abdul Wali Khan University, KPK, Mardan, Pakistan
5 College of Pharmacy, Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul 03760, Korea
Introduction

Paracetamol is widely used as an antipyretic and considered usually safe at therapeutic doses, i.e., the dose up to 4 g per day is well tolerated; however, greater than the recommended dose can trigger hepatotoxicity (Pradhan and Girish 2006). Paracetamol is metabolized primarily via conjugation reaction such as glucuronidation and sulfonation, while 30–55% of paracetamol is excreted in urine as a conjugated form (Jóźwiak-Bebenista and Nowak 2014). Paracetamol is mostly metabolized by phase II conjugating enzymes and converted paracetamol to non-toxic metabolite which is readily excreted via urine, and a small amount is excreted as unchanged in the urine (Yan et al. 2018). The remaining 5–9% of the paracetamol is metabolized via cytochrome p450 enzymes into very reactive metabolite, i.e., N-acetyl-p-benzoquinone imine (NAPQI) (Jóźwiak-Bebenista and Nowak 2014). During therapeutic doses, the NAPQI is neutralized rapidly by glutathione (GSH). However, during paracetamol overdose, the excessive NAPQI formation depletes the GSH, which trigger the covalent interaction of the sulfhydryl groups with the cellular proteins (Yan et al. 2018). These pathological changes result into jaundice, decreased synthetic capacity, bilirubin accumulation, and fulminant hepatic dysfunction (Ullah et al. 2018; Khan et al. 2020c). 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 by-products, 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. 2020a, 2020b, 2020c, 2020d). Cumulative effects of excessive ROS production can be manifested by a variety of injurious events like hepatotoxicity (Lukasiewicz-Hussain and Moniuszko-Jakoniuk 2004; Khan et al. 2020a, 2020b, 2020c, 2020d). The reactive free radical induces the production of pro-inflammatory cytokines such as IL-1β, 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-κB, which interact with the intermediate signaling protein and transcriptional factors such as AP-1 (Rasheed et al. 2018b, Khan et al. 2020a, 2020b, 2020c, 2020d). The body is armed with endogenous antioxidant defense mechanism such as Nrf2 signaling, which are induced during oxidative stress and induce the expression of 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 for the new drug development which are used for the treatment of many disease including inflammation, cancer, and arthritis (Khalid et al. 2018; Sana et al. 2021). Similarly, poncirin is a natural compound obtained from the immature fruit of Poncirus trifoliata and has been reported for various biological activities including inflammation, pain, arthritis, and anti-bacterial (Khalid et al. 2018; Afridi et al. 2019; Ullah et al. 2020). Based on the previously reported biological activities, the present study was aimed to investigate the hepatoprotective activity of poncirin against the paracetamol-induced liver injury using in vivo and in silico analysis (Khalid et al. 2018).

Materials and methods

Chemicals and reagents

Various chemical and reagents used in the present study include paracetamol (Sigma–Aldrich, Germany), ketamine (Sigma–Aldrich, Germany), formalin (Sigma–Aldrich, Germany), xylazine (Sigma–Aldrich, Germany), and poncirin (Sigma–Aldrich, Germany). ELISA kits for the determination of TNF-α, IL-1β, and IL-6 were purchased from 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 hemoglobin 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 g) 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.
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 was 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 (negative control), 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 poncirin was selected based on the previously reported study (Afridi et al. 2019; Khan et al. 2020a, 2020b, 2020c, 2020d; Ullah et al. 2020). All the drugs (silymarin and poncirin) were administered via intraperitoneal (i.p.) route. The liver injury was induced by the toxic dose of paracetamol as reported previously (Liu et al. 2016). The animals in the silymarin (positive control) and poncirin (treatment control) were administered 1 h before the induction of liver injury with paracetamol according to the previously established protocols (Liu et al. 2016).

### 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 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

Food intake was assessed in all the treated groups before the commencement of disease induction and every day after the disease induction (Afridi et al. 2019). The food was weighted and placed for each group, and 24 h later, 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 was compared (Afridi et al. 2019; Ullah et al. 2020).

### Survival rate

The Kaplan-Meier analysis was used for the survival in all the treated groups following paracetamol-induced liver injury. The effect of 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 the experiment, the animals were euthanized with cervical dislocation as reported previously (Khan et al. 2019a, 2019b). 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. 2019a, 2019b; 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. 2019a, 2019b).

### 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).

### Nitric oxide (NO) determination

The NO assay was performed using Griess reagent method and the effect of Poncirin (5, 15, 30 mg/kg, i.p) was evaluated (Atiq 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. 2019a).
Histopathological study of liver tissues

The histological changes in the liver tissue following induction of injury with 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 histopathologists 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. 2020c). The left lateral lobe was selected and sliced. The tissues 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. 2019c). 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 to 4, while 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. 2020c). 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. 2020c).

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 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-1β, IL-6, and TNF-α production

The cytokine production in liver tissue following paracetamol-induced liver injury was 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-1β, IL-6, and TNF-α were studied, and the effect of 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-1β, and IL-6 production was calculated using a commercially available TNF-α, IL-1β, 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-κB, 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), and primary antibodies and placed overnight. The next day, primary antibodies were washed while treated with secondary antibodies and 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 gene 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 GenBank search engine which were implicated in the pathogenesis of the paracetamol-induced liver injury and filtered for Homo sapiens. Similarly, to retrieve the gene list related with poncirin, various search engines were utilized such as Comparative Toxicogenomics Database (http://ctdbase.org/), Therapeutic Targets Database (http://bidd.nus.edu.sg/group/cjttdd/), and Traditional Chinese Medicine System Pharmacology Database and Analysis 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 poncirin. These interacting genes were plotted in the form of the cluster using STRING online software (https://string-db.org/). The Comparative Toxicogenomics database uses disease query to assess the interaction of the genes associated with poncirin (Xinqiang et al. 2020). Similarly, the genes that are related with 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 are 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 AutoDock Vina 4.2 software. The crystal structure of the 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 was prepared, i.e., energy was minimized, water molecules were removed, co-crystallized ligands were removed, and hydrogens were added using Swiss PDB viewer and Discovery Studio Visualizer 16 (Khan et al. 2020a). The active site of the proteins was analyzed using CASTp online software, and the amino acids within the active pockets were assessed within the chain of interest (Khan et al. 2020c). 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. 2020c; 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 and grid parameter files, and configuration file was generated (Khan et al. 2020c; 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. 2020c). 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–σ 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. 2020c). 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 was shown using Discovery Studio Visualizer 16 (Khan et al. 2020c).

Molecular dynamic simulation and analysis

The AMBER20 simulation package was utilized to accomplish molecular dynamic simulation of poncirin–1vkx complex. The protein parameters were generated using ff14SB, whereas general Amber force field was applied to assign poncirin parameters (Khan et al. 2020c). The complex was solvated explicitly in TIP3P water box where counterions were added to achieve charge neutralization. The complex was then forwarded to energy minimization phase and subjected to 2500 rounds of steepest descent and conjugate gradient 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 a 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. 2020c).

Estimating MM-GBSA binding free energies

Molecular mechanic 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. 2020c).
In silico pharmacokinetic analysis and toxicokinetic analysis

The pharmacokinetic analysis of poncirin was performed using SwissADME (absorption, distribution, metabolism, and elimination) software. The various parameters that were studied include physicochemical properties, lipophilicity, water solubility, pharmacokinetics, drug-likeness, and medicinal chemistry aspects (Atiq et al. 2019; Xinqiang et al. 2020). Furthermore, the preADMEDT software was utilized to assess the potential metabolites, and the metabolites were predicted based on their probability such as Rank1, Rank2, and Rank3 (Atiq et al. 2019).

Statistical analysis

The data was analyzed as mean ± S.D. using SPPS 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 various parameters such as body weight variation, liver weight variation, survival rate, and food intake following paracetamol-induced liver injury. 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 poncirin was analyzed on the liver weight variation following disease induction. Poncirin administration dose dependently reduced the liver weight variation compared to paracetamol-induced liver injury. However, poncirin did not show any effect on the body weight variation and survival rate. All values are expressed as mean ± S.D. (n = 8). ###P < 0.05 compared with normal control group; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the paracetamol treated group.

Fig. 1 The effect of poncirin treatment (5 mg/kg, 15 mg/kg, 30 mg/kg) on food intake (A), liver weight variation (B), survival rate (C), and body weight changes (D) was assessed following paracetamol-induced liver injury. Poncirin treatment at the dose of 30 mg/kg improved food intake compared to the control. Similarly, the liver weight variation was dose dependently improved by poncirin administration compared to paracetamol-induced liver injury. However, poncirin did not show any effect on the body weight variation and survival rate. All values are expressed as mean ± S.D. (n = 8). ###P < 0.05 compared with normal control group; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the paracetamol treated group.
variation, and the dose of 30 mg/kg showed maximum response (Fig. 1). The Kaplan-Meier survival analysis showed no significant statistical changes in the mortality rate as shown in 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 Fig. 1.

Effect of 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 poncirin was assessed. Paracetamol markedly increased the liver function tests markers such as ALT, AST, ALP, serum bilirubin, and total protein. However, the poncirin administration markedly attenuated the serum markers compared to the only paracetamol treated groups as shown in Fig. 2. Similarly, the poncirin treatment markedly improved the liver functions tests, and the dose of 30 mg/kg showed maximum response. All values are expressed as mean ± S.D. (n = 8). ###P < 0.05 compared with normal control group; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the paracetamol treated group.

| Sample | WBC (10⁹/L) | NEU (10⁹/L) | MON (10⁹/L) | RBC (10¹²/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 normal control group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with paracetamol treated group.
hematological parameters compared to paracetamol treated group as shown in Table 1.

**Effect of poncirin on histopathological parameters**

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

**Effect of poncirin on the Massons’s trichrome and PAS staining**

Paracetamol treatment showed marked increase in the fibrosis of the liver. However, 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 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 Fig. 5.

**Effect of poncirin on the antioxidants**

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

**Effect of poncirin on NO, LPO, and MPO**

Poncirin was evaluated against NO production in both plasma and liver homogenate following paracetamol-induced liver injury. The NO production was significantly attenuated in the Poncirin treated groups, and the response was dose dependent (Fig. 6). The LPO assay was performed to assess the effect of poncirin on the malonaldehyde (MDA) production. Poncirin treatment significantly reduced the level of MDA as shown in Fig. 6. Similarly, the MPO activity was performed to assess the effect of poncirin on the neutrophilic infiltration marker. The Paracetamol treated group showed sharply increases in the MPO activity; however, the poncirin treatment markedly neutralized the MPO activity compared to the paracetamol-induced group as shown in Fig. 6.

**Effect of poncirin treatment on inflammatory cytokines**

The effect of poncirin treatment (30 mg/kg) on pro-inflammatory cytokines follows establishing paracetamol-induced liver injury. The paracetamol administration markedly enhanced the production of the inflammatory mediators such as IL-1β, IL-6, and TNF-α using ELISA assay. Poncirin treatment significantly attenuated the production of the

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**Fig. 3** The influence of poncirin treatment (5 mg/kg, 15 mg/kg, 30 mg/kg) was investigated on 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. Poncirin treatment dose dependently improved the histological changes compared to the control. The images were taken at 40× and quantified using ImageJ software. All values are expressed as mean ± S.D. (n = 8). ###P < 0.05 compared with normal control group; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the paracetamol treated group.
cytokines compared to the paracetamol-induced group as shown in Fig. 7.

**Immunohistochemistry analysis**

Poncirin treatment (30 mg/kg) showed marked reduction in the expression level of the NF-κB, 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-κB, JNK, and COX-2 level as shown in Fig. 8.

Pathway and gene network related with the paracetamol-induced liver injury

The Cytoscape, STRING, and FunRich software were used to predict the interaction between the proteins and to construct a protein–protein interaction (PPI) network as reported previously (Zeng et al. 2021). 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 imported to the Cytoscape. The proteins retrieved from PUBMED have also been implicated in many other diseases such as inflammatory disorders, cancer, arthritis, renal injuries, and lung injuries. In Cytoscape analysis, three parameters were considered such as degree, betweenness centrality, and closeness centrality threshold to establish the major hub between the nodes as shown in Fig. 9A. Out of 434 genes, the PPI network was developed between 100 genes having confidence level greater or equal to 10. Similarly, another gene enrichment analysis was performed using STRING database with three queries, i.e., poncirin-based (Fig. 9B), disease-based query (Fig. 9C), and disease-poncirin-based query (Fig. 9D). In the STRING analysis, a network of protein based on the compound (poncirin) search (contain network between 23 genes), disease-based query (contain network between 39 genes), and disease-poncirin-based query (contain network between 27 genes) with the confidence level more than 0.4 was constructed. Furthermore, Comparative Toxicogenomics Database and FunRich analysis showed interaction of poncirin-related genes with the different diseases and drugs. In Comparative Toxicogenomics Database, the liver injury-based query showed similarity of gene involvement in digestive diseases, nervous system disease, and cancer to that of liver injury (Fig. 10A). Poncirin-based query revealed the similarity (greater than 0.3 similarity score was selected) with various compounds (active against similar target proteins) based on the protein targets as shown in Fig. 10B. Similarly, the FunRich analysis was performed to develop as biological network of protein and subsequently assess the poncirin target proteins based on P value. The FunRich enrichment was performed based on the genes retrieved from the PUBMED query. The poncirin
FunRich enrichment was constructed based on the three parameters such as cellular component (Fig. 10C), transcriptional factor as target (Fig. 10D), and molecular function (Fig. 10E).

**Molecular docking analysis**

The molecular docking analysis was used to assess the binding interaction with the inflammatory cytokines such as IL-1β, IL-6, and TNF-α. Poncirin showed significant affinity with the cytokines via multiple hydrogen and hydrophobic bonds as shown in Fig. 7. Similarly, the computational approach was used to assess the binding interaction of poncirin with the inflammatory cytokines such as IL-1β, IL-6, and TNF-α. Poncirin formed complexes with these mediators via hydrogen bonds and 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.
413B, Val 358B) and JNK via one hydrogen bond (Asn 114B). While in case of c-fos-DNA complex, poncirin was anchored within the active pocket of the protein–DNA complex and docked within the active pocket of the complex via multiple hydrogens (DT 4D, Arg 157A, Glu 267B) and hydrophobic bonds as shown in Fig. 11. Additionally, the docking validation was commenced to assess the molecular docking process, and the RMSD value was 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 number of hydrogen bonds, interacting amino acid involved, and their respective molecular docking score are shown in the Table 2.

Fig. 8 The immunohistochemistry analysis following paracetamol-induced liver injury. Poncirin treatment (30 mg/kg) 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 normal control group; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the paracetamol treated group.
All-atom molecular dynamic simulation

The NF-κB plays a crucial role in the pathogenesis of the liver injury, which induces the inflammatory cytokines and aggravates the underlying inflammatory condition. Based on its pivotal role in inflammation and hepatic injury, poncirin was simulated with the NF-κB to assess the stability of poncirin and NF-κB complex stability (Sana et al. 2021; Shal et al. 2021). The molecular docking revealed the highest docking score between the NF-κB and poncirin, so this complex was processed for molecular dynamic simulation. The poncirin–NF–κB complex was used in all-atom molecular dynamic simulation to unveil dynamic stability of the complex and to decipher the binding mode and interaction stability of poncirin at the docked site of NF-κB protein. It was important to describe that the compound remains intact with the protein in dynamic environments 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–ILvkx complex (Fig. 13A). As can be noticed, structurally, the complex...
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 a demonstration of the fact the poncirin–NF–κB complex enjoys intermolecular affinity and then 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 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, the 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 NF-κB and their stable nature in dynamics.

Calculating MM-GBSA binding free energies

The MM-GBSA binding free energy method was run for poncirin–NF–κB complex simulation trajectories to determine the different atomic level binding free energies that 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 compound binding affinity. The complex interactions are mainly dominated by van der Waals energy as well as 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 a favorable complex stabilization. The net binding energy of the complex is −63.09 kcal/mol that is a reflection of highly stable nature of the complex. Details about the contribution of each energy component to the overall complex intermolecular interactions are provided in Table 3.
The computational approach was used to assess the binding interaction of poncirin with the molecular signaling proteins such as p65, JNK, and p38. Poncirin showed binding interaction via several hydrogen bonds and 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. Poncirin was docked against the transcriptional factor and DNA complex. 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.

The molecular docking process was validated by re-docking the ligand with the same protein and determining the root mean square deviation (RMSD) of the docked ligand with re-docked 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.
In silico pharmacokinetic analysis

The poncirin pharmacokinetic profile was analyzed using SwissADME, and the studied parameters include the physicochemical properties such as the number of heavy atoms, hydrogen bond donors and acceptor, the number of rotatable bonds, and the number of aromatic heavy atoms. The software also calculated the lipophilicity of poncirin and

| 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-1β (1tib) | 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 |

Fig. 13  Different post-molecular dynamic simulation analysis to evaluate dynamic stability of poncirin-1vkx complex. A RMSD, B RMSF, and C Rg.
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 Fig. 14. Similarly, the preADMET predicted the possible metabolites of 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 synthesis, metabolism (endogenous and exogenous...
substances), and detoxification (Burrel et al. 2003; Khan et al. 2021). 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 sulfonation (Clark et al. 2002; Shal et al. 2018). Similarly, the liver is also a very critical site for the metabolism of xenobiotic, where the liver make the drugs either active or inactive to perform the intended therapeutic effect or increase the clearance from the body (Clark et al. 2002; Hernandez-Gea and Friedman 2011). The plants are an important source for the new drug developments, and numerous plants derived compounds are in clinical practice for the ailment of various diseases. Poncirin is derived from Poncirus trifoliata and has been reported for various biological activities including pain, inflammation, arthritis, and hepatoprotection (Ullah et al. 2020). Poncirin protects against inflammation by reducing the oxidative stress and inflammatory mediator via multiple signaling. Poncirin provided protection against carbon tetrachloride-mediated liver injury by reducing the oxidative stress and inflammatory cytokines (Ullah et al. 2020). Paracetamol is a commonly prescribed analgesic and antipyretic 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 triggers 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 poncirin was evaluated against the paracetamol-induced liver injury in rodents. The effect of poncirin was evaluated against the survival rate, food intake, body weight variation, and liver weight variations. 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 serve 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 the present study, paracetamol administration markedly increase the LFTs; however, poncirin treatment significantly normalized the LFTs compared to the control. Similarly, paracetamol administration markedly alter the

![Fig. 15 The preADMET software was used to assess the potential metabolites of poncirin. The software predicted 5 metabolites and ranked them according to the probability of their production.](image-url)
hematological parameters; however, poncirin treatment significantly improved hepatology 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). Paracetamol administration markedly altered the histopathology of the liver tissue; however, the poncirin treatment dose dependently improved the histology compared to the control. 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. Poncirin has been reported for the reduction in the oxidative stress and provides protection against the oxidative stress-mediated damage (Afridi et al. 2019; Ullah et al. 2020). Furthermore, poncirin has been implicated in inducing the endogenous antioxidant enzymes and maintaining the hemostatic environment of the body (Ullah et al. 2020). In the present study, the effect of the poncirin treatment was evaluated against the paracetamol-induced antioxidants and oxidative stress such as GSH, GST, catalase, SOD, and MDA. Poncirin treatment markedly alleviated the oxidative stress induced by the paracetamol administration via enhancing the antioxidants. Similarly, 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 an indirect marker of the neutrophilic infiltration, and its concentration is enhanced significantly during inflammatory conditions (Mohite 2010; Khan et al. 2020a). 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 et al. 2005; Khan et al. 2020a). Previously, poncirin attenuated the inflammatory pain by reducing the inflammatory cytokines and induced the analgesia following CFA-induced inflammatory pain as reported (Afridi et al. 2019). Similarly, the present study also investigated the effect of poncirin treatment on the paracetamol-induced inflammatory mediator production in liver tissue. Paracetamol administration markedly enhanced the pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. However, poncirin treatment markedly attenuated the production of poncirin treatment compared to the paracetamol-induced group.

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 et al. 2019a). Poncirin and disease-based query using Cytoscape software showed fruitful interaction with the genes commonly involved in the pathogenesis of inflammatory diseases including hepatic injuries, renal dysfunction, colitis, and cancer. Furthermore, the computational approach was used to determine the binding affinity of poncirin with the various protein targets and analyzed the mode of interaction. The various protein targets that were studied computationally include p65, JNK, p38, AP-1, and inflammatory cytokines (IL-1β, IL-6, and TNF-α). Poncirin showed variable interaction with the protein targets, and binding energies varied significantly. Similarly, poncirin also showed variable mode of interaction with the protein targets, i.e., the number of hydrogen bonds, hydrophobic interaction, and amino acid involve. The docking results were further validated through more sophisticated approaches of molecular dynamics simulation and MMGBSA binding free energies. Both of these techniques translate the good binding affinity of poncirin for NF-κB 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 physicochemical properties, lipophilicity, water solubility, metabolites, medicinal chemistry aspects, and drug-likeness behavior were analyzed. Similarly, the potential activities of poncirin were analyzed using in silico approaches, and the best activities against the various diseases were reported.

**Conclusion**

In the present study, poncirin was evaluated against the paracetamol-induced liver injury using in vivo and in silico approaches. Poncirin showed significant improvement in the liver function tests and inflammatory cytokines. Similarly, poncirin treatment improved the histological parameters and antioxidant status and attenuated the oxidative stress. Poncirin treatment also attenuated the expression level of various signaling protein and inflammatory cytokines. Furthermore, 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 poncirin could be promising agent/ligand attenuating liver injuries induced by many factors mainly drugs including paracetamol.

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Author contribution Hu and AUK performed the in vivo activities. SA and AUK performed the computational studies. AUK and SK designed the project. HU, AUK, and TB performed the biochemical assays. EKS, HA, OS, and SK analyzed the results and drafted the manuscript. EKS, HA, OS, and SK revised 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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Declarations

Ethics 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.

Conflict of interest The authors declare no competing interests.

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