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RESEARCH ARTICLE

Pomegranate Peel Extract with Antioxidant Activity Enhances Immunity, Growth Performance, Oxidative Stress and Tissue Histology in CCl_4-Challenged Mice

Kamlah Ali Majrashi[1*]

[1]Biological Sciences Department, College of Science & Arts, King Abdulaziz University, Rabigh, 21911, Saudi Arabia
*Corresponding author: Kmajrashi@kau.edu.sa (Kamlah Ali Majrashi)

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A B S T R A C T

Polyphenolic-enriched extract has antioxidant and anti-inflammatory properties and helps maintain immune system balance; therefore, the aim of the study was to investigate the effects of polyphenol content in pomegranate peel extract on the immune system. Pomegranate (*Punica granatum*) processing generates substantial peel waste, which is rich in bioactive ellagitannins and flavonoids. This study investigates the therapeutic potential of pomegranate peel extract (PPE) to enhance immunity and restore tissue histology in mice challenged with carbon tetrachloride (CCl_4). *In-vivo* experiment, five distinct groups of male Swiss albino mice were as follow; T1, control group, T2, challenged group receiving carbon tetrachloride (CCl_4 at 1mL/kg, i.p., twice a week), T3 and T4 experimental CCl_4-challenged groups and treated with a low dose (100 mg/kg) or high dose (300 mg/kg) of PPE, and T5, positive control group given CCl_4 plus silymarin (50 mg/kg) to evaluate their effects on the immune system. All treatments were administered orally for 28 days. Assessed parameters included oxidative stress markers [malondialdehyde (MDA), protein carbonyl (PC)], antioxidant enzyme activities [superoxide dismutase (SOD), catalase (CAT), peroxidase (GPx), glutathione reduced (GSH)], immunological markers (serum immunoglobulins, IL-6, TNF-α, splenocyte proliferation), and histopathology of liver and spleen. HPLC analysis showed that the main compounds in PPE were punicalagin A (32.1mg/g), punicalagin B (15.6mg/g), ellagic acid (11.3mg/g), gallic acid (8.7mg/g) and catechin (4.2mg/g). PPE (300ug/ml) scavenged 82 and 38% of DPPH and ABTS free radicals, respectively. *In-vivo*, the PPE significantly ($P<0.05$) reversed oxidative stress by improving the activities of hepatic SOD, CAT and GPx and levels of GSH and markedly decreased MDA and PC in comparison with CCl_4. Immunologically, the PPE increased serum IgG/IgM, decreased pro-inflammatory cytokines (IL-6, TNF-α) and enhanced splenocyte proliferation. Histologically, the PPE significantly ($P<0.05$) attenuated the CCl_4-induced hepatocellular necrosis, inflammatory infiltration and splenic lymphoid depletion in a dose-dependent manner, with the high dose restoring near-normal architecture like silymarin. The pomegranate peel exhibits potent antioxidant activity, which ameliorates the oxidative stress induced by CCl_4. It boosts immunity and restores active tissue histology.

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INTRODUCTION

Pomegranate (*Punica granatum* L.) peel is particularly rich in polyphenolic compounds such as punicalagin, ellagic acid, hydrolyzable tannins and flavonoids. Several studies have reported that pomegranate peel has greater antioxidant potential than other parts of the fruit, particularly the arils (Benchagra *et al.*, 2021). Studies have consistently shown that these extracts can effectively scavenge radicals, prevent lipid peroxidation, protect LDL against oxidative modification, and bring ROS levels way down in stressed cells (Benchagra *et al*., 2021; Olchowik-Grabarek *et al.*, 2024). Beyond *in-vitro* assays, pomegranate peel and whole fruit extracts have improved antioxidant status and reduced lipid peroxidation in multiple organs, including liver,

kidney, heart and testes, in toxin-challenged rodent models (Vanani *et al.*, 2020; Jebur *et al.*, 2023). These findings position pomegranate peel as a promising, underutilized agro-industrial waste product with considerable therapeutic potential.

Beyond its generic antioxidant effects, pomegranate peel extract (PPE) exerts well documented anti-inflammatory and anti-apoptotic actions *in-vivo*. In rats subjected to intoxication with acrylamide or pesticides, PPE or pomegranate peel gel extract (PGPE) lowered malondialdehyde (MDA) and ROS, restored reduced glutathione (GSH) and enzymatic antioxidants (superoxide dismutase, catalase, glutathione peroxidase), reduced pro inflammatory cytokines, and modulated apoptosis related markers such as p53, caspase 3, Bcl-2 and transforming growth factor β1 (Sayed *et al.*, 2022; Jebur *et al*., 2023).

In models of metabolic syndrome, pomegranate peel polyphenols modulated splenic oxidative status and immune-related factors, increasing catalase and GPx while lowering ROS/RNS (Rak-Pasikowska *et al.*, 2024). Furthermore, pomegranate extract reduced basal and inducible ROS in leukocytes of aging mice, thereby helping preserve immune cell function (Verdú *et al.*, 2025).

In parallel, carbon tetrachloride (CCl_4) is a well-established hepatotoxin whose metabolic activation by cytochrome P450 enzymes, primarily CYP2E1, generates trichloromethyl radicals ($\bullet CCl_3$) and peroxytrichloromethyl radicals ($\bullet OOCCL_3$). These reactive species drive lipid peroxidation, depletion of cellular antioxidants, and subsequent inflammation, cell death, fibrosis, and multi organ injury (Unsal *et al.*, 2020). Due to its reproducible and progressive pathophysiology, the CCl_4-challenged rodent model remains a benchmark for evaluating hepatoprotective and antioxidant agents. Studies have demonstrated that a broad spectrum of phytochemicals, including poncirin, 4-octyl itaconate, apigenin, tea polyphenols, salidroside, nebivolol, chlorophyllin, AdipoRon, and zerumbone, can effectively safeguard the liver from CCl_4-induced injury in murine models. These agents have been shown to improve serum transaminases (ALT, AST), restore hepatic antioxidant enzyme levels, reduce malondialdehyde (MDA), down-regulate pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and mitigate histopathological liver damage. (Li *et al.*, 2021; Melaibari *et al.*, 2023; Wang *et al.*, 2025).

This work confirms that targeting oxidative stress and inflammatory signaling is an effective strategy in CCl_4-induced injury. However, most studies focus primarily on liver function and do not systematically assess broader immune status, growth performance, or extra-hepatic histopathology.

Despite strong evidence that pomegranate peel is richer in total phenolics and exhibits superior antioxidant and anti-inflammatory activity compared to other fruit fractions (Chukwuma *et al.*, 2020; Benchagra *et al*., 2021; Olchowik-Grabarek *et al*., 2024). Its protective potential in a CCl_4-challenged mouse model has not been directly investigated. Existing in vivo studies with PPE employ other toxicants (acrylamide, fenpropathrin, tert-butyl hydroperoxide) and rarely integrate endpoints such as body weight gain, feed intake, systemic immunity, organ-specific oxidative biomarkers and detailed multi-tissue histology within a single experimental design (Bagheri *et al.*, 2021; Rak-Pasikowska *et al*., 2024).

Consequently, a clear knowledge gap exists regarding whether, and by what mechanisms, pomegranate peel extract with high antioxidant activity can ameliorate CCl_4-induced oxidative stress, modulate immune responses, support growth performance, and protect tissue histology in mice in a single integrated experiment.

Addressing this gap is novel because it combines: (i) a well-characterized oxidative hepatotoxic model (CCl_4) that recapitulates key features of chemically induced oxidative injury (Unsal *et al*., 2020)(ii) a polyphenol-rich, underutilized agro-waste source with superior antioxidant properties (pomegranate peel) (Benchagra *et al*., 2021); and (iii) simultaneous evaluation of performance (body weight, feed intake), systemic immunity (splenic or leukocyte markers), oxidative biomarkers (MDA, GSH, SOD, CAT, GPx) in multiple tissues, and detailed multi-organ histology in CCl_4-challenged mice endpoints that current PPE and CCl_4 antioxidant studies do not jointly provide. Based on this background, the proposed study aims to evaluate the protective effects of pomegranate peel extract in CCl_4-challenged mice by characterizing its impact on growth performance and basic clinical indices, assessing systemic and tissue-specific oxidative stress markers and antioxidant defenses, examining the modulation of immune and inflammatory mediators, and investigating histopathological changes in the liver and spleen to clarify the structural correlates of functional protection.

MATERIALS AND METHODS

Plant material and extraction: Pomegranate (*Punica granatum*) peels were collected, washed, air-dried and ground into fine powder. The powder was subjected to hydroethanolic extraction using 70% ethanol (1:10 w/v) under continuous stirring at room temperature for 72 hours. The extract was filtered through Whatman No. 1 filter paper, and the solvent was evaporated under reduced pressure using a rotary evaporator at 40°C. The resulting crude extract (PPE) was lyophilized and stored at −20°C until further use.

HPLC phytochemical profiling: Qualitative and quantitative analyses of phenolic compounds in PPE were analyzed using high-performance liquid chromatography (HPLC) coupled with a diode-array detector (DAD). Chromatographic separation was achieved on a reversed-phase C18 column (250×4.6mM, 5µm) maintained at 30°C (Li *et al.*, 2015). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile) with a gradient elution program at a flow rate of 1.0mL/min. Detection wavelengths were set at 280 nm for punicalagin A, punicalagin B, gallic acid, catechin and at 360 nm for ellagic acid (Ding *et al.*, 2019). Identification and quantification were performed by comparing retention times and peak areas with authentic external standards. Results were expressed as milligrams of compound per gram of dry extract (mg/g).

***In-vitro* antioxidant assays (DPPH and ABTS):** The radical scavenging capacity of PPE was evaluated using

DPPH and ABTS assays across a concentration (25, 50, 100, 150, 300 and 600 ug/mL). For the DPPH assay, PPE solutions were combined with 100 µg/mL methanolic DPPH solution and incubated in the dark at room temperature for 30 minutes. Absorbance was recorded at 517nM using a UV-Vis spectrophotometer. Scavenging activity was calculated using the following equation:

IC_{50} values were subsequently determined via non-linear regression analysis (Malviya *et al*., 2014; Sihag *et al*., 2022).

Scavenging activity = (*A* control-*A* sample/*A* control) x100

For the ABTS assay, the $ABTS^{+}$ radical cation stock reagent was diluted to an initial absorbance of 0.70 at 734nM. PPE concentrations were then reacted with the ABTS+ solution, and absorbance was measured at 734 nm after a 6-minute incubation.

Experimental set: The study was carried out on male Swiss albino mice (8-10 weeks old; initial body weight 22- 24g) obtained from the institutional animal house. The animals were acclimatized for one week under standard laboratory conditions (temperature 22±2°C; relative humidity 50-60%; 12h light/dark cycle) and were provided with water and commercial mice chow *ad libitum*. After acclimatization, the animals were divided into five experimental groups (n = 10 each). The first group (T1, normal control) received distilled water orally along with intraperitoneal injection of vehicle (olive oil; 1mL/kg body weight) twice weekly. The second group (T2, CCl_4 control) received distilled water orally and intraperitoneally administered CCl_4 (1mL/kg body weight, diluted 1:1 in olive oil) twice weekly. The third and fourth groups (T3, T4) were administered PPE orally at doses of 100 and 300mg/kg body weight, respectively, daily, along with CCl4 intraperitoneal injections twice weekly. The fifth group (T5) was administered the reference compound, silymarin (50 mg/kg body weight, p.o.), daily alongside the CCl_4 treatment. All treatments were carried out over a continuous 28-day period. On the designated dosing days, the intraperitoneal CCl_4 injection was administered at 2-hour intervals following the respective oral administrations (Fig. 1).

Growth performance and mortality: Throughout the four-week testing period, the mice were examined daily to assess mortality and detect any emerging pathological symptoms. Body weights were recorded at the start (initial) and at the end (final) of the study. Feed intake was measured weekly. Total body weight gain was calculated as final weight minus initial weight. Mortality percentage was recorded for each group (Niewiadomska *et al.*, 2023).

Carcass and organ weight determination: At the end of the experimental period, mice were euthanized by cervical dislocation, as described by Davidge *et al.* (2025). The carcass weight was recorded immediately after skinning and evisceration. The dressing percentage was calculated as

$$Dressing\ \% = \frac{Carcass\ weight}{FBW} x100$$

Vital organs (liver and spleen) were carefully dissected, blotted dry, and weighed using an analytical balance. Absolute organ weights were expressed in grams (Marxfeld *et al.*, 2019).

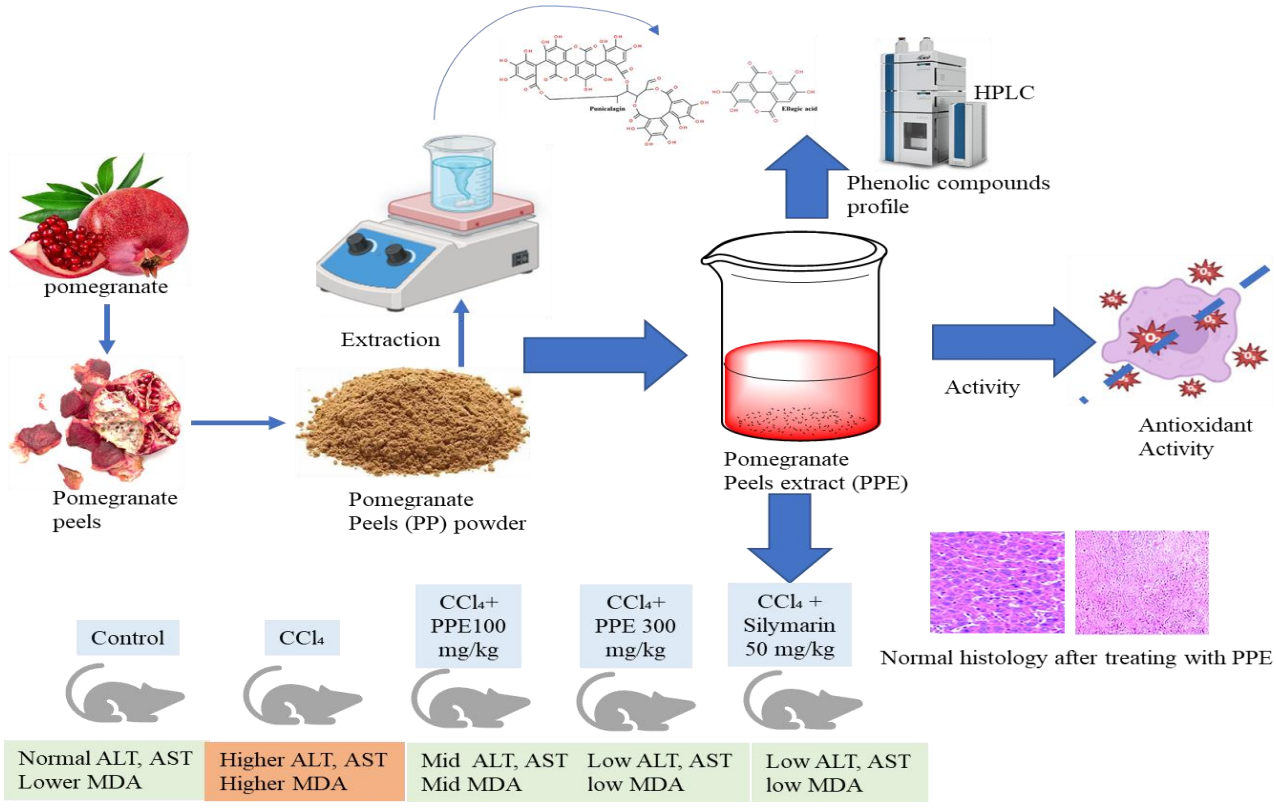

Fig. 1: Experimental setup of the effect of dietary pomegranate peel extract on the CCl_4-induced oxidative stress in male Swiss albino mice model.

Blood collection and hematological analysis: Blood samples were obtained through retro-orbital puncture and divided into two sets of tubes: one containing EDTA as an anticoagulant for hematological analysis and another without anticoagulant for serum separation (Kulkarni *et al.*, 2016; Frohlich *et al.*, 2018). EDTA-whole blood was analyzed within 4 hours using an automated hematology analyzer (BC 3000, Mindray, China) (Fukuda *et al.*, 2017). The blood profile analysis included measurements of red blood cell (RBCs) count, total platelet count, hemoglobin (Hb) levels, platelet distribution width (PDW), and a differential white blood cell count assessing neutrophils, lymphocytes and monocytes. All results are presented in standard units ($\times10^3/\mu L$ or $\times10^6/\mu L$, g/dL) (Schroeder *et al.*, 2024; Andrade *et al.*, 2025).

Serum biochemical assays: Non-anticoagulated blood samples were allowed to clot at room temperature for 30 minutes before centrifugation (4°C, 3,000 rpm, 15 minutes). After separation, serum was stored at -80°C until further analysis. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine were measured using commercially obtained colorimetric kits according to manufacturer's instructions (Sher and Hung, 2013; De Almeida Júnior *et al*., 2020). Absorbances were read using a semi-automated biochemistry analyzer (Lim *et al*., 2023).

Oxidative markers: Liver and spleen tissues were prepared as 10% (w/v) homogenates in cold PBS (pH 7.4, 4°C) and centrifuged at 14000 × g for 15 minutes at 4°C; the supernatants were collected for biochemical analysis. Total protein concentration was quantified using the Bradford assay (Bradford, 1976; Balkan *et al*., 2025). Oxidative damage markers were determined and presented (nmol/mg protein), including lipid peroxidation via thiobarbituric acid reactive substances (TBARS) at 532 nm (De León & Borges, 2020) and protein carbonyl (PC) accumulation following 2,4-dinitrophenylhydrazine (DNPH) derivatization at 370nM (Soglia *et al*., 2016). Antioxidant enzyme activities were evaluated and expressed as U/mg protein: superoxide dismutase (SOD) by the inhibition of nitroblue tetrazolium (NBT) reduction at 560nM (Sun *et al*., 1988), catalase (CAT) by tracking H_2O_2 decomposition at 240nM (Grilo *et al*., 2020) and glutathione peroxidase (GPx) using cumene hydroperoxide as a substrate at 340 nm (Hadwan *et al*., 2024). Finally, reduced glutathione (GSH) levels were measured using Ellman's reagent (DTNB) at 412 nm and expressed as umol/g tissue (Alisik *et al*., 2019).

Immunological assays

Quantification of serum immunoglobulins (IgG and IgM): Circulating IgG and IgM levels were quantified using mouse-specific sandwich ELISA kits according to the manufacturer's instructions. Following color development, optical density was measured at 450 nm, and concentrations were calculated by interpolation from standard curves and expressed as mg/mL (Klein-Schneegans *et al*., 1989; Lalani *et al*., 2018).

Measurement of serum cytokines (IL-6 and TNF-α): Commercial mouse ELISA kits were used to measure serum levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). The resulting cytokine concentrations were quantified and reported in pg/mL (Molaae *et al*., 2017; Muñoz, 2023).

Splenocyte proliferation analysis: Spleens were harvested under aseptic conditions and passed through a 70 µm cell strainer to yield single-cell suspensions. Following red blood cell lysis with ammonium-chloride-potassium (ACK) buffer, splenocytes were washed and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Molaae *et al*., 2017). Cells (5×10^5) were seeded into 96-well plates, activated with 5 ug/mL concanavalin A for 48 hours, and evaluated for proliferation via MTT assay at 570 nm (Koyanagi *et al*., 2016). The stimulation index (SI) was calculated using the following equation: $SI=A_{\text{stimulated}}/A_{\text{unstimulated}}$.

Histopathological examination: Liver and spleen samples were collected at the end of the experiment and fixed in 10% neutral buffered formalin for 48 hours (Slaoui *et al*., 2017; Al-Sabaawy *et al*., 2021). Tissues were processed by dehydration in graded ethanol series, cleared in xylene and embedded in paraffin wax (Al-Sabaawy *et al*., 2021). Sections of 5µM thickness were cut by rotary microtome and placed on glass slides. The sections were stained with hematoxylin and eosin (H&E) for histopathological examination. Visual examination and photomicrography were performed using a light microscope at 400× magnification. Histological scoring was performed by a pathologist blinded to the treatment groups. Hepatocellular necrosis, inflammatory infiltration, and splenic lymphoid depletion were semi quantitatively graded using a 0–3 scale: 0: normal/no pathology; 1: mild (focal changes); 2: moderate (multifocal involvement); 3: severe (diffuse/ extensive changes) (Treuting and Boyd, 2019).

Statistical analysis: All data are expressed as mean±standard deviation (SD) (n = 10 per group). Statistical comparisons among the five experimental groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Differences were considered statistically significant at $P<0.05$. All statistical analyses were performed using SPSS software (version 23.0, IBM, USA).

RESULTS

Phytochemical profile: HPLC profiling successfully detected the major phenolic compounds in PPE, displaying well-separated peaks for the major ellagitannins. Punicalagin A eluted at *tR* [12.4 min] and was identified as the dominant ellagitannin at 32.1mg/g, while punicalagin B eluted at *tR* [14.1 min] with a concentration of 15.6mg/g. Baseline separation between the two isomers was achieved with a chromatographic resolution *Rs* of > 1.8. Together, the combined punicalagin content of 47.7mg/g accounted for over 65% of the total quantified phenolic pool, with ellagic acid and gallic acid also contributing substantially (Table 1).

Table 1: Phenolic compounds profile of pomegranate peel extract (PPE) by HPLC

Compound	Concentration (mg/g extract)
Punicalagin A	32.1±0.8a
Punicalagin B	15.6±0.5b
Ellagic acid	11.3±0.4c
Gallic acid	8.7±0.3d
Catechin	4.2±0.2e

Different superscript letters indicate significant differences among compounds (P<0.05).

***In-vitro* antioxidant activity:** The findings revealed a marked concentration-dependent increase in radical scavenging, with DPPH inhibition surging from 18.4% at 25µg/mL to 92.4% at 600µg/mL, an approximate five-fold increase (Fig. 2A). The DPPH IC_{50} was low (15.2µg/mL), indicating high potency. The ABTS profile paralleled this trend, achieving >93% scavenging at the peak concentration (Fig. 2B). These findings demonstrate that PPE's electron-donating capacity is exceedingly strong.

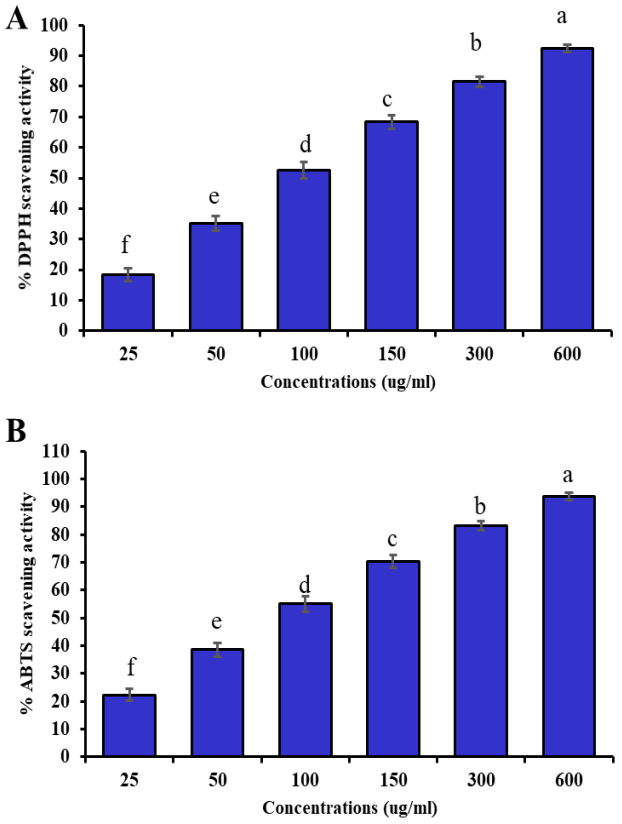

Fig. 2: Scavenging activity percentage of pomegranate peel extract (PPE) at different concentrations on the free radicals (A) DPPH, and (B) cationic ABTS. Lowercase letters above columns indicate significant differences at P<0.05.

Growth performance and mortality: CCl_4 intoxication severely inhibited growth, reducing total body weight gain by a striking 80.8% compared to the normal control, while inducing a 20% mortality rate. Administration of low-dose PPE partially alleviated this, restoring weight gain to 55.1% of normal levels (Table 2). However, the high-dose PPE excelled, recovering weight gain to 92.3% of the normal value and entirely abolishing mortality (0%). This near-complete rescue underscores the extract's capacity to mitigate CCl_4-induced metabolic derangements and systemic toxicity, preserving overall vitality.

Table 2: The effect of pomegranate peel extract on growth performance parameters and mortality rates of CCL_4-challenged male Swiss albino mice

Parameter	T1	T2	T3	T4	T5
Initial body weight (g)	22.4±1.1a	22.6±1.0a	22.5±1.2a	22.3±0.9a	22.7±1.1a
Final body weight (g)	30.2±1.5a	24.1±1.3c	26.8±1.4b	29.5±1.2a	29.8±1.3a
Body weight gain (g)	7.8±0.9a	1.5±0.6c	4.3±0.8b	7.2±0.7a	7.1±0.8a
Feed intake (g/day)	5.2±0.3a	3.4±0.4c	4.1±0.3b	5.0±0.2a	5.1±0.3a
Mortality (%)	0±0b	20±5a	10±3ab	0±0b	0±0b

Values are mean±SD (n = 10 per group). Different superscript letters in the same row indicate significant difference at P<0.05.T1, Normal control; T2, CCl_4 control, T3, CCl_4+PPE low (100mg/kg), T4, CCl_4+PPE high (300mg/kg); and T5, CCl_4+silymarin (50mg/kg)

Carcass properties: CCl_4 exposure induced marked damage to the carcass and spleen, reducing their absolute weights by 28.6% and 41.7%, respectively, while concurrently eliciting a 43.2% pathological increase in liver weight, likely attributable to hepatic lipid accumulation and inflammatory edema. Conversely, high-dose PPE treatment effectively counteracted these aberrations, restoring carcass and spleen weights to within 2.7% and 8.3% of control values, respectively, and decreasing liver weight by 27.0% relative to the CCl_4-treated group (Table 3). Collectively, this normalization of organ indices underscores the extract's efficacy in mitigating hepatocellular steatosis and reinstating splenic lymphoid mass.

Table 3: The effect of pomegranate peel extract on carcass properties of CCL_4-challenged male Swiss albino mice at the end of the experiment

Parameter	T1	T2	T3	T4	T5
Carcass weight (g)	18.5±1.1a	13.2±0.9c	15.6±1.0b	18.0±0.8a	18.2±0.9a
Dressing percentage (%)	61.3±1.5a	54.8±1.8c	58.2±1.6b	61.0±1.4a	61.1±1.5a
Liver weight (g)	1.32±0.08b	1.89±0.11a	1.61±0.09a	1.38±0.07b	1.35±0.08b
Spleen weight (g)	0.12±0.01a	0.07±0.01c	0.09±0.01b	0.11±0.01a	0.12±0.01a

Values are mean±SD. Different superscript letters in the same row indicate significant difference at P<0.05. T1, Normal control; T2, CCl_4 control, T3, CCl_4+PPE low (100mg/kg), T4, CCl_4+PPE high (300mg/kg); and T5, CCl_4+silymarin (50mg/kg)

Hematology, blood biochemistry, and spleen-related parameters: Table 4 and Fig. 3A- D provide critical insights into systemic immune-hematopoietic status and vital organ function. Hematological and splenic-immune indicators: CCl_4 exposure induced a profound anemia, with RBC and hemoglobin levels decreasing by 30.3 % and 31.0%, respectively. More notably, the spleen-specific parameters revealed severe immunosuppression: lymphocyte counts plummeted by 65.4%, mirroring the histologically observed lymphoid depletion, while platelet counts declined by 27.1%. Concomitantly, neutrophil and monocyte counts exhibited marked elevations of 281.8% and 200%, respectively, indicative of an acute systemic inflammatory state. Additionally, the platelet distribution width (PDW) increased by 46.7%, serving as a robust surrogate marker of aberrant platelet morphology, a phenomenon frequently associated with splenic sequestration and subsequent platelet destruction. Notably, administration of high-dose PPE markedly attenuated these hematological perturbations; lymphocyte and platelet counts increased by 172.2% and 33.9% relative to the CCl_4 control group,

effectively restoring both parameters to baseline physiological ranges. Concurrently, neutrophil and monocyte levels were reduced by 66.7% and 55.6%, respectively, accompanied by a 27.4% reduction in PDW—collectively indicating abatement of splenic hyperactivity and resolution of the underlying inflammatory stress. Taken together, these restorative hematological shifts provide compelling evidence for the immunomodulatory efficacy of PPE, specifically through the re-establishment of homeostasis within the splenic microenvironment.

Blood biochemistry: The CCl4 group exhibited severe hepatorenal injury, with ALT and AST skyrocketing by 250 and 247%, while creatinine and BUN rose by 133% each. High-dose PPE attenuated this damage, reducing ALT and AST by 65.3 % and 66.7%, respectively, relative to the CCl_4 group, and lowering creatinine and BUN by 50% each (Table 4). These percentage improvements confirm that PPE not only protects hepatic parenchymal integrity but also preserves glomerular filtration function, outperforming the low dose, which offered only intermediate protection.

Oxidative stress, antioxidant enzymes, immunity and histopathology: Fig. 4A and B show that CCl_4 induced a 3.1-fold increase in MDA and a 3.6-fold increase in protein carbonyls, indicative of rampant lipid peroxidation and protein oxidation. Concurrently, the antioxidant defense (SOD, CAT, GPx) and GSH were depleted by 59–62%, leaving cells defenseless. High-dose PPE reversed this by lowering MDA and PC by 67.8 and 70.8%, while enhancing the levels of SOD, CAT, GPx, and GSH to within 92–94% of normal values, a near-complete enzymatic recovery (Table 5).

Table 4: The effect of pomegranate peel extract on Hematology and blood biochemistry parameters in CCL_4-challenged male Swiss albino mice

Parameter	T1	T2	T3	T4	T5
Hematology					
Lymphocytes (×10³/µL)	5.2±0.4a	1.8±0.3c	3.1±0.4b	4.9±0.4a	5.0±0.3a
Neutrophils (×10³/µL)	1.1±0.2c	4.2±0.5a	2.9±0.4b	1.4±0.2c	1.3±0.2c
Monocytes (×10³/µL)	0.3±0.1b	0.9±0.2a	0.6±0.1a	0.4±0.1b	0.3±0.1b
PDW (fL)	9.2±0.5b	13.5±0.8a	11.8±0.7a	9.8±0.6b	9.5±0.5b
Blood biochemistry					
ALT (U/L)	28±4c	98±8a	65±7b	34±5c	30±4c
AST (U/L)	45±5c	156±12a	102±10b	52±6c	48±5c
Creatinine (mg/dL)	0.6±0.1b	1.4±0.2a	1.0±0.1a	0.7±0.1b	0.6±0.1b
BUN (mg/dL)	18±2b	42±5a	31±4a	21±3b	19±2b

Values are mean±SD. Different superscript letters in the same row indicate significant difference at P<0.05. T1, Normal control; T2, CCl_4 control, T3, CCl_4+PPE low (100mg/kg), T4, CCl_4 + PPE high (300mg/kg); and T5, CCl_4+silymarin (50mg/kg)

From an immunological perspective, CCl_4 exposure elicited a profound suppression of humoral immunity, as evidenced by significant reductions in serum IgG and IgM levels of 57.3% and 57.9%, respectively. Concurrently, it provoked a pronounced hyperinflammatory response, characterized by marked elevations in the pro-inflammatory cytokines IL-6 and TNF-α, which increased by 400% and 387%, respectively. Administration of high-dose PPE potently attenuated this cytokine storm, reducing IL-6 and TNF-α by 73.8% and 75.1%, while concurrently restoring serum immunoglobulin titers to approximately 95% of control values. Furthermore, splenocyte proliferation—which had been suppressed by 48% under CCl_4 challenge—exhibited a robust recovery to 94% of the control level. Collectively, these findings corroborate the immunorestorative capacity of PPE, demonstrating its efficacy in reinstating functional lymphocyte competence and rebalancing both the humoral and cellular arms of the immune response (Table 5).

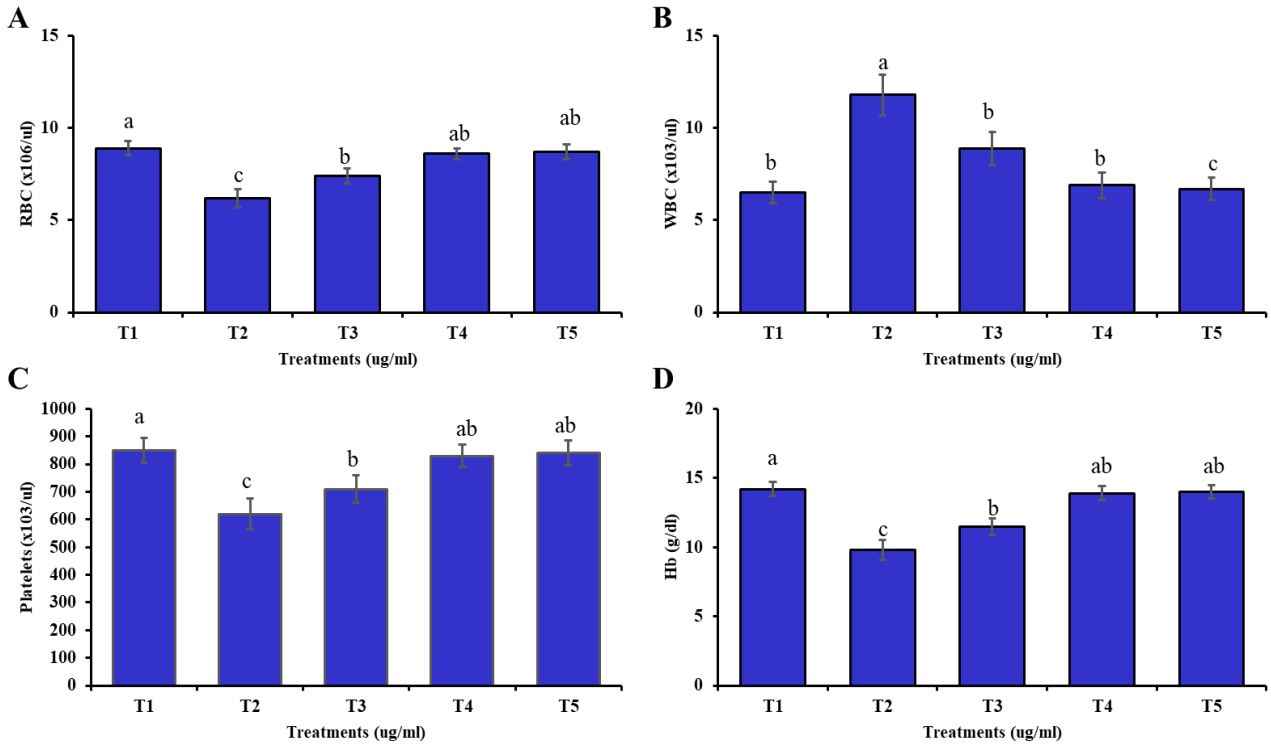

Fig. 3: The effect of supplementing pomegranate peel extract (PPE) in the mice diet on the hematology parameters (A) red blood cells (RBCs), (B) White blood cells (WBCs), (C) platelets, and (D) hemoglobin (Hb) of CCl_4-challenged rats. Lowercase letters above columns indicate significant differences at P<0.05. T1, Normal control; T2, CCl_4 control, T3, CCl_4 + PPE low (100mg/kg), T4, CCl_4 + PPE high (300 mg/kg); and T5, CCl_4 + silymarin (50mg/kg).

Table 5: The effect of pomegranate peel extract on oxidative stress markers, antioxidant enzymes, immunological markers, and histopathology scores

Parameter	T1	T2	T3	T4	T5
Antioxidant enzymes					
SOD (U/mg protein)	78±5a	32±4c	51±5b	72±4a	75±4a
CAT (U/mg protein)	45±4a	18±3c	28±3b	42±3a	43±3a
GPx (U/mg protein)	62±5a	24±4c	38±4b	58±5a	60±4a
GSH (µmol/g tissue)	12.5±1.2a	4.8±0.8c	7.9±1.0b	11.6±1.0a	12.0±1.1a
Immunological markers					
Serum IgG (mg/mL)	8.2±0.6a	3.5±0.5c	5.4±0.5b	7.8±0.5a	8.0±0.6a
Serum IgM (mg/mL)	1.9±0.2a	0.8±0.1c	1.2±0.2b	1.8±0.2a	1.8±0.1a
IL-6 (pg/mL)	42±6d	210±18a	128±12b	55±7c	48±6cd
TNF-α (pg/mL)	38±5d	185±15a	112±10b	46±6c	41±5cd
Splenocyte proliferation	1.00±0.05a	0.52±0.06c	0.73±0.07b	0.94±0.05a	0.97±0.04a

*Values are mean±SD (n = 10). Different superscript letters in the same row indicate significant difference at P<0.05. SI = stimulation index (compared to control). Histopathology scores: 0 = normal, 1 = mild, 2 = moderate, 3 = severe. *T1*, Normal control; T2, CCl_4 control, T3, CCl_4 + PPE low (100mg/kg), T4, CCl_4 + PPE high (300mg/kg); and T5, CCl_4 + silymarin (50mg/kg).

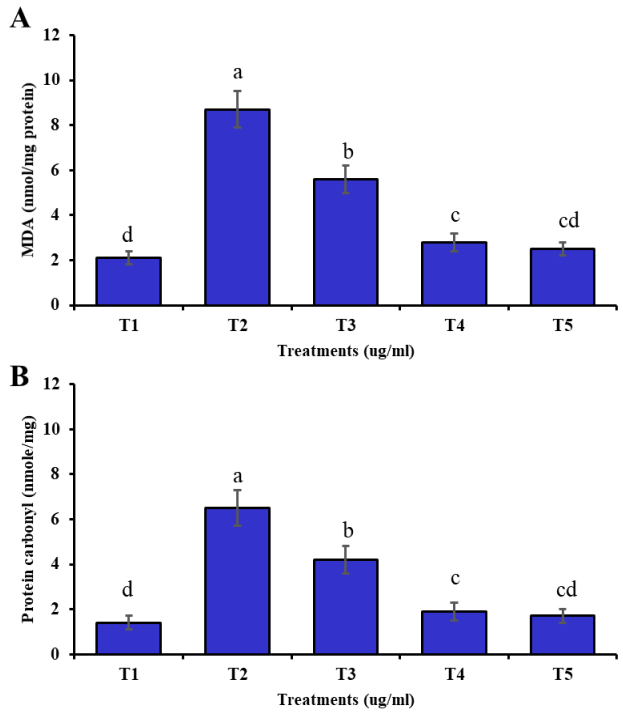

Fig. 4: The effect of supplementing the rat diet with pomegranate peel extract (PPE) on oxidative stress markers: (A) malondialdehyde (MDA) and (B) carbonyl protein in CCl_4-challenged mice. Lowercase letters above columns indicate significant differences at P<0.05. T1, Normal control; T2, CCl_4 control, T3, CCl_4 + PPE low (100mg/kg), T4, CCl_4+PPE high (300mg/kg); and T5, CCl_4+silymarin (50mg/kg).

Histological study: Histopathological scoring corroborated these molecular findings: CCl_4 produced severe hepatocellular necrosis (score ~2.8) and marked splenic lymphoid depletion (score ~2.9). High-dose PPE reduced these scores to 0.3 and 0.2, respectively, a >90% improvement, rendering tissue architecture comparable to silymarin. This morphological restoration is the ultimate proof of PPE's efficacy in preserving organ integrity against oxidative insult (Table 5).

The histological evaluation (Figure 5) revealed stark contrasts among the experimental groups, establishing a clear dose-dependent restorative trajectory for pomegranate peel extract (PPE). Figure 5A (normal control) displayed well-preserved hepatic architecture, with polygonal hepatocytes arranged in orderly cords radiating from central veins, no evidence of steatosis or inflammatory infiltrates, alongside a normal splenic histology featuring distinct white pulp follicles with active germinal centers and an unremarkable red pulp, thereby setting the physiological baseline.

In sharp contrast, Fig. 5B (CCl_4 control) demonstrated severe dual-organ damage: the liver exhibited extensive centrilobular hepatocellular necrosis, pronounced ballooning degeneration, dense mononuclear and polymorphonuclear inflammatory infiltrates, and marked sinusoidal congestion, all reflecting massive oxidative injury; concurrently, the splenic tissue in Fig. 5B showed profound lymphoid depletion with atrophied or absent germinal centers, hypocellular periarteriolar lymphoid sheaths (PALS), and prominent red pulp congestion with reduced megakaryocyte numbers, indicating severe systemic immunosuppression secondary to CCl_4-induced oxidative stress and cytokine dysregulation.

Treatment with low-dose PPE (100mg/kg) in Fig. 5D produced only moderate improvements: the liver displayed visibly reduced centrilobular necrosis and fewer ballooned hepatocytes, although residual inflammatory foci and mild sinusoidal congestion persisted, demonstrating partial cytoprotection; in the spleen, Fig. 5D showed a modest restoration of follicular cellularity with small but recognizable germinal centers, yet scattered areas of lymphoid hypocellularity and mild congestion remained, confirming that the 100mg/kg dose offered only incomplete immunological recovery.

Remarkably, high-dose PPE (300mg/kg) in Fig. 5E elicited a near-complete histopathological rescue: the hepatic lobular architecture was essentially restored, with well-organized hepatocyte cords, minimal inflammatory infiltration, patent sinusoids and intact nuclear morphology, closely approximating the normal control; similarly, the splenic architecture in Fig. 5E was profoundly restored, displaying large, densely cellular germinal centers, well-defined PALS with active lymphocytic zones, and minimal red pulp congestion, signifying robust immuno-restoration and active lymphoid regeneration. The silymarin (50mg/kg) reference group consistently demonstrated near-normal hepatic and splenic architecture, with only occasional very mild vacuolation in the liver and fully intact lymphoid follicles, achieving a protective effect essentially equivalent to that of high-dose PPE in Fig. 5E.

Thus, both high-dose PPE and silymarin effectively repealed CCl_4-induced necrosis, inflammation, and lymphoid depletion. Collectively, the histological findings in Fig. 5 demonstrate a clear dose-dependent mitigative effect of PPE, wherein the 300mg/kg dose achieves therapeutic outcomes statistically and biologically indistinguishable from the conventional hepatoprotective agent, strongly corroborating the biochemical, hematological, and immunological data that establish PPE as a potent antioxidant and immunomodulatory agent capable of restoring active tissue histology against oxidative damage.

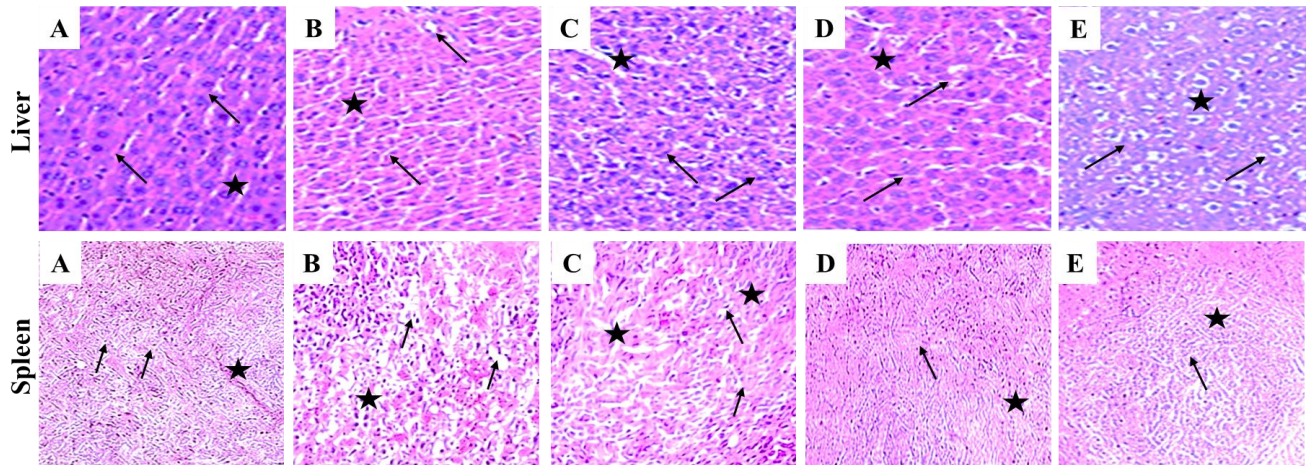

Fig. 5: Representative photomicrographs (H&E, ×400) of liver and spleen sections showing the dose dependent protective effects of pomegranate peel extract (PPE) against CCl_4 induced histopathology. A (normal control): Arrows and stars indicate healthy hepatocytes with euchromatic nuclei and active splenic germinal centers, confirming intact tissue architecture. B (CCl_4 control): Severe hepatic injury with arrows and stars indicating extensive centrilobular necrosis, ballooning degeneration and dense inflammatory aggregates, and splenic lymphoid depletion with arrows indicating atrophied white pulp and congested sinusoids. D (low dose PPE): Arrows and stars indicate noticeably reduced necrotic foci and smaller but identifiable germinal centers, but some residual inflammation and mild congestion remain, indicating only partial recovery of tissue. E (high dose PPE): Near complete restoration with arrows and stars indicating healthy hepatocyte cords, patent sinusoids, and large, densely cellular splenic follicles with active germinal centers, with only occasional inflammatory cells; this architecture closely resembles the normal baseline seen in A. The silymarin reference group (not shown) is histologically equivalent to E confirming that the high dose PPE provides similar protection.

DISCUSSION

The current study aimed to provide an integrative evaluation of the therapeutic potential of pomegranate peel extract (PPE) in ameliorating carbon tetrachloride (CCl_4)-induced oxidative injury, with a particular focus on its immunomodulatory and histoprotective activities beyond the classical hepatoprotective paradigm. Being extremely rich in bioactive ellagitannins and flavonoids, known for their potent electron-donating and metal-chelating properties, pomegranate peel was hypothesized to exert multi-targeted systemic protection, simultaneously counteracting hepatic necrosis, restoring splenic immune integrity, and preserving overall physiological homeostasis (Alami *et al*., 2024; Aydin *et al*., 2025)

The findings strongly support this hypothesis, revealing that PPE, particularly at the high dose (300 mg/kg), dose-dependently ameliorated CCl_4-induced growth retardation, hepatorenal dysfunction, oxidative macromolecular damage, and pro-inflammatory cytokine release, while effectively reinstating humoral immunity (IgG/IgM), splenocyte proliferative capacity and the microarchitecture of both liver and spleen, with a therapeutic efficacy statistically indistinguishable from the standard reference drug silymarin. The following discussion interprets these integrated pharmacological findings within the framework of contemporary literature, starting with the phytochemical basis of the extract's antioxidant potency, proceeding through the mechanistic pathways governing redox balance and immune homeostasis, and concluding with a comparative appraisal against other natural interventions in CCl_4 models, thus reinforcing the translational value of valorizing pomegranate peel waste as a sustainable chemoprotective and immunomodulatory agent.

The dominance of punicalagin A and B, with ellagic and gallic acids, is consistent with recent phytochemical studies indicating that pomegranate peel is the richest fruit fraction in ellagitannins and polyphenolic compounds, with punicalagin being the primary component and the major contributor to antioxidant capacity (Sabraoui *et al*., 2020; Benchagra *et al*., 2021; Rak-Pasikowska *et al*., 2024). The low IC_{50} and high free radical scavenging activity are consistent with reports that peel extracts achieve >90% DPPH inhibition with low IC50 values and exhibit superior reducing power and metal-chelating capacity compared with aril extracts (Benchagra *et al*., 2021; Abdel-Daem *et al*., 2025). These data support the interpretation that the in vivo protection is driven by electron-donating and metal-chelating ellagitannins.

CCl_4 is a classical hepatotoxin that produces trichloromethyl radicals, increases ALT/AST, increases MDA, but suppresses SOD, CAT & GPx, upregulates IL 6/TNF α and induces histological necrosis (Alabbad *et al*., 2023; Algefare *et al*., 2024). Similar profiles are observed with a variety of natural antioxidants, the administration of which restores liver enzymes, antioxidant defenses and histology in CCl_4 models (El-Shabasy *et al*., 2022; Garmroodi *et al*., 2024).

The increased body weight gain, carcass and organ indices with the high dose PPE closely match studies reporting that phenolic-rich products categorized by high TPC and free radical scavenging capacity provide the greatest hepatoprotection and histological improvement against CCl_4 (Garmroodi *et al*., 2024). Punicalagin and related polyphenolic compounds were the primary contributors to the antioxidant and hepatoprotective effects (Venusová *et al*., 2021; Saparbekova *et al*., 2022). The growth biomarkers in CCl_4-intoxicated mice are mechanistically consistent with enhanced hepatic metabolism and reduced systemic toxicity.

The marked increase in MDA and protein carbonyls with concurrent decrease of SOD, CAT, GPx, and GSH is characteristic of CCl_4 induced oxidative stress (Algefare *et al*., 2024; Garmroodi *et al*., 2024). The high-dose PPE almost completely reversed these parameters, similar to reports where pomegranate peel extract in other toxicant

models (acrylamide, metabolic syndrome) decreased MDA and ROS/RNS levels while increasing SOD, CAT, GPx, and GSH (Čolić *et al*., 2022; Sayed *et al*., 2022; Rak-Pasikowska *et al*., 2024). Punicalagin A/B, the major pomegranate ellagitannins, are poorly absorbed intact and are hydrolyzed in the stomach and intestine to ellagic acid, after which gut microbes convert ellagic acid into urolithins through microbiota-dependent reactions; tannase activity and producers such as *Gordonibacter* and *Ellagibacter* help explain this biotransformation, and interindividual metabotypes partly determine how much urolithin is formed. Because intact ellagitannins have low systemic bioavailability, multiple reviews argue that the better-absorbed urolithins are likely major mediators of *in vivo* bioactivity, although responses vary by host microbiome composition and the precise active forms reaching target tissues remain uncertain (Alalawi *et al*., 2026).

Mechanistically, punicalagin itself activates Nrf2/Keap1 and increases HO-1 expression in obesity, brain-injury, and keratinocyte inflammation models, while urolithins, especially urolithin A and B, also enhance Nrf2/ARE- or Keap1/Nrf2-linked antioxidant signaling and improve redox balance (Huang *et al*., 2024). In parallel, ellagic acid and urolithins suppress NF-κB signaling and downstream inflammatory mediators: ellagic acid inhibits NF-κB in intestinal models, urolithin A and B reduce NF-κB nuclear translocation and cytokine-associated outputs such as *COX*-2, mPGES-1, PGE2, TNF-α, IL-6, iNOS, MCP-1 and punicalagin likewise downregulates NF-κB alongside MAPK-related inflammation pathways (Sayed *et al*., 2022; Huang *et al*., 2024).

The robust recovery of IgG and IgM, enhancement of cytokines and restoration of splenocyte proliferation are very consistent with emerging data on complex immunoregulatory actions of pomegranate peel polyphenols and punicalagin (Čolić *et al*., 2022; Huang *et al*., 2024) where peel extract or punicalagin suppress pro inflammatory cytokines (TNF α, IL 6, IL 1β, IL 17) and NF κB/MAPK signaling and promote regulatory IL 10 responses at appropriate doses in human PBMC and T cell models.

PPE increased splenic CAT and GPx in rats with metabolic syndrome and altered apoptotic regulators, supporting an immunoprotective effect on secondary lymphoid organs (Rak-Pasikowska *et al*., 2024). Restoration of lymphocyte counts, platelets and splenic architecture in CCl_4-challenged mice therefore dovetails with these immunomodulatory observations and highlights that PPE acts beyond the liver to preserve humoral and cellular immunity.

Many recent CCl_4 studies using various phytochemicals (apigenin, geraniol, mangiferin, grape-seed nanoparticles) demonstrate a recurring triad: correction of serum enzyme levels, normalization of oxidative stress markers, and histological improvements in necrosis and inflammation (Alabbad *et al.*, 2023; Algefare *et al.*, 2024; Shi *et al.*, 2024). The >90% reduction in necrosis and lymphoid depletion scores with high-dose PPE, comparable to silymarin, places PPE among the most potent hepatoprotective and immuno-restorative interventions described to date, particularly given its dual action on the liver and spleen.

Furthermore, dietary PPE has been shown to improve growth, survival and haemato-immunological indices in fish challenged with bacterial pathogens, with optimal doses enhancing weight gain, survival and cytokine responses (Gupta *et al.*, 2022). Together with mammalian data in hepatotoxicity and metabolic syndrome models, these findings corroborate the present observation that PPE can simultaneously support growth performance, survival, antioxidant status and immune competence under intense oxidative challenge.

Conclusions: This study confirms that pomegranate peel extract (PPE), particularly at 300 mg/kg, exerts potent dose-dependent chemoprotective, antioxidant, and immunomodulatory effects against CCl_4-induced toxicity. Dominated by punicalagins and ellagic acid, PPE effectively counteracted oxidative stress by reducing MDA and protein carbonyls while restoring endogenous antioxidants (SOD, CAT, GPx, GSH) and hepatorenal function. Critically, PPE uniquely rejuvenated splenic immunity by reversing lymphopenia, reducing pro-inflammatory cytokines (IL-6, TNF-α), restoring immunoglobulin levels, and repairing splenic lymphoid architecture. The high dose achieved near-complete histological and biochemical recovery, matching the standard drug silymarin. Future Studies rely on conducting clinical trials to validate PPE's chemoprotective and immunomodulatory efficacy across diverse populations. Isolate and test individual constituents (punicalagins, ellagic acid) to identify the primary bioactive drivers. Evaluate chronic administration safety, optimal dosing regimens, and potential drug–PPE interactions. Develop standardized extraction protocols to ensure consistent punicalagin and ellagic acid content across preparations.

Declaration of interests: The author declares that she has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors contribution: Kamlah Ali Majrashi: Conceptualization, visualization, methodology, writing the original draft, writing-review, and editing.

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