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# *The Role of Pomegranate Peel Extract in Improving Hepatotoxicity, and hMSH2 Expression in $CCl_4$ -Treated Rats*

**Dalia Mostafa Mohammed Domiaty[1]***

*[1]Department of Biology, College of Science, University of Jeddah, Jeddah, Saudi Arabia.*

***Email:** ddomiaty@uj.edu.sa*

## ABSTRACT

*Recently, pomegranate fruit and its leaves have found wide usage as a natural treatment for several ailments. In this study, we investigated the restorative potentials of pomegranate (Punica granatum) peel extract against hepatic damage brought on by calcium tetrachloride ($CCl_4$) in rats and its effects on the human mutS homolog 2 (hMSH2) gene expression. Four groups of twenty male Wistar rats (n =5) were created at random. Group I was the untreated control group. Group II was given a dose of 0.4 ml $CCl_4$ intraperitoneally (IP) for two consecutive days/week for 3 weeks. Group III was given a daily dose (500 mg/ kg b.w) of pomegranate peel extract (PPE) for 3 weeks. Group IV received an IP of $CCl_4$ for two consecutive days/week for 3weeks followed by a daily oral dose of (500 mg/ kg b.w) of PPE for 3 weeks. Bodyweight, relative liver weight, serum AST, ALT, bilirubin, tissue GSH, and MDA, the expression of the hMSH2 gene, liver histology, and immunohistochemistry were assessed. Our results showed that the administration of $CCl_4$ to rats did not affect their body weight and relative liver weight. However, $CCl_4$ administration resulted in a decrease in tissue GSH, an increase in serum AST, ALT, bilirubin, lipid peroxidation, modification of liver histology, and immunohistochemistry, and the downregulation of the expression of the hMSH2 gene. Intriguingly, PPE treatment following $CCl_4$ administration to rats attenuated these changes. Taken together, our study reveals the potential of PPE for its use in the treatment of liver damage.*

**Key words:** *Hepatotoxicity, Antioxidant, Oxidative stress, Free radicals, Toxicant*

## INTRODUCTION

The liver is both the largest gland and the largest organ in the mononuclear phagocyte system of the human body [1]. It is composed of kupffer cells, sinusoidal endothelial cells, and hepatic stellate cells and is situated in the upper right region of the abdomen beneath the diaphragm [2]. The liver is essential for the body's detoxification processes, digestion, metabolic control, and maintaining the flow, and safety of compounds that are taken from the digestive system before they enter the circulatory system [1, 3].

Hepatotoxicity can be caused by agents called hepatotoxins or hepatotoxicants [2]. These agents include drugs, (anti-cancer and anti-tubercular drugs, paracetamol, and anti-cancer drugs), chemicals like galactosamine and chloroform, and oxidative species. Anti-tubercular drugs usually result in toxic hepatitis and liver necrosis which could cause encephalopathy and death [4]. Hepatotoxicity can be caused by both direct toxicities of primary compounds and by reactive metabolite/immunologically mediated response [3, 4]. Vaccines, steroids, and antiviral drugs used in liver disease therapy sometimes produce adverse effects [5]. Hepatocytes are damaged by hepatotoxicants by inducing lipid peroxidation and causing oxidative stress to the liver due to chronic treatment or toxic doses [6]. Age, gender, obesity, drug-drug interactions, kidney damage, genetics, and alcohol consumption, are risk factors for hepatotoxicity [7].

Carbon tetrachloride ($CCl_4$) is a clear, colorless, volatile, and fireproof liquid made up of carbon and four chlorine atoms. The chemical compound occurs both naturally and artificially and has been used in the production of cleaning agents and solvents and in the synthesis of chlorofluorocarbons [8]. Due to its strong hepatotoxic, nephrotoxic, and prooxidant nature, $CCl_4$ is used to induce hepatotoxicity in animal research studies and is used to create hepatocellular carcinoma, hepatic cirrhosis, and liver injury [9]. Inhalation, ingestion, and dermal absorption are the different routes through which $CCl_4$ can easily enter the body [9]. Furthermore, $CCl_4$ is a hepatotoxin that is believed to form free radicals and caused peroxidation after entering the hepatocytes which can lead to the disruption in the liver structure and function [10, 11].

One of the oldest fruits still in existence in the world is pomegranate (*Punica granatum* L.). It belongs to the family *Punicaceae* and the genus *Punica*. The fruit is native to the Middle East and can be found in countries like Iran, India, Afghanistan, and some other Mediterranean countries [12]. The pomegranate is a pharmacologically precious fruit and has been used numerously for cooking and medical purposes. Over the years, research studies have discovered the many health benefits of this plant (fruit, seed, and peel) [13]. The pomegranate peel extract (PPE) is a part of the fruit known to contain high levels of phytochemicals, phenolic acids, flavonoids, and tannins. These phytochemicals and compounds are responsible for the antioxidant, antimicrobial, anticancer, antiulcer, and anti-inflammatory properties of the pomegranate peel extract [14, 15]. The flavonoids, phenolic acids, and tannins present in the PPE are very important in preventing damage caused by free radicals in the human body [12].

The DNA mismatch repair pathway includes the mismatch repair gene, the human mutS homolog 2 (hMSH2) gene [16]. This gene encodes an important nuclear protein that plays an essential role in nucleotide mismatch recognition in the DNA repair pathway. However, studies have shown that mutations in the hMSH2 gene are associated with the progression in the development of liver disease including hepatocellular carcinoma [17]. Consequently, in this research, we looked at the potential advantages of PPE extract on liver injury induced by $CCl_4$ and determined its effects on the expression levels of the hMSH2 gene.

## MATERIALS AND METHODS

*Plant material and preparation of extract*

The dried peels of pomegranates were obtained from an herbal and folk medicine market in Jeddah, Saudi Arabia. These peels were washed, air-dried, and powdered followed by the dissolution of 10 g from this powder in 500 ml distilled water. The extract was filtered, concentrated to 8.5 mg/ml of pomegranate peel extract, and kept till usage at 4 °C.

*Animals*

Male Wistar rats weighing 150-250 g were purchased from the King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. Animals were left to acclimatize to the lab ambiance for one week (12hr/12hr light off/on) and fed on a lab animal diet with freely available water. Declaration by Bioethics Committee of Scientific and Medical Research approved this animal experiment with No. HAP-02-J-094.

*Chemicals*

Carbon tetrachloride ($CCl_4$) was purchased from Sigma-Aldrich, (Missouri, United States) and diluted in olive oil (1:1 v/v). All other chemicals were of analytical grade.

*Experimental design*

Rats were randomly placed into four groups (n = 5) after acclimatization and given the following treatment:

Group I (Control): the control group, received no treatment. Group II ($CCl_4$): This group received $CCl_4$ in olive oil (1:1) intraperitoneally (IP) at a dose of 0.4 ml for two consecutive days/week for 3weeks. Group III (PPE): Animals in this group received pomegranate peel extract (PPE) orally at a dose level of (500 mg/ kg b.w) daily/for 3 weeks. Group IV ($CCl_4$ + PPE): This group received IP of $CCl_4$ for two consecutive days/week for 3weeks followed by a daily oral dose of (500 mg/ kg b.w) of PPE for 3 weeks.

After the three weeks experimental period, food was withdrawn from the animals overnight and they were later euthanized under diethyl ether anesthesia. Following this, blood was drawn from the animals and the liver tissue was removed, rinsed in normal saline, and weighed. Part of the liver was either stored in 10% buffered formalin for histological analysis and immunohistochemical analysis or kept at -80˚C for extraction of RNA. The leftover liver tissue was blended in a 100 mM phosphate buffer with a pH of 7.4 at 14,000 rpm for 30 min.

*Assessment of liver damage biomarkers*

Using a commercial kit (Diagnostic System Laboratories Inc., USA), the serum concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin were measured.

*Assessment of oxidative stress biomarkers*

For this, the supplier's instructions were followed when measuring the amounts of glutathione (GSH) and malondialdehyde (MDA) in the supernatant collected after centrifugation at 14,000 rpm using a commercial kit (MyBioSource, California, USA).

*RNA Extraction and Real-time quantitative PCR (RT-qPCR)*

According to the supplier's recommendations, total RNA was extracted from the liver tissues using a (QIAgen RNeasy mini kit, cat # 74104). Next, 200ng of the extracted RNA was used in cDNA synthesis by the use of the M-MLV Reverse Transcriptase System (Promega, USA), and the qPCR reaction was made up of the following components: cDNA, 3 mL; right and left primers **(Table 1)**, 0.5 mL (500 nM); purified water, 1 mL; SYBR Green Master Mix (Applied Biosystems, USA). To assess the relative mRNA expression of the hMSH2 gene, the $2^{-\Delta\Delta CT}$ method was applied and normalized to the expression of GAPDH.

**Table 1.** Primer sequences

| Primers | Primers sequence (5`-3`) |
|---|---|
| hMSH2 - left | 5'-TGCTCAAGGACAAAGGCTG-3' |
| hMSH2 right | 5'-GGAAATCGGCGAAGTAAATC-3' |
| GAPDH - left | 5'-GAT GGT GAA GGT CGG TGT G-3' |
| GAPDH -right | 5'-ATG AAG GGG TCG TTG ATG G-3' |

*Histopathological studies*

The fixation of liver tissue was carried out in 10% buffered formalin, then embedded in paraffin wax after being dried out graded ethanol for a day at room temperature. To evaluate histopathological alterations, hematoxylin, and eosin (H&E) were used to stain the sections of tissue blocks that were cut into thin sections. Light microscope images of stained liver sections were taken at 400x magnification.

*Immunohistochemistry analysis*

Immunohistochemical analysis was done for anti- CK7 and CK19 antibodies using streptavidin-biotin. The liver sections of a thickness of 5 µm and at room temperature were deparaffinized followed by incubation in hydrogen peroxide (0.3%) prepared in methanol for half an hour. The liver sections were incubated with anti- CK7 and CK19 antibodies at a dilution of 1:100 respectively followed by counterstaining with hematoxylin and eosin.

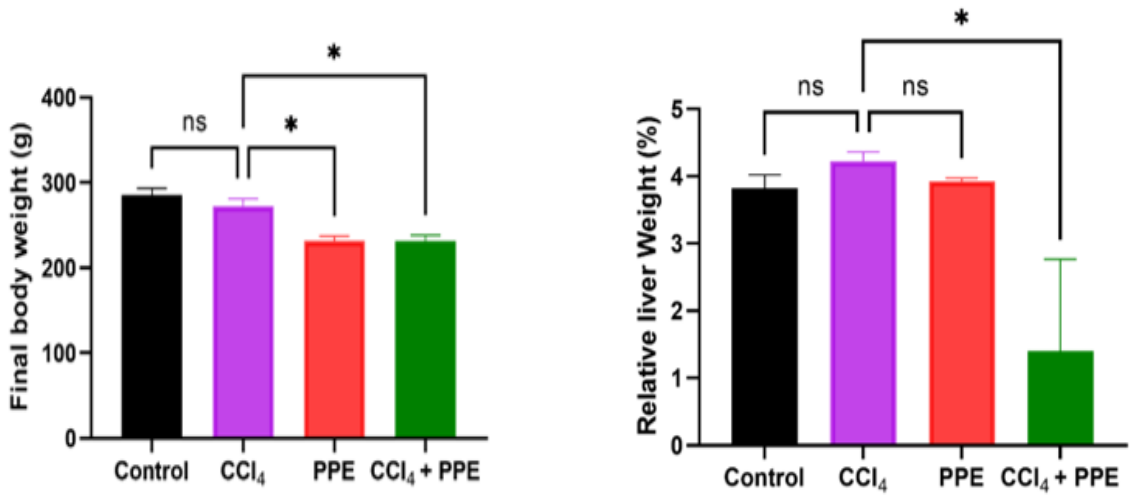
*Statistical analysis*

The statistical analyses for this study were conducted using one-way ANOVA, and the data are presented as mean SEM. Dunnett's multiple comparisons test was used to compare means, and a significance level of p less than 0.05 was chosen.

## RESULTS AND DISCUSSION

*Body weight and relative liver weight alterations induced by $CCl_4$ administration*

The administration of $CCl_4$ to rats has no significant effect on the body weight of rats compared to animals in the control group **(Figure 1a)**. On the other hand, when compared to rats given $CCl_4$, PPE treatment to the rats resulted in a significant ($p < 0.05$) drop in body weight. In addition, rats treated with PPE after $CCl_4$ administration saw a significant ($p < 0.05$) reduction in body weight when compared to animals just given only $CCl_4$ **(Figure 1a)**. Furthermore, $CCl_4$ administration to rats resulted in liver weight increased by 9% and 7% in comparison with the PPE-treated group and the control group respectively. Moreover, rats treated with PPE after receiving $CCl_4$ showed a significant reduction in relative liver weight compared to those who received only $CCl_4$ **(Figure 1b)**.

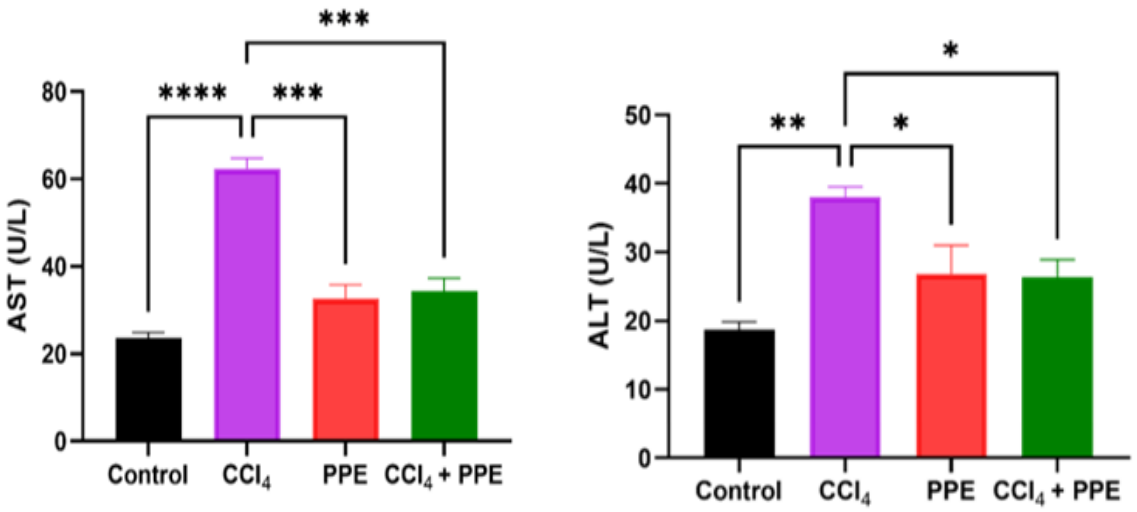



a) b)

**Figure 1.** Body weight and relative liver weight alterations induced by $CCl_4$ administration. a) final body weight. b) relative liver weight

*Protective effects of PPE against liver damage induced by $CCl_4$*

To assess the effect of $CCl_4$ on the health status of the liver, we quantified the serum AST and ALT levels, two biomarkers of liver damage. As shown in **Figure 2**, $CCl_4$ administration to rats, led to a significant increase in the serum AST and ALT levels in comparison to the control ($p < 0.0001$ for AST and $p < 0.01$ for ALT) and PPE treated groups ($p < 0.001$ for AST and $p < 0.05$ for ALT) respectively. However, PPE administration to rats following $CCl_4$ led to a significant decrease ($p < 0.001$) in serum AST levels when compared with rats administered only with $CCl_4$. Similarly, rats treated with PPE after receiving $CCl_4$ revealed a significant reduction in the serum levels of ALT in comparison with $CCl_4$-only administered rats **(Figure 2b)**.



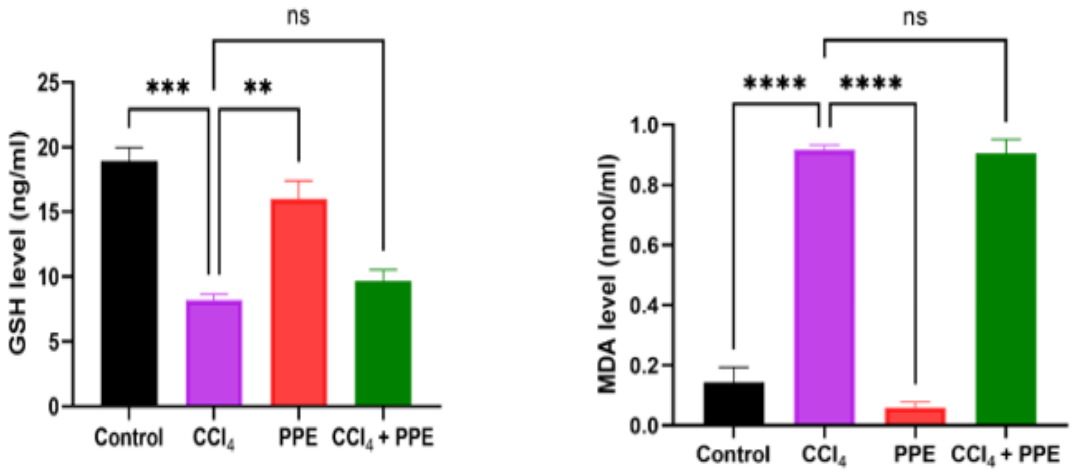

a) b)

**Figure 2.** Effects of $CCl_4$ on serum levels of AST and ALT. a) Serum AST level. b) Serum ALT level.

*Antioxidant effects of PPE against $CCl_4$-induced oxidative stress*

When compared to the animals in the control group, treatment with $CCl_4$ caused a significant ($p < 0.001$) drop in the amount of GSH present in the liver tissue. However, when compared to animals given $CCl_4$, rats given PPE saw a considerable rise ($p < 0.01$) in the GSH content **(Figure 3a)**. In addition, the GSH contents showed no discernible difference from the rats treated with PPE following $CCl_4$ administration and $CCl_4$-only administered rats. Furthermore, $CCl_4$ administration to rats resulted in a significant elevation ($p < 0.0001$) in the liver MDA content in comparison to the rats in the control group, and the PPE-only administered to rats **(Figure 3b)**.

However, PPE treatment to rats after $CCl_4$ administration resulted in non-significant changes in the MDA contents in comparison with rats administered with only $CCl_4$ **(Figure 3b)**.

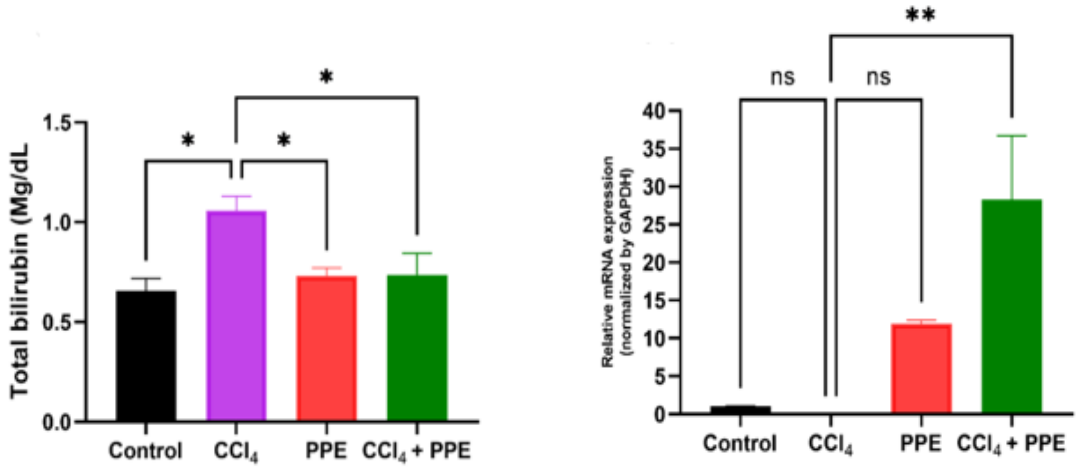



a) b)

**Figure 3.** Effects of CCl4 and PPE on GSH and MDA levels. a) Serum glutathione (GSH) level. b) Serum malondialdehyde (MDA) level.

*Effects of $CCl_4$ and PPE on serum bilirubin levels and hMSH2 gene expression*

Rats administered with $CCl_4$ saw a substantial rise ($p < 0.05$) in blood bilirubin levels when compared to the control group and rats given PPE, respectively. Interestingly, rats given PPE following $CCl_4$ administration showed a significant reduction in the bilirubin level when compared to the rats in the $CCl_4$-only group. This decrease in serum bilirubin by PPE treatment to the $CCl_4$ administered rats was near normal **(Figure 4a)**. Furthermore, the hMSH2 gene expression levels between the control group and the $CCl_4$-treated group did not differ significantly. Although PPE administration to rats, upregulated the expression of the hMSH2 gene in comparison to the $CCl_4$-only treated group, this does not reach a significant level **(Figure 4b)**. Interestingly, rats receiving both $CCl_4$ and PPE treatment demonstrated significantly higher levels of hMSH2 gene expression ($p < 0.01$) as compared to rats receiving $CCl_4$ only.




a) b)

**Figure 4.** Effects of $CCl_4$ and PPE on serum bilirubin levels and hMSH2 gene expression. a) serum testosterone level. b) Relative hMSH2 gene expression.

*PPE administration improved liver histology in $CCl_4$-induced liver-injured rats*

Hematoxylin and eosin staining revealed that the liver sections from the animals in the control group presented a normal liver histological architecture with normal hepatic lobules, central vein, visible hepatic sinusoids, and

hepatic cells **(Figure 5a)**. However, rats administered with $CCl_4$ presented a visible liver injury with an altered liver architecture, and an increased necrotic infiltration **(Figure 5b)**. In addition, rats administered with PPE only showed similar liver architecture when compared to the liver sections from the animals in the control group **(Figure 5c)**. Furthermore, the liver sections from the animals who received treated $CCl_4$ treatment followed by the administration of PPE revealed an improved liver architecture with visibly radiating hepatic cells as compared with the liver sections from $CCl_4$-only administered rats **(Figure 5d)**.

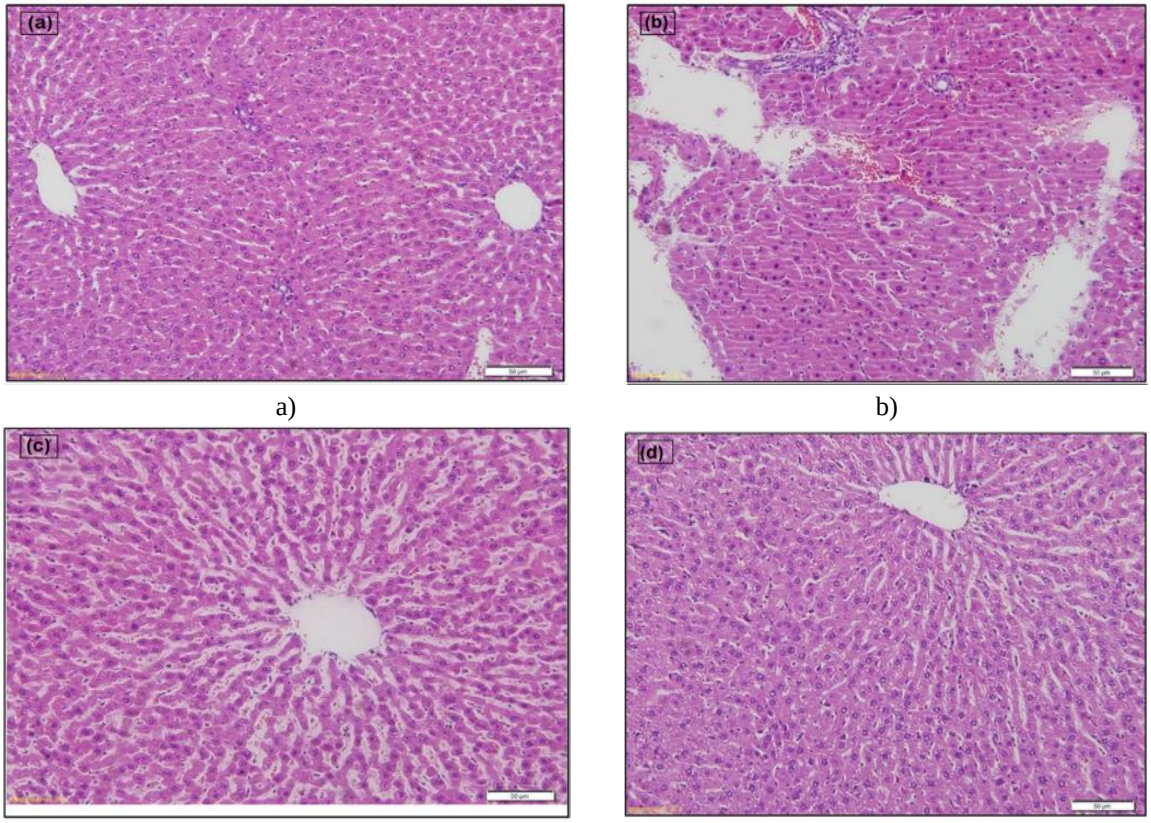
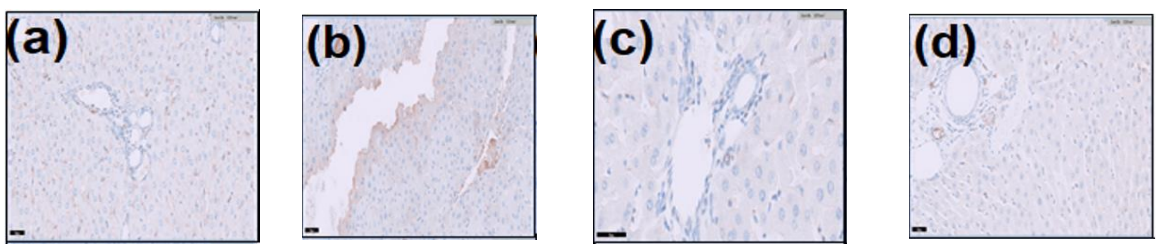
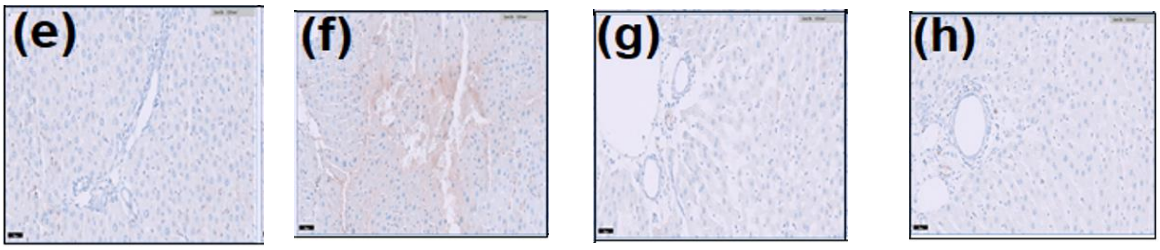



**Figure 5.** Histology of the representative liver sections $CCl_4$ and ART on liver tissue architecture. a) Histology of the liver in the normal control group showing normal liver architecture. b) Histology of the liver sections of the animals in the $CCl_4$-administered group shows damaged liver sections. c) PPE only administered group, showing normal liver architecture. d) $CCl_4$ + PPE group showing improved liver architecture to near normal with visible radiating hepatic cells.

*Impact of $CCl_4$ administration on oval cell regeneration*

**Figure 6** showed the immunohistochemical staining of the cell's response to damage induced by $CCl_4$ administration against anti-CK7 and anti-CK19 antibodies. The liver sections from animals in the control group for both CK7 and CK19 antibodies showed no visible presence of oval cells **(Figures 6a and 6e)**. However, the liver sections from rats administered with $CCl_4$ showed growing oval-shaped cells located around the periportal region **(Figures 6b and 6f)**. In addition, the sections of the liver of rats administered with CCl4 and treated with PPE showed little oval cells for CK7 antibodies **(Figure 6e)** but little or no oval cells for CK19 antibodies **(Figure 6h)**.






e) f) g) h)

**Figure 6.** Immunohistochemical staining (a, b, c, d) against CK7 and Immunohistochemical staining (e, f, g, h) against CK19. a) Immunohistochemistry against CK7 of the liver in the normal control group showed no visible oval cells. b) Immunohistochemistry against CK7 of the liver sections of the animals in the $CCl_4$-administered group showed growing oval cells. c) Immunohistochemistry against CK7 for PPE only administered group, showing no visible oval cells. d) Immunohistochemistry against CK7 for $CCl_4$ + PPE group showed improved liver architecture to near normal with visible radiating hepatic cells. e) Immunohistochemistry against CK19 of the liver in the normal control group showed no visible oval cells. f) Immunohistochemistry against CK19 of the liver sections of the animals in the $CCl_4$-administered group showed growing oval cells. g) Immunohistochemistry against CK19 for PPE only administered group, showing no visible oval cells. h) Immunohistochemistry against CK19 for $CCl_4$ + PPE group showed improved liver architecture to near normal with visible radiating hepatic cells.

The production of reactive oxygen species by aerobic organisms during respiration is unavoidable. However, when the production of ROS becomes abnormally high, it can lead to injury or damage to the cells and tissues. Unregulated lipid peroxidation is responsible for the occurrence of diseases such as liver damage, cardiovascular diseases, and myocardial infarction among others [18]. Due to the major and important roles the liver plays in the body, it is prone to many diseases and toxic agents [19].

This study aims to evaluate the hepatoprotective property of pomegranate peel extract on $CCl_4$-induced liver damage by accessing the body weight and liver weight changes, serum biomarkers, oxidative stress, liver histology, and immunohistochemistry. In contrast to the control group and the rats were given PPE, our findings indicated that there was no appreciable gain in body weight in the $CCl_4$ treatment group. On the impact of $CCl_4$ on body weight, various earlier studies have found a significant decrease. For example, Gazwi *et al*. [20], and Dutta *et al*. [21] recorded a reduction in the body weight of the group receiving $CCl_4$ treatment. However, a significant decrease in the body weight of the $CCl_4$ treated group was observed when the PPE was administered to the rats **(Figure 1a)**. This result confirms the body weight-lowering potentials of pomegranate as reported in various previous studies [22, 23]. Interestingly, despite the non-significant change in body weight in the $CCl_4$-treated rats in comparison to the control and PPE-only administered rats, an increase in the liver weight was observed which was ameliorated by the administration of PPE. This increase in liver weight was in agreement with the study of Gazwi *et al*. [20].

To further determine the hepatotoxic effects of $CCl_4$ and the hepatoprotective effect of PPE, the AST and ALT biomarkers were tested. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are enzymes that are found in the liver, kidney, muscle, and heart. These enzymes' main functions are to metabolize protein and break down food to produce energy. An increase in their concentration is a symbol of liver injury and disease [24]. Our results showed that the serum levels of AST and ALT significantly increased in response to $CCl_4$ and this agreed with previous studies [18, 25]. This increase in the serum levels of these enzymes has been associated with liver damage since they are usually produced from hepatocytes during hepatonecrosis [26]. The increase in the AST and ALT levels was ameliorated with the administration of PPE which agrees with a previous study that showed that pomegranate peel extract decreased the ALT and AST serum levels [27].

Glutathione is an antioxidant that helps in the detoxification of drugs and xenobiotics. It helps to improve any abnormality in the liver [28]. The GSH protects the cells and tissues against oxidative stress. The inhibition of the production of GSH causes an increase in the quantity of $O_2$ and $H_2O_2$ which can result in oxidative stress and ultimately lead to liver damage [21]. In this study, the treatment of rats with $CCl_4$ reduced the GSH level in the liver tissue of these rats in comparison to the control group. This agrees with the results of Ullah *et al*. [29]. In addition, our results showed that PPE administration to rats increases the levels of GSH in the liver of these rats. However, no significant increase was recorded when PPE was administered to the $CCl_4$-treated group. This result was in disagreement with the study of Ghamry [30] and Ali *et al*. [31] where PPE significantly elevated the GSH

level of the $CCl_4$-treated group. The non-significant increase recorded in our study might likely be due to the short treatment duration. A longer treatment duration might result in a significant increase in the GSH levels.

Furthermore, lipid peroxidation occurs when free radicals or oxidative species attack lipids with carbon-carbon double bonds resulting in cell injury. Malondialdehyde (MDA) is one of the most mutagenic products of lipid peroxidation and has always been used as a biomarker for cell injury or damage [32]. It is an oxidative stress marker that is used to monitor the level of lipid peroxidation [29]. According to our findings, there was no discernible change in the liver MDA content in $CCl_4$-treated rats when administered with PPE and compared to the untreated control rats. However, other studies found a significant decrease in the MDA concentration in the liver homogenate of rats treated with $CCl_4$ and given PPE for 4 weeks [33]. As noted earlier, this non-significance in the MDA levels in PPE + $CCl_4$ rats recorded in our results might likely be due to the short PPE treatment duration.

Bilirubin is a yellowish waste product that is produced during the breakdown of the red blood and excreted through the liver in the urine. An increase in its concentration can result in jaundice and anemia and may signify a liver problem [24]. When the serum bilirubin level was tested, a significant increase was recorded in the group that was given $CCl_4$. The elevation in the bilirubin level could be a result of hepatocellular injury and cholestatic liver diseases [34]. This elevation in the serum bilirubin level was ameliorated significantly when PPE was administered to the $CCl_4$-only treated group.

Furthermore, our results showed that $CCl_4$ administration in rats led to a complete loss of the hMSH2 gene in the $CCl_4$-only treated group, signifying potential genomic instability. However, When PPE was administered to $CCl_4$-treated rats, the expression levels of the hMSH2 gene significantly increased, activating the DNA repair pathway in the liver cells and restoring the structural integrity of the liver.

Finally, the histology and immunohistochemistry of the liver showed that the administration of $CCl_4$ resulted in damage to the liver architecture and the proliferation of oval cells. However, the treatment of rats with PPE after $CCl_4$ administration restored the changes to the liver histology and immunochemistry resulting from $CCl_4$ administration. This result agrees with the decrease in the levels of serum biomarkers of liver damage **(Figures 2 and 4a)**.

## CONCLUSION

In this study, we assessed the role of pomegranate peel extract in improving hepatotoxicity, immunohistochemistry, and the expression of the MSH2 gene in $CCl_4$-treated rats. We showed that the administration of $CCl_4$ to rats did not affect their body weight and relative liver weight. However, $CCl_4$ administration on the other hand resulted in a decrease in tissue GSH, led to an increase in serum AST, ALT, bilirubin, lipid peroxidation, and modification of liver histology and immunohistochemistry. Moreover, PPE treatment following $CCl_4$ administration to rats resulted in a decrease in serum liver damage biomarkers, restoration of liver histology and immunohistochemistry, an increase in the relative liver weight, and the upregulation of the hMSH2 gene expression. Taken together, our study reveals the potential of PPE for its use in the treatment of liver damage.

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**CONFLICT OF INTEREST :** None

**FINANCIAL SUPPORT :** None

**ETHICS STATEMENT :** Declaration by Bioethics Committee of Scientific and Medical Research approved this animal experiment with No. HAP-02-J-094.

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