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RESEARCH ARTICLE

# Pomegranate Peel Extract and Quercetin Possess Antioxidant and Hepatoprotective Activity against Concanavalin A-induced Liver Injury in Mice

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**A B S T R A C T**

Pomegranate peel extract (Ppe) is reported to possess antioxidant, and anti-inflammatory activity due to presence of bioactive constituents like quercetin (Qrt). The goal of this study was to explore the protective effects of Ppe, Qrt and their combination PQ (Ppe + Qrt) against concanavalin A (Con A)-induced liver injury in mice. *In vitro* antioxidant activities including 2,2-diphenyl-1-picrylhydrazyl-radical scavenging activity, peroxidase, superoxide dismutase (SOD), and catalase (CAT) were determined for different treatment groups. To evaluate the protective effects of Ppe, Qrt and PQ, BALB/c mice were pretreated with Ppe (150 mg/kg, i.g.), Qrt (100 mg/kg, i.g.) and PQ (Ppe; 150 mg/kg + Qrt; 100 mg/kg, i.g.) for fourteen days, prior to administration of a single dose of Con A (20 mg/kg, i.v.). The HPLC analysis indicated that Ppe possessed high contents of Qrt. Moreover, PQ demonstrated higher antioxidant enzymes activity than Ppe and Qrt alone. The *in vivo* study showed that PQ ameliorated Con A-induced liver injury more effectively than alone pretreatments by significantly decreasing the levels of serum transaminases. Also, PQ restored the increased levels of oxidative stress markers (TOS and MDA) and decreased levels of antioxidant markers (TAC, SOD, CAT and GSH) upon Con A administration. Histopathological studies revealed that Con A-treated mice group presented hepatic lesions and leukocytes infiltration which were not observed in PQ pretreatment group. Together, these results showed that the Ppe and Qrt combination group (PQ) showed better hepatoprotective effects than alone treatment groups suggesting additive action that might provide therapeutic potential against liver injury involving oxidative stress.



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## INTRODUCTION

Hepatitis is characterized by elevated transaminase enzymes, programmed cell death, and necrosis that lead to fibrosis and cirrhosis. Excessive immune stimulation plays a pivotal role in the pathogenesis of hepatitis caused by various factors like virus, drugs, toxins, alcohol, and metabolic disorders (Liu *et al*., 2016; Ishtiaq *et al.*, 2018; Khalid *et al.*, 2018). The incidence of liver failure and resultant death is increasing worldwide (Ishtiaq *et al.*, 2019). Pakistan presents the highest prevalence rate of hepatitis C worldwide (Bhatti *et al.*, 2016). Generally, immune-mediated liver injury is treated with immunosuppressive drugs (prednisolone and azathioprine) which are associated with severe side effects like osteoporosis, hyperglycemia, and impaired immunity (Sun *et al.*, 2017; Wang *et al.*, 2018). Thus, the search for new preventive/therapeutic strategies is an urgent need by understanding the underlying molecular mechanism of hepatitis (Zhang *et al.*, 2017).

Concanavalin A (Con A; a mannose/glucose-binding lectin) induces liver injury in mice. Con A is used as a reliable experimental model to explore the mechanistic pathway of immune cell-mediated hepatitis (Li *et al*., 2015). This model mimics the clinical characteristics of viral and autoimmune hepatitis in human (Tiegs *et al.*, 1992; Zhai *et al.*, 2016; Mo *et al.*, 2018; Ye *et al.*, 2018). Lectins bind to glycated residues present on the outer

surface of a variety of cell types resulting in immunogenic stimulation of T lymphocytes and the production of hepatotoxic inflammatory cytokines like interferons, tumor necrosis factor, and interleukins. Increased serum level of liver transaminases is frequently observed due to immense hepatocyte cell death (apoptosis and necrosis) and associated oxidative stress (Sun *et al.*, 2017).

Traditional medicines have got much attention as their protective and curative effects against many diseases (Hussain *et al.*, 2019a; Khaskheli *et al*., 2020; Taha *et al*., 2020). Among these, pomegranate is a safe food nutrient. Decades of research have proven the antioxidant and anti-inflammatory effects of pomegranate in general and its peels extract in particular (Wang *et al.*, 2018). Pomegranate peel is reported to possess bioactivity in regulating blood glucose levels and has antioxidant, antifungal, antiparasitic and anti-tumor activities (Wang *et al.*, 2018; Hassan *et al*., 2020). Pomegranate juice revealed its activity in down-regulating the level of NF-κB (Deng *et al.*, 2017). Moreover, pomegranate extract ameliorates fatty liver by decreasing triglyceride contents.

Quercetin (Qrt), a flavonoid, is found in numerous plants (apples, green tea, onions, grape skin and pomegranate). Qrt has the potential to treat excessive oxidative stress-induced cell damage because of its antioxidant and anti-inflammatory activities (Wu *et al.*, 2017). It increases activities of antioxidant enzymes like superoxide dismutase and glutathione peroxidase, along with inhibition of malondialdehyde activity (Zhu *et al.*, 2017). The present study was planned to examine the potential protective effects of pomegranate peel extract (Ppe) and quercetin (Qrt) against liver injury induced by Con A in mice.

## MATERIALS AND METHODS

**Reagents:** Lyophilized powder of Con A from *Canavalia ensiformis* (Jack Bean) was purchased from Sigma-Aldrich (L7647). Qrt was purchased from Avonchem limited, United Kingdom (Reference # 5488Q754). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) test kits were obtained from Diasys diagnostic System GmbH, Germany. Glutathione (GSH) was measured in liver tissues by using Reduced glutathione (GSH) colorimetric assay kit of Elbascience (E-BC-K051), USA. All other reagents were of analytical grade.

**Preparation of 80% ethanolic and aqueous extract of pomegranate peel:** At 30℃, 400 g powder of pomegranate peel was macerated separately in 2 L of 80% ethanol and distilled water for three consecutive days. The extracts were filtered by using Whatman No.1 filter paper and subjected to the rotatory evaporator at 50℃. Finally, a semi-solid paste was collected and stored in an amber bottle at -20℃ (Wang *et al.*, 2018).

***In vitro* determination of TFC, TPC, and DPPH activity:** Total phenolic contents (TPC) and Total flavonoid contents (TFC) of both extracts were measured according to the methods described by Sultana *et al* (2007). DPPH-radical scavenging activity was assessed as described with slight modifications (Yen and Chen, 1995).

**HPLC analysis of the hydroalcoholic extract of pomegranate peel (Ppe):** High performance liquid chromatography (HPLC) equipped with UV-Vis detector (SPD-10.AV, Shimadzu, Japan) and pump (LC-10AT, Shimadzu, Japan) was used for identification and quantification of quercetin, gallic acid, vanillic acid and *p*-coumaric acid. The sample was diluted to a concentration of 1.25 mg/mL (solvent 40% methanol) and filtered using syringe filter of 0.2 µm pore size. The standard solution was prepared (1 mg/mL) and its serial dilutions from 10 to 200 µg/mL were used as a reference. A volume of 20 µL was used for HPLC analysis (Wang *et al.*, 2018).

***In vitro* determination of the antioxidant enzymatic activities of Ppe, Qrt, and PQ:** DPPH-radical scavenging activity was assessed as described by Yen and Chen (1995) with slight modifications. Briefly, 1 mL of freshly prepared 0.004% DPPH solution was added to 3 mL of plant extracts solution (25 µg/mL) and the absorbance was noted at 517 nm. Superoxide dismutase (SOD) activity was performed by calculating the rate of inhibition in the photoreduction of nitro blue tetrazolium (NBT) by the SOD enzymes at 560 nm as the method described previously (Onoja *et al.*, 2014). Peroxidase (POD) activity as unit per gram was performed by adding 100 µL of extract (1 mg/mL) into 100 µL of the reaction mixture [800 µL potassium phosphate buffer (pH 5.0), 20 mM guaiacol, and 40 mM hydrogen peroxide] and absorbance was noted at 470 nm (Khatun *et al.*, 2012; Ulfat *et al.*, 2012). Catalase activity (µmole of consumed $H_2O_2$/min/mg of total protein) was measured by following method reported previously (Mohebbi *et al.*, 2012). ELISA plate was filled with the mixture of 100 µL of extract and 100 µL of $H_2O_2$ and absorbance was recorded at 240 nm wavelength. A reduction in the absorbance after 5 min was recorded at the same wavelength.

**Animals grouping and drug-administration protocol:** BALB/c mice of male sex of age 8-10 weeks (25-30 g) were maintained at animal research facility, Institute of Physiology and Pharmacology, University of Agriculture, Faisalabad under standard laboratory conditions of 25℃, 12 hours of light and dark cycle, and 40-60% of relative humidity. The animals were given a standard pellet diet and had free access to autoclaved water.

A pilot study was carried out to develop an animal model of acute liver injury of optimal dose of Con A that produces maximum effects at specific time interval. For this, mice were divided randomly into four groups (n=3). An intravenous dose of Con A was given at 10 mg/kg, 15 mg/kg, and 20 mg/kg. After 8 hours of injection, mice were sacrificed, and blood was collected for biochemical assays. For determining time-dependent effect of Con A, post-Con A injection (20 mg/kg, i.v.) was administered and mice were sacrificed at different intervals (2 h, 8 h, and 24 h). Serum was collected for biochemical assays (Ye *et al.*, 2018).

For final study, mice were administered intragastrically with Ppe (150 mg/kg), Qrt (100 mg/kg), and PQ (150 mg/kg) for 14 days. Then Con A was injected intravenously at 20 mg/kg. After 8 hours of Con A injection, all the mice were sacrificed and serum and liver samples were collected for biochemical and histopathological assays (Wang *et al.*, 2018).

**Serum biochemical analysis:** The clear serum was used for the estimation of ALT, AST, and ALP. All parameters were measured by an automated Bio-lab 310 serum analyzer using biochemical kits. Serum total antioxidant capacity (TAC) and total oxidant status (TOS) were determined by method described by Erel, (2005). Serum catalase activity and malondialdehyde (MDA) levels were determined through colorimetric method as reported previously (Aliahmat *et al.*, 2012). Reduced glutathione (GSH) activity was measured in liver tissue by using reduced glutathione assay kit (Choi *et al.*, 2015).

**Histopathological analysis of liver tissues:** After paraffin embedding, liver tissues were sectioned into 4-5 µm thickness. The histopathological examination was performed after H&E stain using light microscope (Model IM-910 IRMECO Gmbh & Co; Germany) at 400x using camera (TOUPCAM, ToupTek Photonics Co., Ltd; China) (Wang *et al.*, 2018; Hussain *et al.*, 2019b).

**Statistical analysis:** The results are presented as mean ± standard error. Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Tukey's test using GraphPad Prism 7 with *p*-values ≤0.05 considered statistically significant.

## RESULTS

**In vitro analysis of the aqueous and hydroalcoholic extract:** Results showed that hydroalcoholic extract possessed more phenolic (255.76 mg/g ± 5.60) and flavonoids (198.99 mg/g ± 0.76) contents as compared to that of aqueous extract (TPC: 146.47 mg/g ± 11.40 & TFC 167.33 mg/g ± 9.80).

**HPLC-based quantification of quercetin in Ppe:** The HPLC analysis showed that Ppe possessed important bioactive compounds like quercetin (36.63 ppm), gallic acid (24.91 ppm), vanillic acid (6.15 ppm), and *p*-coumaric acid (1.14 ppm) (Fig. 1, Table 1).

***In vitro* antioxidant enzyme activities of Ppe, Qrt and PQ:** The PQ showed significantly higher DPPH and SOD activities than that of Ppe and Qrt. While Qrt showed significantly higher POD and CAT activities than that of Ppe and PQ as shown in Fig. 2.

**Dose and time dependent Con A induced liver injury:** The optimized dose of Con A to induce liver damage was detected at a dose of 20 mg/kg (Fig. 3A). Moreover, maximum liver damage was observed at 8 hours of post-Con A injection (Fig. 3B, 3C). Therefore, we selected a dose of 20 mg/Kg and a time interval of 8 hours for a maximum effect of Con A.

**Table 1:** Identification of bioactive compounds by HPLC analysis

| Compound Name | Reten. Time (min) | Area (mV.s) | Area [%] | ppm |
|---|---|---|---|---|
| Ethanol | 2.193 | 4.113 | 0.1 | --- |
| T-Butanol | 2.713 | 188.559 | 3.0 | --- |
| Quercetin | 3.020 | 196.541 | 3.1 | 36.63 |
| Gallic acid | 4.347 | 1015.484 | 15.9 | 24.91 |
| Vanillic acid | 13.440 | 97.661 | 1.5 | 6.15 |
| p-coumeric acid | 17.640 | 38.055 | 1.2 | 1.14 |

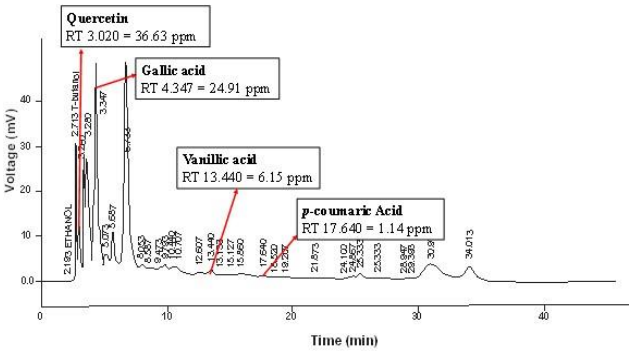


**Fig. 1:** Identification of bioactive compounds by HPLC analysis: Extract at a concentration of 1.25 mg/mL (solvent 40% methanol) was injected using a syringe of 20 µL. Standards were injected at 10-200 µg/ mL. Identification of quercetin, gallic acid, vanillic acid and *p*-coumaric acid was performed based on the retention time.

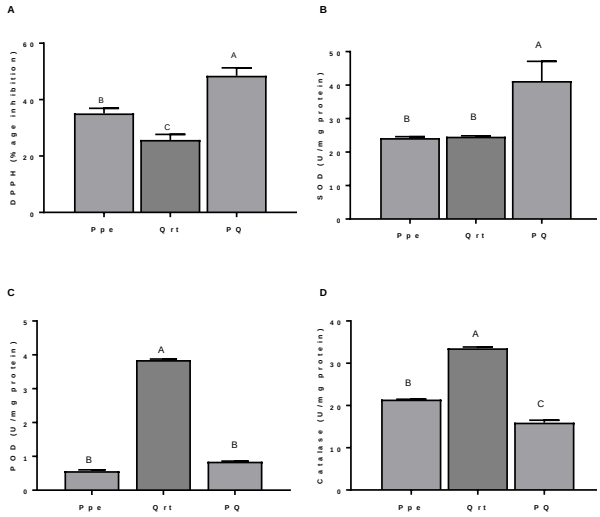


**Fig. 2:** *In vitro* antioxidant activity of Ppe, Qrt and PQ: The antioxidant activity (DPPH scavenging activity, SOD, POD and CAT) of Ppe, Qrt and PQ (n=5) was performed. $^{A-C}$ Mean values within the column, bearing a different superscript vary significantly from each other ($p \leq 0.05$).

**Ppe, Qrt and PQ normalize the ALT, AST and ALP levels after Con A challenge:** Con A challenge increased the serum ALT, AST, and ALP levels that were normalized when pretreated with PQ, Qrt and Ppe (Fig. 4).

Con A challenge increased serum TOS and MDA levels while decreased the serum TAC, CAT, SOD and liver lysate GSH. Moreover, the PQ pretreatment proved effective in enhancing the level of serum TAC, CAT, SOD and liver lysate GSH and decreasing the TOS and MDA levels (Fig. 5).

**Ppe, Qrt and PQ pretreatments attenuate Con A-induced liver injury:** Histopathological study showed that Con A significantly increased the scars in the liver tissue. However, Ppe, Qrt, and PQ pretreatments significantly decreased the incidence of the hepatic lesions (Fig. 6). Similarly, necrosis was observed in extensive areas and an increased inflammatory infiltration were detected in liver tissues in Con A-treated group (arrows). However, PQ pretreatment markedly reduced the pathological alterations to normal hepatic parenchyma.

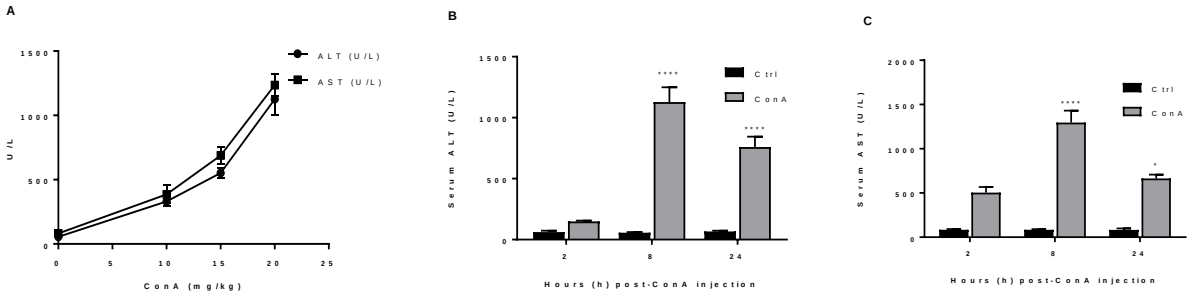


**Fig. 3:** Dose and time-dependent Con A induced liver injury: A) Three groups (n=3) were given different doses (10 mg/kg, 15 mg/kg, and 20 mg/kg) of Con A (intraorbital i.v.). Serum ALT and AST levels were measured; B & C) 20mg/kg dose of Con A dose was given and mice were sacrificed at different intervals (2 h, 8 h, and 24 h). Time-dependent effect was evaluated by serum ALT and AST levels (n=3). Symbol (x) shows level of significant difference when compared with Con A (Veh) group.

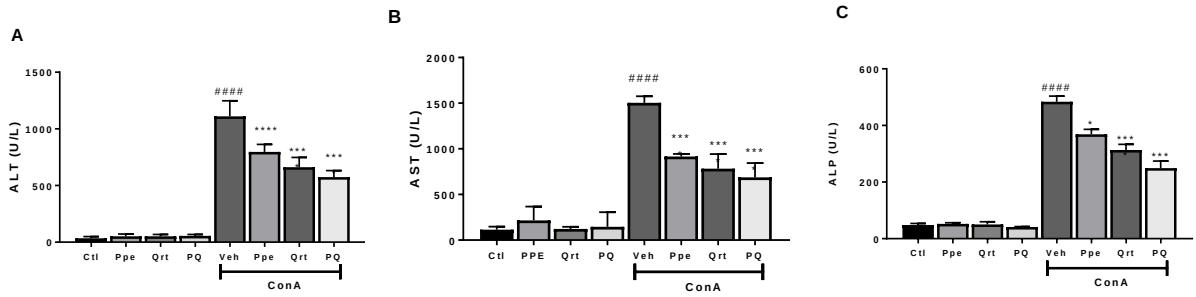


**Fig. 4:** Effect of Ppe, Qrt and PQ against Con A-induced liver injury: A-C) Different treatments of bioactive compounds were given to mice (n=5) for 14 days, prior to the challenge of Con A (20 mg/Kg). Mice were sacrificed 8 hours of post-injection of Con A. Serum levels of ALT, AST, and ALP were measured. Hash symbol (##### as $p$<0.0001) shows the level of significance from negative control (Ctrl) while asterisk symbol (*) shows level of significance from Con A (Veh). *** shows $p$<0.001, ** shows $p$<0.01, and * shows $p$<0.05.

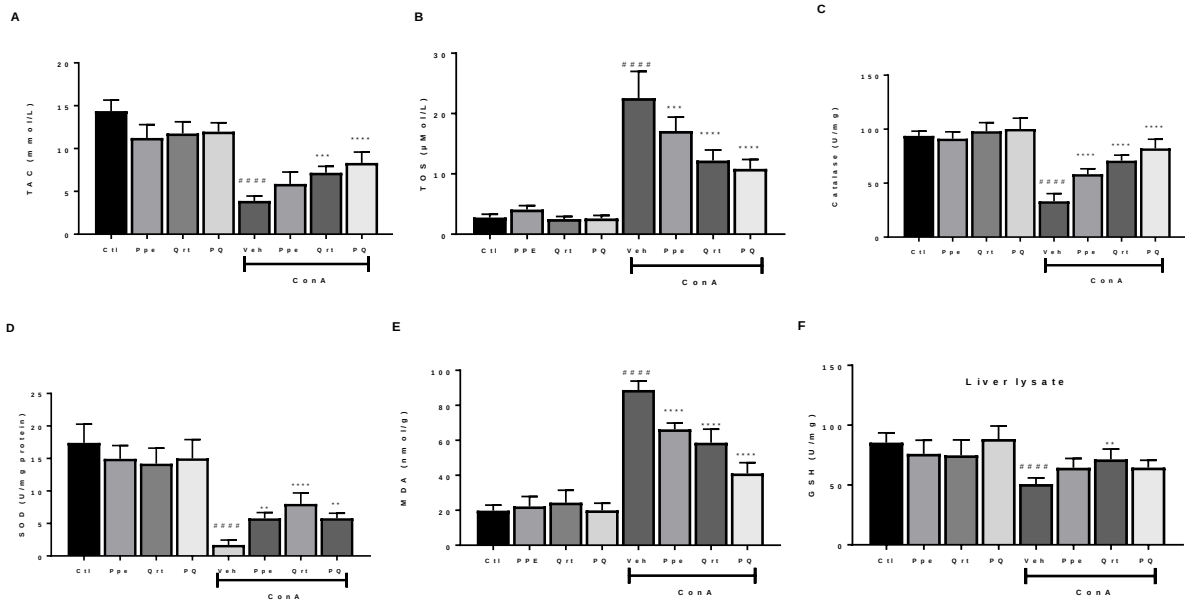


**Fig. 5:** Effect of Ppe, Qrt and PQ on antioxidant enzymes activity against Con A injection: Ppe, Qrt and PQ were given to mice (n=5) for 14 days, prior to the challenge of Con A (20 mg/Kg). Mice were sacrificed 8 hours of post-injection (intra-orbital, i.v.) of Con A. A – E ) serum levels of TAC, TOS, catalase, SOD and MDA were measured; F) Liver tissues were homogenized and GSH level was determined. Hash symbol (##### as $p$<0.0001) shows the level of significance from negative control (Ctrl) while asterisk symbol (*) shows level of significance from Con A (Veh). **** shows $p$<0.0001, *** shows $p$<0.001, ** shows $p$<0.01, and * shows $p$<0.05.

## DISCUSSION

The protective effects of Ppe are reported due to the presence of phenolics and flavonoid compounds (Wang *et al.*, 2018). It is revealed that hydroalcoholic extract of Ppe possessed more phenolics and flavonoids than aqueous extract thus possessing more antioxidant activity. Similarly, the HPLC analysis showed that hydroalcoholic extract of Ppe possesses higher contents of quercetin than vanillic acid, gallic acid, and *p*-coumaric acid. Therefore, the current study was performed to explore the protective effects of Ppe and Qrt, alone and in combination, against Con A induced hepatitis. The *in vitro* analysis of antioxidant activity of Ppe and Qrt, alone and in combination, showed that PQ significantly decreased the free radicals and increased the antioxidant enzymes activity as compared to that of ppe or Qrt, alone.

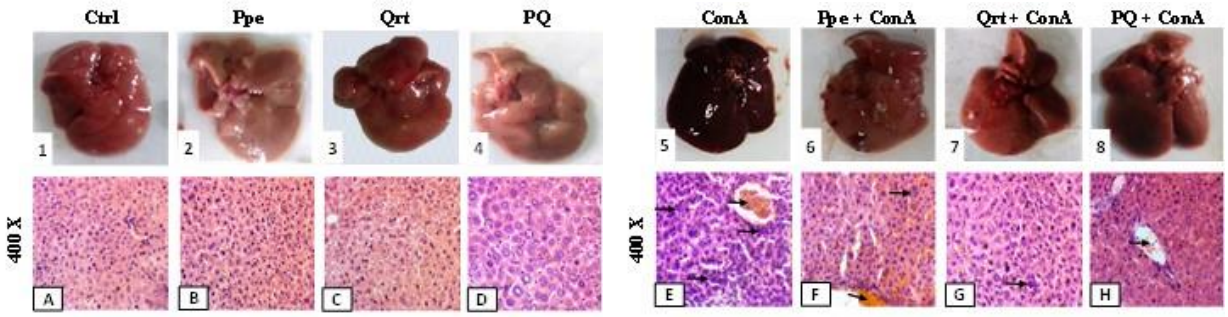


**Fig. 6:** Histopathological examination of mouse liver tissues: Ppe, Qrt and PQ were given to mice (n=5) for 14 days, prior to the challenge of Con A (20 mg/Kg). Mice were sacrificed 8 hours of post-injection (intra-orbital, i.v.) of Con A. Liver tissues were collected and subjected to H&E staining. Images were taken at 400X.

Con A was used to establish the hepatitis model in mouse that mimics the characteristics of clinical viral and autoimmune hepatitis in human (Tiegs *et al.*, 1992; Zhai *et al.*, 2016; Mo *et al.*, 2018; Ye *et al.*, 2018). Lectins bind to glycated residues present on the surface of the variety of cell types resulting in immunogenic stimulation of T lymphocytes and the production of hepatotoxic inflammatory cytokines (interferon, tumor necrosis factor, and interleukin). Thus, along with immense cell death (apoptosis and necrosis) of hepatocytes and oxidative stress, elevated serum transaminase was observed (Sun *et al.*, 2017). Maximum liver damage was observed at 8 hours of post-Con A injection at a single dose of 20 mg/kg as maximum serum ALT and AST levels were measured in the preliminary study. Upon Con A challenge, the serum ALT, AST and ALP levels were increased that were normalized when pretreated with Ppe and Qrt and PQ.

Oxidative stress causes various types of liver injuries. Overproduction of reactive oxygen species (ROS) increases the release of inflammatory cytokines that leads to the acute inflammation and hepatitis. Elevated ROS levels along with suppressed antioxidant capacity are involved in causing Con A-induced hepatitis (Shirin *et al.*, 2010; El-Agamy, 2016; Wang *et al.*, 2016). In our study, upon Con A administration, oxidative stress markers were significantly increased (serum TOS and MDA levels) while the antioxidant enzymes activities (serum TAC, SOD, CAT and liver GSH) were significantly decreased. However, PQ significantly increased the antioxidant system by enhancing the antioxidant activities of serum TAC, SOD, CAT, liver GSH and decreasing the oxidative stress markers (serum TOS and MDA levels) than Ppe and Qrt, alone. These findings were supported by histopathological studies showing hepatic lesions and leukocytes infiltration in Con A-treated group while such changes were not observed in PQ pretreatment group. The present work provides new insights for further research on elucidating the mechanism of hepatoprotection by pomegranate peel extract and quercetin.

**Conclusions:** In conclusion, our results show that pomegranate peel extract contains considerable amount of quercetin, a polyphenol flavonoid. Comparative analysis of pomegranate peel extract and its phytoconstituent quercetin alone or in combination showed that the combination group presented better hepatoprotective effects than alone treatments suggesting additive action that might provide therapeutic potential against liver injury involving oxidative stress.

**Declarations**

**Ethics approval:** The study was approved by Institutional Biosafety and Bioethics Committee, University of Agriculture, Faisalabad vide approval no. 3455/ORIC. The animals were handled according to the guidelines of National Biosafety Committee, 2005 and Punjab Biosafety Rules 2014.

**Authors contribution:** SM conducted all the experiments, processed data, drew graphs, and wrote manuscript. JAK, BA, and MNF designed the experiment, analyzed data and reviewed the article.

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