

Evaluating the potential antioxidant and *in vivo* hepatoprotective properties of *Praecitrullus fistulosus* against CCl₄-induced hepatic injury in rats

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Abstract

Chronic liver disease (CLD) progressively impairs liver function, leading to cirrhosis, which has limited available treatments and a bleak prognosis. The current investigation aims to evaluate the hepatoprotective potential of the methanolic extract of *Praecitrullus fistulosus* in mitigating carbon tetrachloride (CCl₄)-induced hepatic injury in a rat model. The methanolic *P. fistulosus* extract was prepared, qualitatively analyzed for phytochemical composition, followed by assessments of its total phenolic contents, total flavonoid contents and *in vitro* antioxidant activity. The male albino rats (n=36) were assigned into six groups: normal control, CCl₄-treated group, standard group with silymarin and three treatment groups receiving *P. fistulosus* extract orally at doses of 200, 400, and 600 mg/kg body weight, respectively, for 30 consecutive days. Except for the normal control, all groups were co-administered with CCl₄ (1 mL/kg) intraperitoneally every 72 hours. *P. fistulosus* extract has indicated the significant presence of flavonoids, phenols and glycosides, further supported by the total phenolic content (TPC) of 1748 mg GAE/g, total flavonoid content (TFC) of 1573 mg QE/g and potent antioxidant capacity. The hepatoprotective potential of *P. fistulosus* extract was demonstrated by its ability to significantly reduce the elevated ALT, AST, ALP levels, oxidative stress markers, and pro-inflammatory cytokines compared to the CCl₄-treated group. A marked increase in serum protein levels, including total protein and albumin, along with endogenous antioxidants such as total antioxidant capacity and catalase, was observed. Lastly, histopathological findings indicated a substantial decline in cellular swelling and tissue congestion in the *P. fistulosus* extract treated groups. These findings suggest that *P. fistulosus* extract at doses of 400 mg/kg and 600 mg/kg possesses optimal hepatoprotective properties by attenuating oxidative stress and markedly declining inflammation mediated by the suppression of inflammatory cytokines, leading to reduced hepatocyte necrosis.

Keywords: antioxidant enzymes, hepatoprotective activity, oxidative stress, *Praecitrullus fistulosus*, pro-inflammatory cytokines.



Introduction

Chronic liver diseases account for 4% of global deaths, claiming nearly two million lives annually (GBD 2019 Diseases and Injuries Collaborators 2020). This ranks it as the eleventh leading cause of mortality worldwide (Griffin et al. 2023). Liver, being the most metabolically active organ, is responsible for several crucial processes, including protein and hormone metabolism, synthesis, biotransformation and detoxification (Mahadevan 2020). This increases the liver's susceptibility to injury from toxin exposure, drug abuse, viral infection (Higuchi et al. 2003) and inflammation caused by poor dietary habits and excessive alcohol consumption (Nivukoski et al. 2020). Furthermore, oxidative stress, mitochondrial insult and immune dysregulation accelerate the pathogenesis of liver injury (Yu 2021). Although fibrosis is regarded as a general reaction to ongoing liver injury, yet liver anomalies rapidly progress from fibrosis to cirrhosis. Advanced stage cirrhosis is irreversible, with adverse clinical effects and poor prognosis (Mokdad et al. 2014).

Carbon tetrachloride (CCl_4) is a well-known hepatotoxin responsible for inducing free-radical-mediated toxicity. Bioactivation of CCl_4 via cytochrome P-450 in the liver makes it a major target of toxicity. Upon activation of CCl_4 , two free radicals are produced which cause lipid peroxidation, covalent bonding with DNA and structural proteins leading to hepatocellular injury, hepatic degeneration and carcinoma (Li et al. 2015). At the cellular level, CCl_4 activates Kupffer cells in the liver, which subsequently recruit neutrophils. These neutrophils generate reactive oxygen species (ROS), further exacerbating inflammation through the release of pro-inflammatory cytokines, resulting in hepatotoxicity. The sub-chronic hepatic injury model is widely used in *in vivo* experiments to explore the protective and therapeutic potential of bioactive compounds in foods (Erdemli et al. 2018). This model involves repeated intraperitoneal administration of CCl_4 at 1 mL/kg every 72 hours for 28–30 days, effectively simulating gradual toxicant exposure and pathophysiology of human liver injury (Abdelgalil et al. 2024).

The scarcity of successful treatment modalities for the cure and management of hepatic diseases has shifted the focus on exploring the bioactive compounds exhibiting hepatoprotective effects which can serve as a promising alternative for both prevention and treatment (Abdel-Moneim et al. 2015). *Praecitrullus fistulosus* is a member of the Cucurbitaceae family and is primarily cultivated in Pakistan, India, and Afghanistan. This small, flattened-round fruit, widely known as Indian pumpkin, apple gourd and Tinda, is utilized in South Asian cuisine as a cooked vegetable and is also

candied and pickled (Mukherjee et al. 2022). In addition to its nutritional benefits, *P. fistulosus* contains a diverse array of secondary metabolites including flavonoids, alkaloids, saponins, tannins, phytosterols, diterpenes, glycosides and phenols which may contribute to its pharmacological properties (Bollavarapu et al. 2016). Scientific consensus holds that edaphic conditions, agronomic factors, different cultivars and growing seasons can significantly impact the phytochemical profile of the plant (Ogundola et al. 2022, Hu et al. 2023). To date, it has been explored for its anti-diabetic (Karandikar et al. 2014), anticancer (Shivamadhu et al. 2017), anti-helminthic properties (Ishnava et al. 2020) and short-term hepatoprotective effect (Madhu et al. 2019). However, its sustained efficacy and response to higher doses over prolonged periods against hepatotoxicity remained uninvestigated. The present study intended to explore the phytochemical profile and hepatoprotective potential of *P. fistulosus* methanolic extract against CCl_4 -induced hepatic injury in the rat model over 30 days. The investigation focused on assessing liver function tests, oxidative stress markers, inflammatory cytokines and histological alterations.

Materials and Methods

Chemicals and reagents

Analytical grade chemicals and reagents were utilized in the study. Methanol and CCl_4 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) however, Silymarin (Abbott Laboratories Pak Ltd) was procured from local pharmacy in Lahore. 2, 2-Diphenyl-1-picrylhydrazyl, gallic acid (anhydrous), Folin-Ciocalteu's phenol reagent and quercetin were obtained from Merck, Darmstadt, Germany. Analytical kits for ALT, AST and ALP were acquired from Response® 910, Diagnostic System-SIEMENS, Munich, Germany. ELISA kits were obtained from Thermo-SCIENTIFIC, Rockford, IL, USA.

Plant authentication

Whole *P. fistulosus* fruit was procured during the mid-summer season from a local market in Lahore, Punjab, Pakistan. The species was authenticated from the Botany Department, G.C University, Lahore, Pakistan with an identification number (GC. Herb. Bot. 3974).

Extract preparation

P. fistulosus was peeled, diced, and then air-dried under shade (35°C) for 7 days. The sample was dried and ground to a fine consistency. This powder was

placed in a shaking incubator suspended in 98% methanol (1:10 w/v) at room temperature and agitated at 120 rpm for 72 hrs. Subsequently, the extract was filtered through filter paper (Whatman No. 1). An excess solvent was evaporated using a rotary evaporator at 50°C (Chuah et al. 2020). The concentrated crude extract was preserved at 4°C for future analysis.

Phytochemical screening and qualitative profiling

Methanolic Extract was prepared and analyzed using eight qualitative phytochemical tests including alkaloids (Mayer's and Wagner's), flavonoids (alkaline reagent), glycosides (Keller-Killiani), terpenoids (Liebermann-Burchard), steroids (Salkowski's), saponins (froth test), tannins and phenols (ferric chloride) by using standardized protocols established by (Harborne 1973). Phytochemical presence in each assay was indicated through precipitate formation and distinct color change.

Total phenolic content (TPC) determination

The total phenolic content (TPC) was quantified by the Folin-Ciocalteu method (Slinkard et al. 1977). A standard calibration curve was established with six different series of gallic acid dilutions from 1 mg/mL stock solution. The absorbance was measured at 725 nm using a spectrophotometer. The TPC values were reported in terms of gallic acid equivalent (mg GAE) per gram of plant extract.

Total flavonoid content (TFC) determination

The total flavonoid content (TFC) was quantified by (Mohdaly et al. 2013) with some modifications. In this method, TFC is assessed by the development of a flavonoid-aluminum complex with highest absorbance at 430 nm using a spectrophotometer. The calibration curve was generated with quercetin (QE) as the reference compound, and TFC was reported as (mg QE/g) of plant extract.

DPPH radical scavenging activity assay

The *in vitro* antioxidant activity was assessed by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay using the method reported in (Blois 1958, Xie et al. 2014). This assay operates on the principle of a color change from purple (DPPH solution) to yellow, which can be quantitatively measured at 515 nm using a spectrophotometer and compared with a blank control (methanol). Ascorbic acid served as the antioxidant standard to evaluate and compare the antioxidant activity of the test samples. The percentage of inhibition per 50 µL of the extract was compared to the inhibition rates achieved

by 1 mg/mL of ascorbic acid. The percentage of inhibition was determined using the following formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Experimental design and animal grouping

Thirty-six male albino rats weighing in the range of 187-193 g aged 6 weeks were purchased from the animal house of the Faculty of Biosciences, University of Veterinary and Animal Sciences, Lahore, Pakistan. The animals were kept under standard conditions of a 12-hour light/dark cycle at $24 \pm 2^\circ\text{C}$. The rats were allowed access to fresh water and a standard rat diet. After a 7-day acclimation period, the rats were assigned to six groups, with each group consisting of six rats. This study was carried out following the approval from the ethical review committee of the University of Veterinary and Animal Sciences, Lahore (Reg No. DR/158; April 2023).

For induction of hepatotoxicity, CCl_4 (1.0 mL/kg body weight) was dissolved in olive oil with 1:1 v/v and injected intraperitoneally (i.p) every 72 hrs. for 30 days. The treatments and silymarin were administered by oral gavage once daily. The rat groups were as follows: (1) The normal control group was administered normal saline (2) CCl_4 -treated group received CCl_4 i.p. (3) Standard group: CCl_4 + silymarin (100 mg/kg b.w) (4) PFE 200 (CCl_4 + 200 mg/kg *P. fistulosus* extract) (5) PFE 400 (CCl_4 + 400 mg/kg *P. fistulosus* extract) (6) PFE 600 (CCl_4 + 600 mg/kg *P. fistulosus* extract). After an overnight fast, rats were euthanized under sodium pentobarbital anesthesia. Blood was drawn through cardiac puncture and processed to quantify serum biochemical markers, and the liver was weighed. Liver lobes were excised and then preserved for histology, and both liver lobes and serum were preserved in an ultra-low temperature freezer (at -80°C) for further analysis.

Body weight and liver index determination

The body weight of rats in all experimental groups was measured at both the beginning and the end of the study, and the average weight for each group was calculated. Liver index was determined by comparing the liver weight of each rat with its body weight before sacrifice.

$$\text{Liver Index} = \frac{\text{Liver weight (g)}}{\text{Body weight (g)}} \times 100$$

Biochemical analyses

The serum was separated by the centrifugation of blood samples at 3500 g at 4°C for 20 min. Biochemical quantification of albumin, bilirubin, total protein con-

centration, and enzyme activity levels of AST, ALT and ALP were measured with commercial kits using a blood chemistry analyzer (Response® 910, Diagnostic System-SIEMENS, Munich, Germany) as per supplier instructions.

Antioxidant parameters

Lipid peroxidation in liver tissue and serum was assessed colorimetrically by determining thiobarbituric acid reactive substances (TBARS) in supernatant at 490 nm wavelength following (Ohkawa et al. 1979) method expressed as malondialdehyde (MDA). Catalase (CAT) of tissue homogenate and serum sample was determined by (Hadwan et al. 2016) method, based on the reaction of residual hydrogen peroxide with ammonium molybdate to form a yellow complex. Absorbance was taken at 374 nm wavelength thrice at 3 mins intervals. Total Antioxidant Capacity (TAC) of serum was assessed using the FRAP assay as described by (Benzie et al. 1996). It quantifies antioxidant ability by measuring the formation of a blue Fe^{2+} -TPTZ complex at 593 nm following ferric ion reduction. Hepatic Reactive oxygen species (ROS) activity in liver tissues was measured by using an enzyme-linked immunosorbent assay (ELISA) kit.

Serum TNF- α and IL-6 levels

TNF- α and IL-6 in serum were estimated by using ELISA kits (Invitrogen, Thermo SCIENTIFIC, Rockford, IL, USA) following the manufacturer's standard protocol.

Histopathological assessment

Once rats were sacrificed, their liver pieces were rinsed with saline and then fixed with 10% formalin. Afterward, tissues were gradually dehydrated using a sequence of ethanol solutions, cleared in xylene and then embedded in paraffin wax. The liver tissues were sectioned into thickness of 5 μm followed by staining with H & E (Hematoxylin & Eosin) dye. Tissue morphology was subsequently examined under a microscope (Olympus CX 31 with DP 20 software) to identify histopathological changes.

Statistical analysis

Data was analyzed using SPSS version 26 (Statistical Package for Social Sciences; SPSS Inc., Chicago, IL, USA). Results were presented as mean $\pm$ standard deviation (SD). Group differences were analyzed using one-way ANOVA, with Tukey's post-hoc test applied for pairwise comparisons. A p-value of <0.05 was

regarded as significant. Figures were generated using GraphPad Prism software (version 10.2.0; GraphPad Software, San Diego, CA, USA).

Results

Qualitative phytochemical screening

Methanolic extract of *P. fistulosus* was subjected to the qualitative screening of phytochemicals as summarized in Table 1. This phytochemical profiling revealed that the methanolic extract had the highest concentration of flavonoids, glycosides and phenols followed by a significant proportion of alkaloids, tannins and saponins. These compounds are noteworthy for their potential therapeutic and protective roles.

TPC, TFC and DPPH determination

As indicated in Table 2, the methanolic extract of *P. fistulosus* has a total phenolic content of 1748.04 mg GAE/g DW and a total flavonoid content of 1572.56 mg QE/g DW. Additionally, the extract exhibited strong DPPH radical scavenging activity with an IC_{50} value of 55.36 $\mu\text{g/mL}$. The reduction of the DPPH radical, indicated by a decrease in absorbance, reflects the concentration of plant-derived antioxidants required to inhibit 50% of DPPH radicals, with lower IC_{50} values signifying higher radical scavenging efficiency. Each test was repeated three times, and the results were averaged to reduce the potential for both systematic and random errors in the experiment.

Effect of *P. fistulosus* extract on body weight and liver index

The body weights of rats in all experimental groups were measured at both the beginning and the end of the study, as presented in Table 3. Weight gain in the CCl_4 -treated group (4.33 ± 0.49 g) was not as progressive as normal control group (19.67 ± 1.60 g). Nonetheless, weight reduction was not observed among the experimental groups, suggesting that neither CCl_4 nor subsequent treatments had adverse effects on body weight. Fig. 1 illustrates the markedly raised liver index in the CCl_4 -treated group compared to the normal control ($p \leq 0.01$). However, this effect was mitigated by administering *P. fistulosus* extract, with the most pronounced results exhibited at 400 mg/kg, comparable to the standard drug.

Table 1. Qualitative phytochemical screening of *Praecitrullus fistulosus* extract.

Phytochemicals	Interface	Observation
Alkaloids	++	Creamy white or reddish brown precipitates appear
Flavonoids	+++	Intense yellow color that disappears on acidification
Glycosides	+++	Bluish green layer and reddish brown ring at the interface formation
Phenols	+++	Intense green coloration appears
Steroids	+	A reddish brown ring forms at the interface
Saponins	++	Stable froth formation upon shaking
Tannins	++	Blue black coloration appears
Terpenoids	-	Reddish brown coloration

In the phytochemical profile “+++” indicates a strong presence, “++” signifies a moderate presence, “+” reflects a weak and “-” indicates the absence of the respective phytochemical components.

Table 2. Total phenolic, total flavonoid content and DPPH of *P. fistulosus* extract.

	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)	DPPH IC ₅₀ (50µg/ml)
<i>P. fistulosus</i> extract	1748.04 ± 2.53	1572.56 ± 1.09	55.36 ± 0.09

Values are reported as Mean±SD calculated from three independent experiments. DPPH; 2,2-diphenyl-1-picrylhydrazyl assay. TPC and TFC were expressed on a dry weight basis.

Table 3. Weight interpretation of rats in a CCl₄-induced hepatic injury model.

	Initial Weight (g)	Final Weight (g)	Weight Gain (g)
Control	191.2 ± 2.7	210.8 ± 6.6 ^a	19.7 ± 1.6 ^a
CCl ₄	190.0 ± 3.2	194.3 ± 10.8 ^c	4.3 ± 0.5 ^c
SD	192.8 ± 2.6	204.8 ± 8.7 ^{ab}	12.0 ± 1.2 ^{abc}
PFE 200	189.0 ± 3.6	199.2 ± 4.3 ^{bc}	10.2 ± 1.8 ^{bc}
PFE 400	189.5 ± 3.4	201.0 ± 4.8 ^{bc}	11.5 ± 1.2 ^{abc}
PFE 600	187.8 ± 4.1	202.7 ± 6.2 ^{abc}	14.8 ± 2.5 ^{ab}
<i>p</i> -value	0.492	< 0.001	< 0.001

Mean±SD values were calculated using data from six rats per group. Means labelled with distinct letters within columns show, a significant difference ($p < 0.05$). SD; Standard drug, PFE; *P. fistulosus* extract.

Effect of *P. fistulosus* extract on hepatic biomarkers

The effect of *P. fistulosus* extract on hepatic biomarkers is tabulated in Table 4. Significantly elevated levels of liver enzymes AST, ALT, and ALP were observed in the CCl₄-treated group compared to the normal control group ($p < 0.001$). Additionally, this group exhibited markedly decreased levels of albumin and total proteins. Different doses of *P. fistulosus* extracts demonstrated a highly significant hepatoprotective effect ($p < 0.001$), especially in 400 mg/kg and 600 mg/kg treatment groups, comparable in potency to the standard group. Moreover, all extracts were proven to be as effective as silymarin in preventing the rise in bilirubin levels. However, the changes in the levels of globulin and the albumin to globulin (A/G) ratio were insignificant.

Effect of *P. fistulosus* extract on oxidative stress parameters

As shown in Fig. 2 (A, B), tissue and serum MDA levels in the CCl₄-treated group were notably elevated compared to the normal control group ($p < 0.05$), indicating raised oxidative stress. However, *P. fistulosus* extract at doses of 400 mg/kg and 600 mg/kg markedly decreased the level of lipid peroxidation, resulting in lower MDA levels. The serum and tissue activity of catalase was noticeably reduced in the CCl₄-treated group in comparison to the normal control group ($p < 0.05$). Moreover, treatment groups have prevented the decline in catalase activity Fig. 2 (C, D).

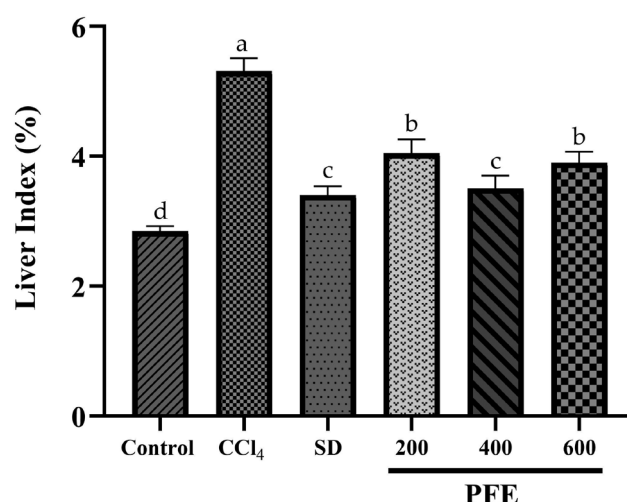


Fig. 1. Effect of *P. fistulosus* extract on liver index in CCl₄-induced hepatic injury rat model.

Mean $\pm$ SD values were calculated using data from six rats per group. Means labelled with distinct letters show a significant difference ($p < 0.05$). SD; Standard drug, PFE; *P. fistulosus* extract.

Table 4. Impact of *P. fistulosus* extract on hepatic biomarkers in the CCl₄-induced hepatic injury rat model.

	AST (U/L)	ALT (U/L)	ALP (U/L)	TP (g/dL)	ALB (g/dL)	GLO (g/dL)	A/G Ratio	Bilirubin (mg/dl)
Control	98.24 $\pm$ 1.60 ^d	46.31 $\pm$ 0.81 ^c	115.01 $\pm$ 1.11 ^d	6.41 $\pm$ 0.29 ^a	4.46 $\pm$ 0.17 ^a	2.21 $\pm$ 0.07	2.02 $\pm$ 0.03	0.81 $\pm$ 0.09 ^b
CCl ₄	457.00 $\pm$ 2.22 ^a	288.55 $\pm$ 1.30 ^a	571.13 $\pm$ 1.24 ^a	4.89 $\pm$ 0.15 ^d	3.69 $\pm$ 0.21 ^b	2.06 $\pm$ 0.15	1.79 $\pm$ 0.06	2.14 $\pm$ 0.12 ^a
Silymarin	174.02 $\pm$ 1.08 ^c	79.71 $\pm$ 0.46 ^d	159.21 $\pm$ 0.90 ^c	6.06 $\pm$ 0.22 ^{ab}	4.35 $\pm$ 0.27 ^a	2.19 $\pm$ 0.03	1.99 $\pm$ 0.08	0.97 $\pm$ 0.04 ^b
PFE 200	272.81 $\pm$ 1.30 ^b	132.45 $\pm$ 0.92 ^b	198.30 $\pm$ 1.44 ^b	5.40 $\pm$ 0.16 ^{cd}	4.18 $\pm$ 0.13 ^{ab}	2.13 $\pm$ 0.11	1.96 $\pm$ 0.03	0.86 $\pm$ 0.07 ^b
PFE 400	210.04 $\pm$ 1.54 ^{bc}	91.06 $\pm$ 0.60 ^{cd}	173.90 $\pm$ 1.31 ^c	5.85 $\pm$ 0.08 ^{bc}	4.27 $\pm$ 0.29 ^a	2.15 $\pm$ 0.04	1.99 $\pm$ 0.08	0.99 $\pm$ 0.05 ^b
PFE 600	186.73 $\pm$ 0.70 ^c	115.00 $\pm$ 0.11 ^{bc}	212.08 $\pm$ 0.46 ^b	5.83 $\pm$ 0.65 ^{bc}	4.24 $\pm$ 0.32 ^{ab}	2.18 $\pm$ 0.17	1.95 $\pm$ 0.05	1.05 $\pm$ 0.04 ^b
p-value	< 0.001	< 0.001	< 0.001	< 0.001	0.007	0.351	0.355	< 0.001

Mean $\pm$ SD values were calculated using data from six rats per group. Means labelled with distinct letters within columns show significant difference ($p < 0.05$).

Effect of *P. fistulosus* extract on free radical burden and antioxidant capacity

To further validate the antioxidant potential of *P. fistulosus* extract, ROS and TAC levels were assessed. The highest ROS (2100 $\pm$ 60.7 RFU/mg) and the lowest TAC levels (0.6 $\pm$ 0.09 IU/g) were recorded in the CCl₄-treated group, indicating severe oxidative stress. Administration of silymarin and *P. fistulosus* extracts reduced the ROS production concomitantly augmenting the TAC levels, in a dose-dependent manner, restoring the redox balance. Among all treatments, doses 400 mg/kg and 600 mg/kg have proven the most potent antioxidant activity, ROS (1000 $\pm$ 74.3 and 1250 $\pm$ 42.9 RFU/mg) and TAC (3.42 $\pm$ 0.19 and 3.03 $\pm$ 0.14 IU/g), respectively, exhibiting comparable efficacy to silymarin, as demonstrated in Fig. 3 (A, B).

Effect of *P. fistulosus* extract on pro-inflammatory cytokines

Compared to the control group, intermittent administration of CCl₄ significantly elevated the serum levels of TNF- α (190 $\pm$ 11.8 pg/mL) and IL-6 (246 $\pm$ 10.3 pg/mL), indicating the activation of inflammatory pathways Fig. 4 (A, B). However, treatment with *P. fistulosus* extract led to a highly significant, dose-dependent reduction in pro-inflammatory cytokine levels in comparison to the CCl₄-treated group ($p < 0.01$).

Histopathological assessment

The histological assessment of rats under study is shown in Fig. 5. (A) The normal control group exhibited typical histoarchitecture with well-preserved hepatic cords, sinusoids, central veins and portal area. (B) The CCl₄-treated group indicated significant morphological damage, including disorganized hepatic

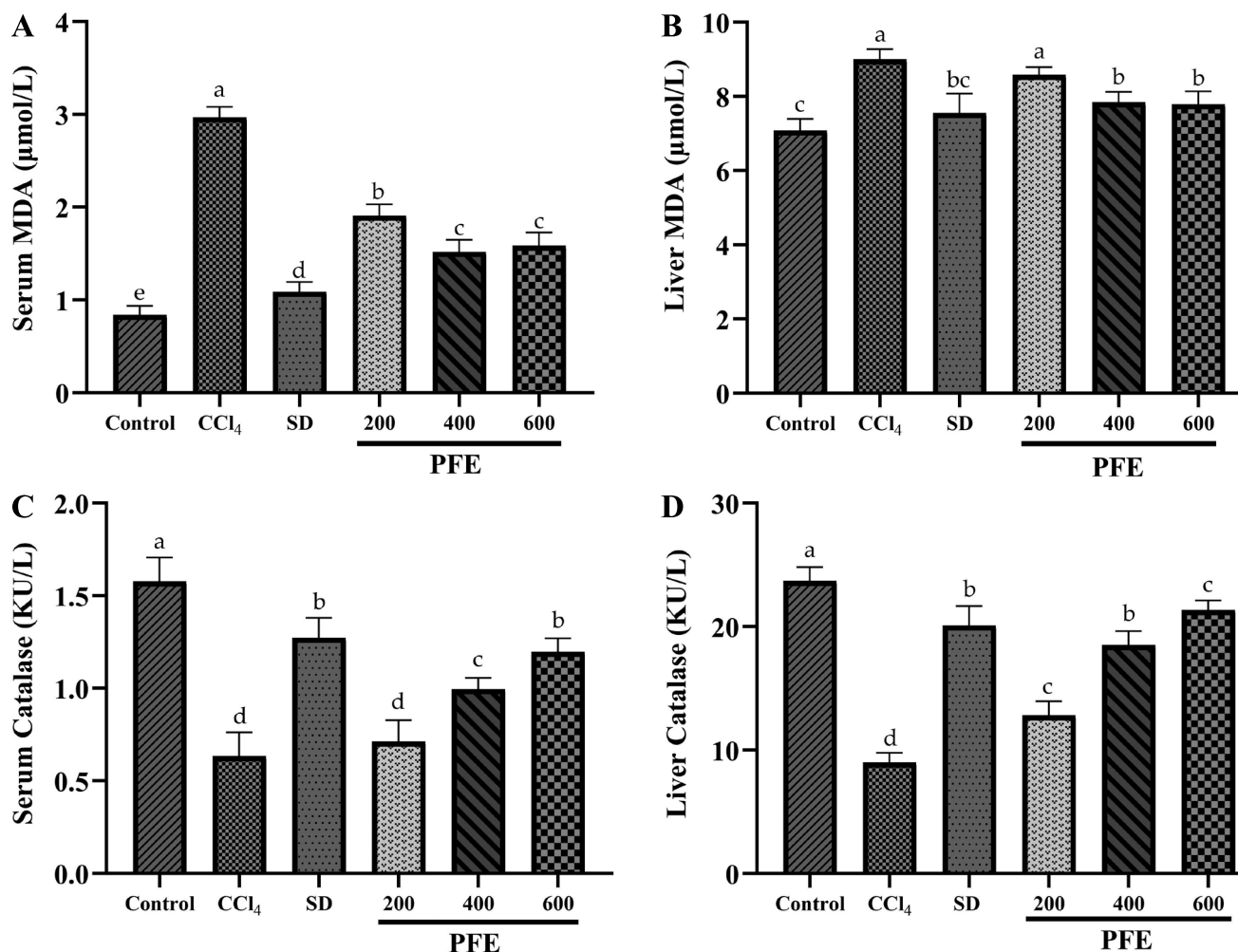


Fig. 2. Effect of *P. fistulosus* extract on oxidative stress level. A) Serum MDA, level B) Liver MDA, level C) Serum Catalase activity, D) Liver Catalase activity in CCl₄-induced hepatic injury model.

Mean $\pm$ SD values were calculated using data from six rats per group. Bars labelled with distinct letters show a significant difference ($p < 0.05$).

cords and mild to moderate cellular swelling in hepatocytes. (C) In the standard group, where most of hepatocytes appeared morphologically unaltered, some revealed low-grade coagulative necrosis with pyknotic nuclei. (D) After four weeks of administration of *P. fistulosus* extract at 200 mg/kg, signs of cellular stress, such as mild cellular swelling in hepatocytes, was evident. (E, F) *P. fistulosus* extract at doses of 400 mg/kg and 600 mg/kg resulted in predominantly intact hepatocytes, with no detectable signs of tissue congestion.

Discussion

Hepatotoxicity is an intricate interplay between prolonged oxidative stress and chronic low-grade inflammation. In this study, hepatotoxicity was induced by CCl₄, a well-established hepatotoxin that generates trichloromethyl free radicals ($\bullet\text{CCl}_3$ or $\text{CCl}_3\text{OO}\bullet$) and ROS, leading to oxidative stress, lipid peroxidation,

and hepatic membrane damage (Fareed et al. 2024). The present study evaluated the hepatoprotective potential of the methanolic extract of *Praecitrullus fistulosus* in mitigating these effects through its antioxidant and anti-inflammatory mechanisms. Medicinal plants are a rich source of bioactive compounds, primarily in the form of secondary metabolites such as phenolics and flavonoids, which are reported to exhibit potent antioxidant and anti-inflammatory activities (Adegbola et al. 2017, Unuofin et al. 2020).

The qualitative phytochemical profiling of methanolic *P. fistulosus* extract revealed a high concentration of phytochemicals except steroids, which were minimally present, aligning with a previous report (Karandikar et al. 2014). Despite being reported earlier (Bollavarapu et al. 2016), terpenoids were not detected in the methanolic extract, owing to their poor solubility in polar solvents such as methanol (Jiang et al. 2016). Furthermore, the current extract showed markedly higher total phenolic (TPC) and flavonoid content

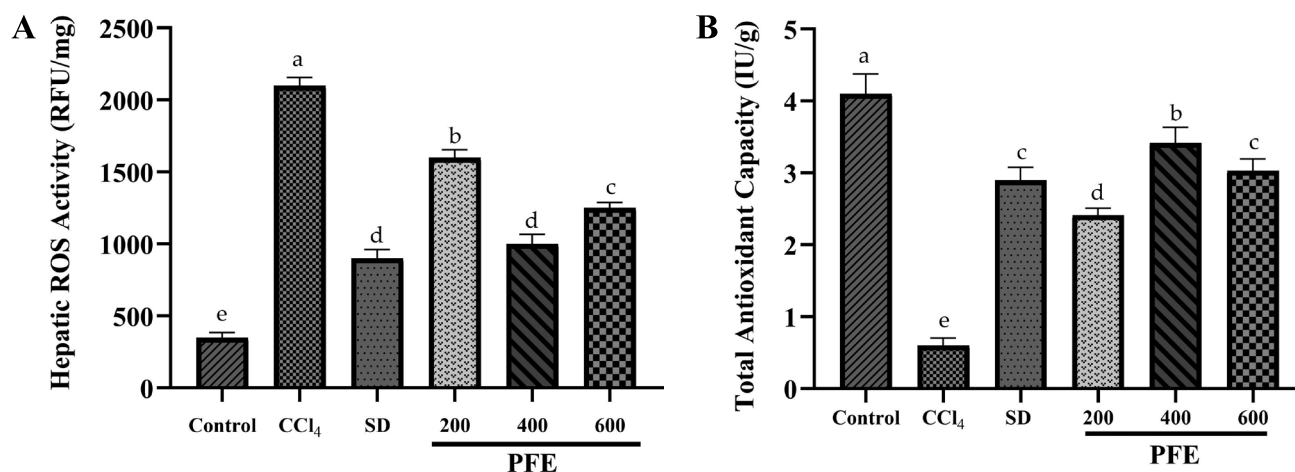


Fig. 3. Effect of *P. fistulosus* extract on A) Hepatic ROS activity in liver tissues, B) Serum total antioxidant capacity (TAC) in CCl₄-induced hepatic injury model.

Mean $\pm$ S.D values were calculated using data from six rats per group. Bars labelled with distinct letters show a significant difference ($p < 0.05$).

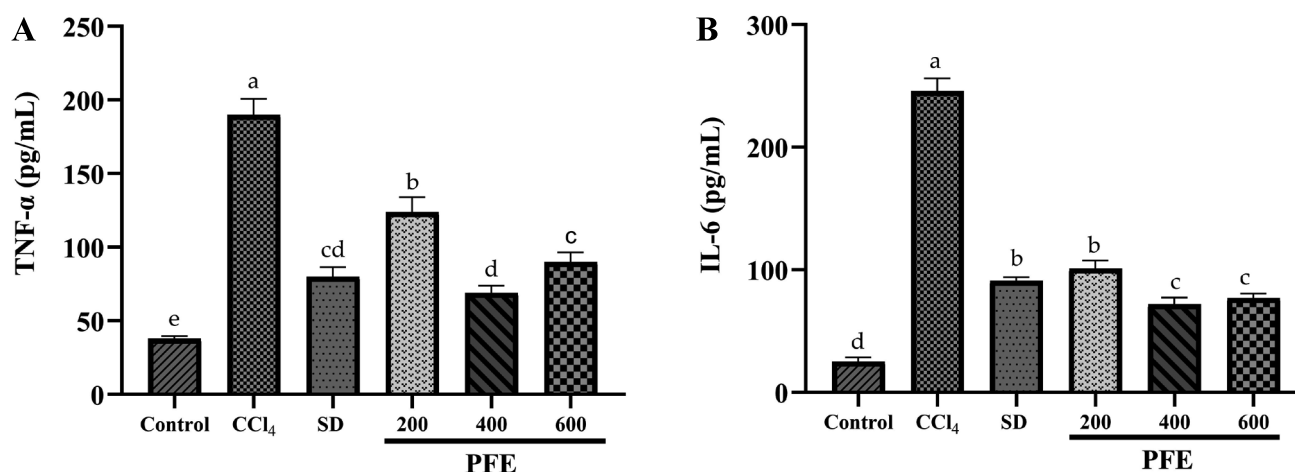


Fig. 4. Effect of *P. fistulosus* extract on (A) TNF- α (B) IL-6 in liver tissues of a CCl₄-induced hepatic injury model.

Mean $\pm$ SD values were calculated using data from six rats per group. Bars labelled with distinct letters show a significant difference ($p < 0.05$).

(TFC) compared to previously reported solvent extracts for the Indian variety of *P. fistulosus*, confirming methanol as an efficient solvent for extracting antioxidant constituents (Bollavarapu et al. 2016). These findings were further supported by the DPPH assay, where methanolic extract exhibited the highest antioxidant activity reported to date, exceeding previously documented values 57% (Aqueous), 59% (Ethyl acetate), 66% (Chloroform) and 73% (Hexane). This is attributed to the superior extraction and radical scavenging efficiency of methanol compared to other solvents. (Mehmood et al. 2022).

In this study, hepatotoxicity was induced by sub-chronic intermittent administration of CCl₄. The effect of *P. fistulosus* extract was explored and compared with the CCl₄-treated group. Silymarin is a well-documented herbal medicinal product recognized for its ability to counteract the impact of CCl₄

through the inhibition of lipid peroxidation (El Rabey et al. 2021). A silymarin-treated group was therefore included in the study as a standard therapeutic control. Body weight variation serves as an indicator of general toxicity and health status (Saganuwan 2017), therefore, body weight was used as a complementary marker to evaluate their health condition. All rat groups exhibited weight gain, with the CCl₄-treated group showing the lowest increase, consistent with earlier findings (Shameenii et al. 2021). Liver index is an indirect measure of inflammation and damage to hepatocytes. The elevated liver index in CCl₄-treated rats reflected hepatomegaly, while co-administration of silymarin or *P. fistulosus* extract normalized the values, indicating restoration of hepatic health. Similar findings have been reported previously in the literature (Govindan et al. 2021), indicating the antioxidant and hepatoprotective potential of plant-derived compounds.

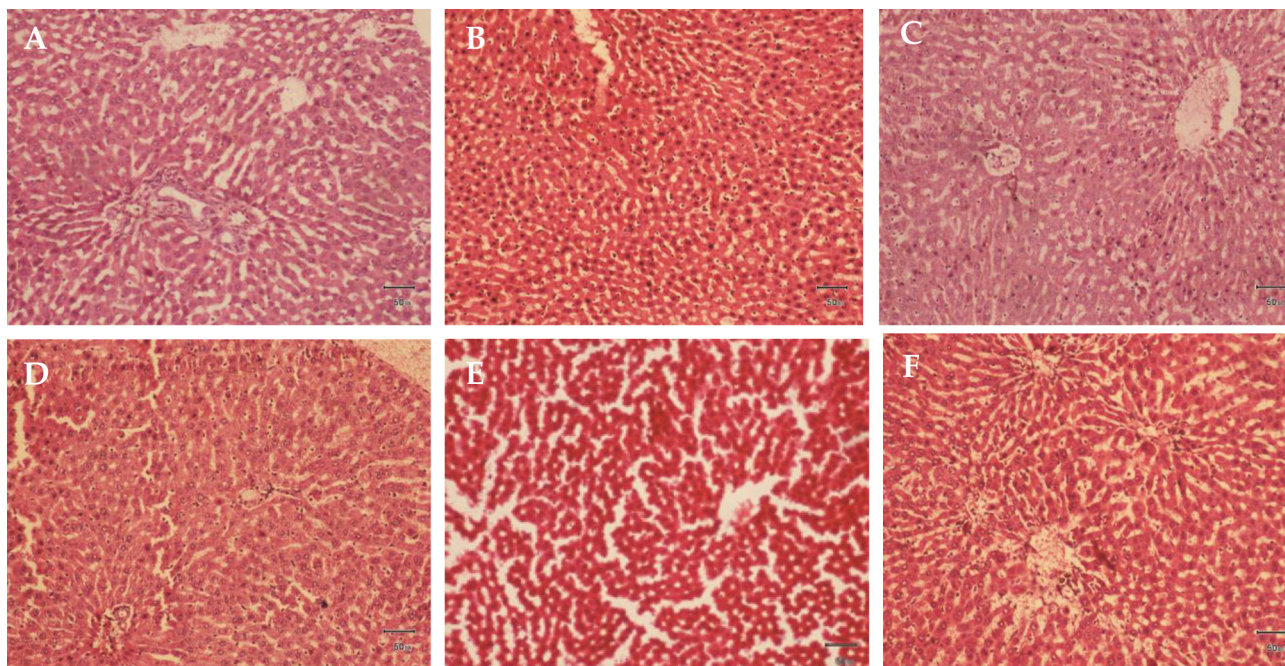


Fig. 5. Effect of *P. fistulosus* extract on histopathology of liver in CCl_4 -induced hepatic injury model using H&E.

Histopathological findings of CCl_4 -treated groups was compared with normal control and all other groups with CCl_4 -treated groups. H&E; Hematoxylin & Eosin; scale bar = 50 μm .

Elevated levels of hepatic enzymes serve as a biomarker of liver injury. Leakage of cytoplasmic enzymes such as AST, ALT, and ALP into circulation reflects hepatocellular membrane disruption (Ouassou et al. 2021), while elevated bilirubin levels indicate compromised bile function (VC et al. 2000). In this investigation, hepatic biomarkers were drastically raised while total protein and albumin levels were decreased in the CCl_4 -treated group relative to the normal group, confirming the hepatic damage. All three doses of *P. fistulosus* extract significantly reduced the elevated ALT, AST and ALP, with the 400 and 600 mg/kg showing efficacy comparable to the silymarin. In addition, restoration of total protein, albumin and bilirubin levels in *P. fistulosus* extract-treated groups advocates improved hepatocellular function by alleviating the effects of CCl_4 . Similar findings were reported in a 3-day acute injury model using lower doses (100 and 200 mg/kg), confirming dose-dependent hepatoprotection (Madhu et al. 2019). This effect is plausibly linked to the significant presence of flavonoids in *P. fistulosus* extract, contributing to hepatocyte membranes stabilization against lipid peroxidation, it confer anti-apoptotic protection through regulation of Bcl-2 and caspase signaling pathways (Saha et al. 2019).

Reactive oxygen species (ROS) play a crucial role in maintaining cellular homeostasis by facilitating redox signaling at optimal levels, however, they are detrimental at elevated concentrations (Zhang et al. 2022). Excessive ROS production signals redox imbalance and oxidative stress, indicated by markers like MDA,

a byproduct of lipid peroxidation (J et al. 2016). Our results indicated that CCl_4 exposure significantly increased ROS and MDA levels while decreasing CAT activity, reflecting oxidative stress-mediated hepatocellular damage (Liu et al. 2002), consistent with (Elbakry et al. 2019) findings. Administration of *P. fistulosus* extract showed strong antioxidant properties, evidenced by reduced ROS and MDA levels and enhanced CAT and TAC activity (a critical marker of antioxidant activity). This dose-dependent effect reinforces previous findings on its antioxidant potential (Karandikar et al. 2014, Bollavarapu et al. 2016, Madhu et al. 2019). This antioxidant-mediated hepatoprotection mirrors the MOS-driven effects reported by (Duan et al. 2025) during cadmium exposure, underscoring the multi-model defense of phytochemical-rich extracts against chemically induced hepatic insults. The extract's efficacy in sustaining redox homeostasis likely stems from the synergistic action of its phytochemicals. Phenolic compounds and saponins reinforce hepatic antioxidant defense by enhancing endogenous enzyme activity and neutralizing reactive oxygen species. Alkaloids further contribute by activating the Nrf2–ARE signaling pathway, which induces the transcription of antioxidant and phase II detoxifying enzymes, thereby promoting glutathione biosynthesis and redox regulation (Belka et al. 2024). Flavonoids complement these effects by upregulating key enzymes such as glutathione-S-transferase and superoxide dismutase, collectively sustaining cellular redox homeostasis (Ma et al. 2025).

At a molecular level, CCl_4 -mediated oxidative

stress activates inflammatory cells, leading to the excessive release of pro-inflammatory cytokines such as TNF- α and IL-6 (Hamid et al. 2017). TNF- α triggers neutrophil activation, resulting in free radical release, causing hepatocyte necrosis or apoptosis. *P. fistulosus* extract significantly reduced ($p < 0.01$) TNF- α and IL-6 levels, with the strongest anti-inflammatory effects observed at 400 and 600 mg/kg. This further strengthens the evidence supporting the antioxidant potential of *P. fistulosus*, emphasizing its effectiveness in scavenging hydroxyl radicals and superoxide anions (Ishnava et al. 2020). Flavonoids and saponins attenuate inflammation by suppressing NF- κ B and TLR4 signaling, thereby downregulating pro-inflammatory cytokines including TNF- α and IL-6. Phenolic compounds complement this action by modulating these pathways and epigenetically regulating cytokine expression to maintain hepatic immune stability (Prasad et al. 2023). At another mechanistic level, previous reports indicate that TNF- α and IL-6 downregulate hepatic albumin synthesis during systemic inflammation as part of the negative acute-phase protein response. Although correlation analysis was beyond the scope of the present data set, the observed restoration of albumin levels in extract-treated groups may indirectly reflect cytokine modulation, indicating the anti-inflammatory potential of *P. fistulosus* extract (Hadžimusić et al. 2025).

At the histopathological level, CCl₄ caused hepatic cord disruption, necrosis, and inflammatory infiltration, consistent with earlier reports (Sayed et al. 2019). Treatment with *P. fistulosus* extract significantly improved liver histology, showing only mild necrotic changes at 200 mg/kg, while nearly normal hepatocellular architecture at 400 and 600 mg/kg. These histological findings reinforce the biochemical results, confirming the extract's restorative effect on liver tissue. These observations align with earlier investigation in which, at doses 100 and 200 mg/kg, mild cellular necrosis was depicted (Madhu et al. 2019). Consistent with the melatonin-mediated histopathological improvements reported by (Şengül et al. 2024), our findings indicated that *P. fistulosus* extract effectively mitigated liver injury caused by CCl₄ in a dose-dependent manner. Similar findings have been reported in previous studies involving various plant extracts (Alsahli et al. 2021, Johra et al. 2023). Both CCl₄ and cadmium exemplify environmental toxicants that provoke oxidative stress; notably, cadmium-induced activation of the MLKL-Drp1 signaling cascade contributes to nephrotoxicity and can be mitigated by targeting this pathway using mitochondria-targeted antioxidants or Drp1 inhibitors to restore mitochondrial homeostasis and reduce oxidative stress-mediated damage (Lian et al. 2025). While *P. fistulosus* attenuates oxidative stress, its mechanistic

action pathway has yet to be fully elucidated, necessitating further investigation into gene and protein expression modulation.

Conclusion

The current findings demonstrated the substantial antioxidant potential of *P. fistulosus* extract. Moreover, it possesses anti-inflammatory and hepatoprotective effects in rats with CCl₄-induced sub chronic hepatotoxicity. It was evident by a significant reduction in serum hepatic biomarkers, oxidative stress markers, further confirmed by histopathological analysis. The effects of silymarin and *P. fistulosus* extract were comparable, particularly at doses of 400 and 600 mg/kg. Overall, the findings from this study present new possibilities for utilizing the *P. fistulosus* fruit cultivated in Pakistan in the treatment of chronic hepatic damage. However, the underlying mechanism remains to be clarified.

References

- Abdel-Moneim AM, Al-Kahtani MA, El-Kersh MA, Al-Omair MA (2015) Free radical-scavenging, anti-inflammatory/anti-fibrotic and hepatoprotective actions of taurine and silymarin against CCl₄ induced rat liver damage. *PLoS One* 10: e0144509.
- Abdelgalil MH, Elhammamy RH, Ragab HM, Sheta E, Wahid A (2024) The hepatoprotective effect of 4-phenyltetrahydroquinolines on carbon tetrachloride induced hepatotoxicity in rats through autophagy inhibition. *Biol Res* 57: 32.
- Adegbola P, Aderibigbe I, Hammed W, Omotayo T (2017) Antioxidant and anti-inflammatory medicinal plants have potential role in the treatment of cardiovascular disease: a review. *Am J Cardiovasc Dis* 7: 19.
- Alsahli MA, Almatroodi SA, Almatroodi A, Khan AA, Anwar S, Almutary AG, Alrumaihi F, Rahmani AH (2021) 6-Gingerol, a major ingredient of ginger attenuates diethylnitrosamine-induced liver injury in rats through the modulation of oxidative stress and anti-inflammatory activity. *Mediators Inflamm* 2021: 6661937.
- Belka M, Gostyńska-Stawna A, Stawny M, Krajka-Kuźniak V (2024) Activation of Nrf2 and FXR via Natural Compounds in Liver Inflammatory Disease. *Int J Mol Sci* 25: 11213.
- Benzie IF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 239: 70-76.
- Blois MS (1958) Antioxidant determinations by the use of a stable free radical. *Nature* 181: 1199-1200.
- Bollavarapu A, Rapaka G, Tamanam R (2016) Phytochemical evaluation and antioxidant potential of *Pracitrrullus fistulosus* fruit extracts. *IOSR J Environ Sci Toxicol Food Technol* 10: 23-28.
- Chuah PN, Nyanasegaram D, Yu K-X, Razik RM, Al-Dhalli S, Kue CS, Shaari K, Ng CH (2020) Comparative conventional extraction methods of ethanolic extracts of *Clinacanthus*

- nutans leaves on antioxidant activity and toxicity. *Br Food J* 122: 3139-3149.
- Duan E, Gao W, Liu C, Wang J, Wang J, Su L, Qiao H, Wang P, Huang J, Gan L (2025) Protective effect of mannose oligosaccharides on cadmium-induced hepatic oxidative damage in rats. *Pol J Vet Sci* 28: 311-320.
- El Rabey HA, Rezk SM, Sakran MI, Mohammed GM, Bahattab O, Balgoon MJ, Elbakry MA, Bakry N (2021) Green coffee methanolic extract and silymarin protect against CCl₄-induced hepatotoxicity in albino male rats. *BMC Complement Med Ther* 21: 1-11.
- Elbakry MA, Rabey HAE, Elremaly W, Sakran MI, Almutairi FM (2019) The methanolic extract of *Moringa oleifera* attenuates CCl₄ induced hepatonephro toxicity in the male rat. *Biomed Res* 30: 23-31.
- Erdemli M, Yigitcan B, Gul M, Bag H, Aksungur SGZ (2018) Thymoquinone is protective against 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin induced hepatotoxicity. *Biotech Histochem* 93: 453-462.
- Fareed MM, Khalid H, Khalid S, Shityakov S (2024) Deciphering Molecular Mechanisms of Carbon Tetrachloride-Induced Hepatotoxicity: A Brief Systematic Review. *Curr Mol Med* 24: 1124-1134.
- GBD 2019 Diseases and Injuries Collaborators (2020) Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. *Lancet* 396: 1204-1222.
- Govindan S, Jayabal A, Shanmugam J, Ramani P (2021) Antioxidant and hepatoprotective effects of *Hypsizygus ulmaris* polysaccharide on alcoholic liver injury in rats. *Food Sci Hum Wellness* 10: 523-535.
- Griffin C, Agbim U, Ramani A, Shankar N, Kanwal F, Asrani SK (2023) Underestimation of Cirrhosis-Related Mortality in the Medicare Eligible Population, 1999-2018. *Clin Gastroenterol Hepatol* 21: 223-225. e223.
- Hadwan MH, Abed HN (2016) Data supporting the spectrophotometric method for the estimation of catalase activity. *Data in Brief* 6: 194-199.
- Hadžimusić N, Hadžijunuzović-Alagić D (2025) Mediators of the acute phase response: the role of cytokines. *Int J Agric Environ Res* 11: 337-350.
- Hamid M, Liu D, Abdulrahim Y, Liu Y, Qian G, Khan A, Gan F, Huang K (2017) Amelioration of CCl₄-induced liver injury in rats by selenizing *Astragalus* polysaccharides: role of proinflammatory cytokines, oxidative stress and hepatic stellate cells. *Res Vet Sci* 114: 202-211.
- Harborne JB (1973) *Phytochemical methods: a guide to modern techniques of plant analysis*. 1st ed., Chapman and Hall, London, pp 5-11.
- Higuchi H, Gores GJ (2003) Mechanisms of liver injury: an overview. *Curr Mol Med* 3: 483-490.
- Hu G, Li X, Lai A, Liu Y, Zhang Y, Wang J, Sun S, Zhu J, Yang M (2023) Comparative analysis of the nutritional quality of *Zizania latifolia* cultivars harvested in different growing seasons. *Foods* 13: 30.
- Ishnava KB, Patel KS (2020) In vitro study of *Praecitrullus fistulosus* (Stocks) Pangalo (Cucurbitaceae) fruit – a potential candidate of anthelmintic activity. *Bull Natl Res Cent* 44: 1-10.
- JA, E R-T, PM (2016) Redox state and methods to evaluate oxidative stress in liver damage: From bench to bedside. *Ann Hepatol* 15: 160-173.
- Jiang Z, Kempinski C, Chappell J (2016) Extraction and analysis of terpenes/terpenoids. *Curr Protoc Plant Biol* 1: 345-358.
- Johra FT, Hossain S, Jain P, Bristy AT, Emran T, Ahmed R, Sharker SM, Bepari AK, Reza HM (2023) Amelioration of CCl₄-induced oxidative stress and hepatotoxicity by *Ganoderma lucidum* in long evans rats. *Sci Rep* 13: 9909.
- Karandikar A, Prasath GS, Subramanian S (2014) Evaluation of Antidiabetic and antioxidant activity of *Praecitrullus fistulosus* fruits in STZ Induced Diabetic Rats. *Res J Pharm Technol* 7: 196-203.
- Li C, Yi LT, Geng D, Han YY, Weng LJ (2015) Hepatoprotective effect of ethanol extract from *Berchemia lineate* against CCl₄-induced acute hepatotoxicity in mice. *Pharm Biol* 53: 767-772.
- Liu DH, Su YP, Zhang W, Lu SF, Ran XZ, Gao JS, Cheng TM (2002) Changes in glucocorticoid and mineralocorticoid receptors of liver and kidney cytosols after pathologic stress and its regulation in rats. *J Crit Care Med* 30: 623-627.
- Ma X, Xiong S, Xiang F, Li Y, Lin Y, Liu Y, Lin L, Xie J (2025) The Protective Effect of Phenolic Acids on Liver Disease: A Review of Possible Mechanisms. *Antioxidants* 14: 1247.
- Madhu CS, Balaji KS, Sharada AC (2019) GC/MS profile, DNA protectant and hepatoprotective effects of *Praecitrullus fistulosus* fruit methanol extract. *Biocatal Agric Biotechnol* 21: 101272.
- Mahadevan V (2020) Anatomy of the liver. *Surgery* 38: 427-431.
- Mehmood A, Javid S, Khan MF, Ahmad KS, Mustafa A (2022) In vitro total phenolics, total flavonoids, antioxidant and antibacterial activities of selected medicinal plants using different solvent systems. *BMC Chemistry* 16: 64.
- Mohdaly AAA, Hassanien MFR, Mahmoud A, Sarhan MA, Smetanska I (2013) Phenolics extracted from potato, sugar beet, and sesame processing by-products. *Int J Food Prop* 16: 1148-1168.
- Mokdad AA, Lopez AD, Shahrz S, Lozano R, Mokdad AH, Stanaway J, Murray CJ, Naghavi Mohsen (2014) Liver cirrhosis mortality in 187 countries between 1980 and 2010: a systematic analysis. *BMC Med* 12: 1-24.
- Mukherjee PK, Singha S, Kar A, Chanda J, Banerjee S, Dasgupta B, Halder PK, Sharma N (2022) Therapeutic importance of Cucurbitaceae: a medicinally important family. *J Ethnopharmacol* 282: 114599.
- Nivukoski U, Niemelä M, Bloigu A, Bloigu R, Aalto M, Laatikainen T, Niemelä O (2020) Combined effects of lifestyle risk factors on fatty liver index. *BMC Gastroenterol* 20: 1-10.
- Ogundola AF, Bvenura C, Ehigie AF, Afolayan AJ (2022) Effects of soil types on phytochemical constituents and antioxidant properties of *Solanum nigrum*. *S Afr J Bot* 151: 325-333.
- Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 95: 351-358.
- Ouassou H, Bouhrim M, Daoudi NE, Mekhfi H, Ziyat A, Legssyer A, Aziz M, Bnouham M (2021) Evaluation of Hepatoprotective Activity of *Caralluma europaea* Stem Extract against CCl₄-Induced Hepatic Damage in Wistar Rats. *Adv Pharmacol Pharm Sci* 2021: 8883040.
- Prasad S, Kumar V, Singh C, Singh A (2023) Crosstalk between phytochemicals and inflammatory signaling pathways. *Inflammopharmacology* 31: 1117-1147.

- Saganuwan SA (2017) Toxicity studies of drugs and chemicals in animals: an overview. *Bulg J Vet Med* 20: 291-318.
- Saha P, Talukdar AD, Nath R, Sarker SD, Nahar L, Sahu J, Choudhury MD (2019) Role of natural phenolics in hepato-protection: a mechanistic review and analysis of regulatory network of associated genes. *Front Pharmacol* 10: 509.
- Sayed HESAE, Morsy LES, Emara TMA, Galhom RA (2019) Effect of carbon tetrachloride (CCl₄) on liver in adult albino rats: histological study. *Egypt J Hosp Med* 76: 4254-4261.
- Şengül SA, Taşkın İİ, Aşır F, Şakar AE, Şengül GP (2024) Melatonin prevents nicotine-induced hepatotoxicity by modulating apoptosis and histopathological changes in rats. *Pol J Vet Sci*: 589-597.
- Shameenii A, Vun-Sang S, Iqbal M (2021) Hepatoprotective effects of *Pandanus amaryllifolius* against carbon tetrachloride (CCl₄) induced toxicity: a biochemical and histopathological study. *Arab J Chem* 14: 103390.
- Shivamadhu MC, Srinivas BK, Jayarama S, Chandrashekaraiah SA (2017) Anti-cancer and anti-angiogenic effects of partially purified lectin from *Praecitrullus fistulosus* fruit on in vitro and in vivo model. *Biomed Pharmacother* 96: 1299-1309.
- Slinkard K, Singleton VL (1977) Total phenol analysis: automation and comparison with manual methods. *Am J Enol Vitic* 28: 49-55.
- Unuofin JO, Lebelo SL (2020) Antioxidant effects and mechanisms of medicinal plants and their bioactive compounds for the prevention and treatment of type 2 diabetes: an updated review. *Oxid Med Cell Longev* 2020: 1356893.
- VC K, ZK I (2000) Plasma erythropoietin level in rats after kidney proximal tubular impairment. *Folia Med* 42: 41-45.
- Xie J, Schaich K (2014) Re-evaluation of the 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH) assay for antioxidant activity. *J Agric Food Chem* 62: 4251-4260.
- Yu L (2021) Pathogenesis of Liver Injury and Hepatic Failure. In: *Artificial Liver*. Springer Nature Singapore. pp 105-166.
- Zhang B, Pan C, Feng C, Yan C, Yu Y, Chen Z, Guo C, Wang X (2022) Role of mitochondrial reactive oxygen species in homeostasis regulation. *Redox Report* 27: 45-52.