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Steroidal Saponins as Antioxidant and Alleviator of CCl_4-Induced Oxidative Damage in Albino Rats

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Abstract: Liver toxicity is a common condition that can be induced by environmental pollutants. The present study explored the hepatoprotective activity of steroidal saponins extracted from the yam plant versus $CC1_4$-induced hepatotoxicity in albino rats. Twenty-five albino rats were classified into 5 groups. Rats of group (G1) were provided a basal diet and drinking water and served as un-treated controls. Other groups were administered $CC1_4$ orally twice a week at a dose of 400 mg/kg. The second group (G2) did not receive any further treatment and served as positive controls while rats in the groups G3, G4 and G5 were administered saponins (50,100 and 200 mg/kg body weight, respectively) for six weeks in the remaining groups. The hepatoprotective activity of saponins was assessed by measurement of liver enzymes, kidney function tests, malondialdehyde content, and antioxidant defense enzymes activities in serum of these rats. Saponins administration improved liver and renal function and significantly increased the activities of catalase, glutathione peroxidase (GSH-PX), glutathione reductase GSH-RD and superoxide dismutase SOD. These increases were linked to a considerable decrease in serum malondialdehyde levels, indicating that lipid peroxidation was being mitigated. Thus, the concentration of saponins (200 mg/kg) is the best concentration of protection against $CC1_4$-induced hepatic injury, improved liver and renal function, and reduced oxidative stress in rats.

1 Introduction

The liver is extremely important organ in protein synthesis, drug and toxin detoxification, bile secretion, bilirubin excretion, hormone metabolism and storage, and fat and carbohydrate metabolism (Amol and Tushar 2013, Trefts et al 2017, Unsal 2020, Michalopoulos and Bhushan 2021). Hepatic diseases are among the most common death causes, as well as a direct economic burden. According to data, approximately 5% of all patients admitted to a general hospital suffer from drug-induced liver injuries (Zhao et al 2018, Zhou et al 2021).

Liver disease is an important global health issue (Williams 2006). Hepatic dysfunction can be caused by infection with viruses, exposure to pollutants (Sun et al 2011). Hepatotoxic ($CC1_4$), is widely employed to cause liver injury in experimental models (Jamshidzadeh et al 2005). Many anti-inflammatory and liver-protective medicines are available; however, some display harmful effects, especially in the immune system. Natural compounds from herbal plants have thus been examined for efficacy and safety as chemical alternatives for liver protection (Ma et al 2009).

Saponins are glycosides consisting of one or more sugar moieties (glycon) and an aglycone (sapogenin), which can be a steroid or triterpenoid (Rocha et al 1995, Haralampidis et al 2001). Saponins are synthesized in many plant species, such as garlic, onion, fenugreek, and ginseng and also found in some marine organisms including starfish and sea cucumbers (El Aziz et al 2019). Steroidal saponins are effective natural therapeutic compounds used as anti- inflammatory treatment due to (i) their ability to react directly with pro-inflammatory cytokins, like interleukin-6 and tumor necrosis factor-alpha (TNF-α) (Lin et al 2012), (ii) their inhibitory effect on macrophages (Dong et al 2013), and (iii) their effects on the arachidonic acid pathway (Li et al 2021). Saponins also reduce cholesterol levels, improve the immune system (Huang et al 2019), prevent diabetes, inhibit tumor growth, improve lipid metabolism and may help in the prevention and treatment of obesity. Saponins are considered antioxidants and prevent fatty liver induced fibrosis in rats (Yuan et al 2018).

The present study evaluated different doses of steroidal saponins extracted from yam (*Dioscorea* spp.) for their protective effects against liver toxicity induced by carbon tetrachloride ($CC1_4$) in rats. In addition, the effects of saponins on the activity of antioxidant enzymes were investigated.

2 Materials and Methods

2.1 Carbon tetrachloride and pure saponins

Carbon tetrachloride ($CC1_4$) and pure steroidal saponins (C_{27}) extracted from yam were supplied by Sigma Chemical Company (St. Louis, MO, USA). All other chemicals used in this work were of analytical grade.

2.2 Animals and Experimental design

Male rats (n = 25) were obtained from the Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo Rats were quarantined for two weeks to allow accommodation to laboratory conditions. Animals were kept under healthy laboratory conditions and fed a standard essential diet containing 20% protein, 5% fat, 4.5% fiber, 8% ash, 2% calcium, 0.6% phosphorus, 3.4% glucose, 2% vitamins; water was provided ad libitum. After accommodation, rats were randomly classified into 5 groups as:

Control (G1): rats were solely fed basal diet and drinking water.

CCl_4-treated (G2): rats fed as above and treated with 400 mg/kg body weight (BW) twice a week for 6 weeks

CCl_4 + saponin 50 mg/kg BW treated (G3): rats treated as G2 animals and administrated saponins daily at a dose of 50 mg/kg BW for 6 weeks.

CCl_4 + saponin 100 mg/kg BW treated (G4): rats treated as G2 animals and administrated saponins daily at a dose of 100 mg/kg BW for 6 weeks.

CCl_4 + saponin 200 mg/kg BW treated group (G5): rats treated as G2 animals and administrated saponins daily at a dose of 200 mg/kg BW for 6 weeks.

Heparinized tubes were used to collect serum samples, 50 U/mL, after 6 weeks of treatment.

2.3 Biochemical analysis

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total proteins (TP), albumin (ALB) Globulins (GLs) and urea levels in the serum were tested using widely available diagnostic kits, from Biomed Company, While creatinine levels in the serum were tested using kit was obtained from Diamond Company.

2.4 Measurement of antioxidant enzyme activities

Serum samples were directly used to assay the activities of superoxide dismatase (SOD), catalase, glutathione peroxidase (GSH-Px), and glutathione reductase (GSH-Rd), the activities of these antioxidant enzymes were measured using kits from Abcam Company.

2. 5 Determination of malondialdehyde levels

Lipid peroxidation levels were assayed by measurement of malondialdehyde (MDA), a final product of peroxidation of unsaturated fatty acids, using kit from Abcam Company. The concentrations of MDA were estimated in nmol / ml.

2.6 Molecular Docking of Saponins (e.g. Diosgenin) in glutathione peroxidase

The molecular docking was performed using autodock4 (Morris et al 2009). Glutathione peroxidase 4 structure 5I71 (Janowski et al 2016) was obtained from protein data bank. A 40 × 34 × 40 Å grid box with -35.571× 11.830× 1.540 the distance between grid points is 0.375 A˚ was applied. The structure of steroidal saponins (the ligand) was drawn using the

draw structure tool in the website (https://pubchem.ncbi.nlm.nih.gov/#draw=true). Ten docking poses were obtained by setting the default parameters of autodock4. The most representative pose was chosen regarding to the energy score.

2.7 Statistics

One-way analysis ANOVA was carried out using SPSS software. The obtained results from five animals from each group were combined and presented as means ± SD. Duncan's multiple range test ($p \leq 0.05$) was used to determine the significant differences between the different treatment groups.

3 Results and Discussion

3.1 Steroidal saponins mitigate CCl_4-induced hepatotoxicity

Activities of liver enzymes (AST, ALT and ALP) in serum of $CC1_4$ intoxicated rats (G2) were substantially higher ($P < 0.05$) than those in serum of rats of normal control group (G1) as shown in **(Fig 1)**. This substantial increase of liver enzymes activities in serum of rats of group (2) reflected the hepatotoxic effect of CCl_4 in these rats. CCl_4-intoxicated rats showed significantly lower activities of liver enzymes after saponins treatment. The protective effect of saponins against liver toxicity induced by CCl_4 was dose-dependent. The lowest levels of transaminases and ALP activities in intoxicated rats of G5 were recorded after treatment with saponins at a dose of 200 mg/kg. The liver harm caused by $CC1_4$ is a classic example of testing drugs' hepatoprotective activity (Luper 1998, Manibusan et al 2007). $CC1_4$ is known to cause degenerative changes and hepatic cell injury leading to the release of hepatic enzymes into the bloodstream (Jaramillo-Juárez et al 2008). Because these enzymes are generally situated in the hepatic cell's cytosol and emitted into the bloodstream following cellular harm, $CC1_4$-induced rises in hepatic enzymes have been related to the significant liver injury. (Singh et al 1998, Ozturk et al 2009). As shown in **Fig 1**, administration of G2 rats with $CC1_4$ resulted in severe hepatocellular damage, and consequently showed high levels of ALT, AST and ALP enzymes in serum of these rats. Comparatively, the activities of these enzymes in serum of rats intoxicated by CCl_4, and treated with saponins reduced significantly, indicating the ability of saponins to regenerate the liver after damage. This protective effect of saponins against liver toxicity induced by CCl_4 indicated its efficacy for treatment of liver dysfunction and promotion of liver regeneration after damage (Huang et al 2021).

3.2 Steroidal saponins enhance serum protein profiles in CCL_4- intoxicated rats

Rats treated with CCl_4 (G2) showed significantly decreased hepatic total protein, albumin and globulin levels ($p < 0.05$) compared to controls (G1) and to rats in groups G4 and G5 as presented in **(Fig 2)**. Group (3) that administered saponins at a dose of 50 mg/kg showed significant ($p < 0.05$) decrease in serum total protein, albumin and globulin levels similar to rats of G2. Higher doses of saponins (G4 and G5) restored these parameters to levels close to those obtained in rats of normal control group (G1). The decrease in total protein may reflect liver harm which results in decreased amino acid absorption or hepatic protein synthesis (Tamim et al 2021). Reduced serum albumin concentrations might result from leakage due to necrosis and damage in both glomeruli and tubules. Moreover, saponins have a hepatoprotective effect against $CC1_4$ -induced hepatic injury and are safe up to 200 mg/kg. Thus, saponins are hepatoprotective. This activity might be mediated by antioxidant properties. Further, the increase in globulins after saponin administration could be attributed to immune-stimulation (Wang et al 2020).

3.3 Effects of Steroidal saponins on serum renal functions

Rats treated with CCl_4 (G2) showed significant ($p < 0.05$) increase of serum creatinine and urea levels compared to rats of normal control (G1) as shown in **(Fig 3)**. Administration of rats with several doses of saponins improved kidney function as evidenced by maintenance in normal range of creatinine and urea in G3, G4, and G5. Administration of $CC1_4$ caused marked renal dysfunction and induced significant oxidative stress in the kidney tissues. Other studies are consistent with the above results in showing detrimental changes in serum biochemical markers of liver and kidney toxicity in rodents (Suzuki et al 2015, Jan and Khan 2016). $CC1_4$ caused cellular injury in the kidney due to the presence of cytochrome P450 in the renal cortex (Abraham et al 1999, Khan et al 2010). Saponins can protect kidney tissues against these toxic effects.

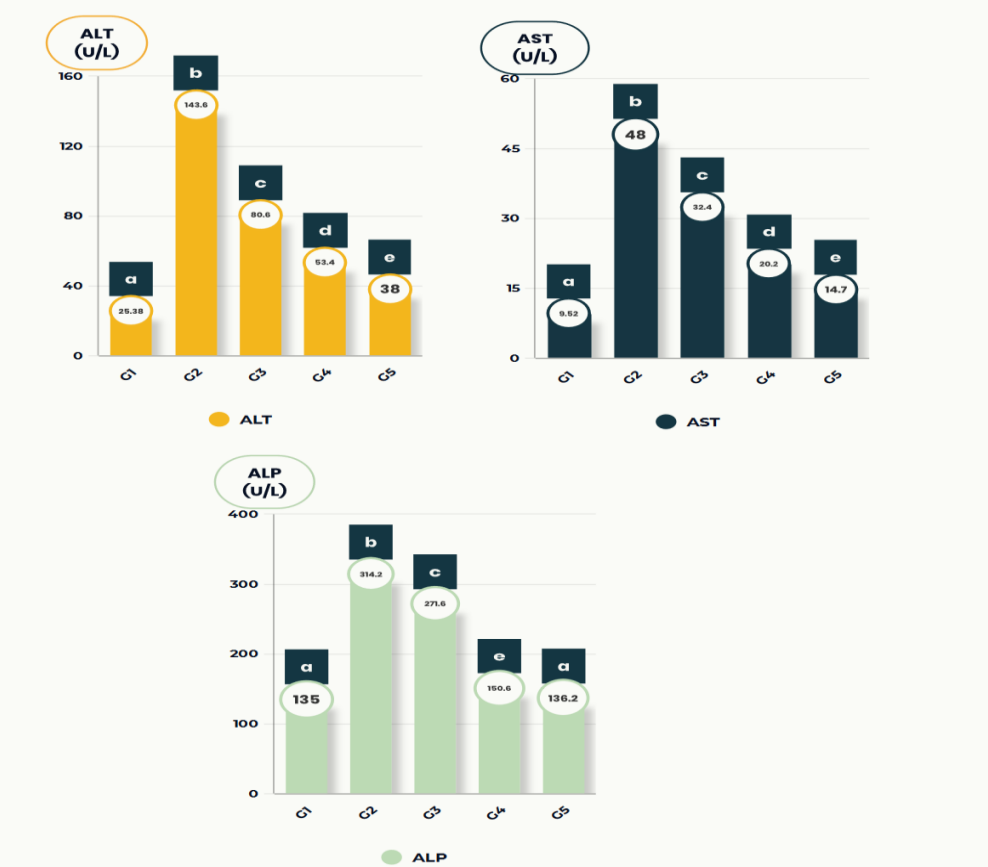

Fig 1. Effects of saponins treatment on liver function (AST, ALT, and ALP) of experimental CCl_4-injured liver rats. Control (G1): rats were solely fed basal diet and drinking water; while groups G2-G5 were with 400 mg/kg BW CCl_4 two times weekly for 6 weeks. G2 did not receive any further treatment and served as a positive control while groups (G3-G5) were treated with saponins (50, 100, and 200 mg/kg, respectively) every twenty-four hours for 6 weeks. The data are presented as means ± SD calculated from five replicates. Different letters refer to significant differences at ($P<0.05$).

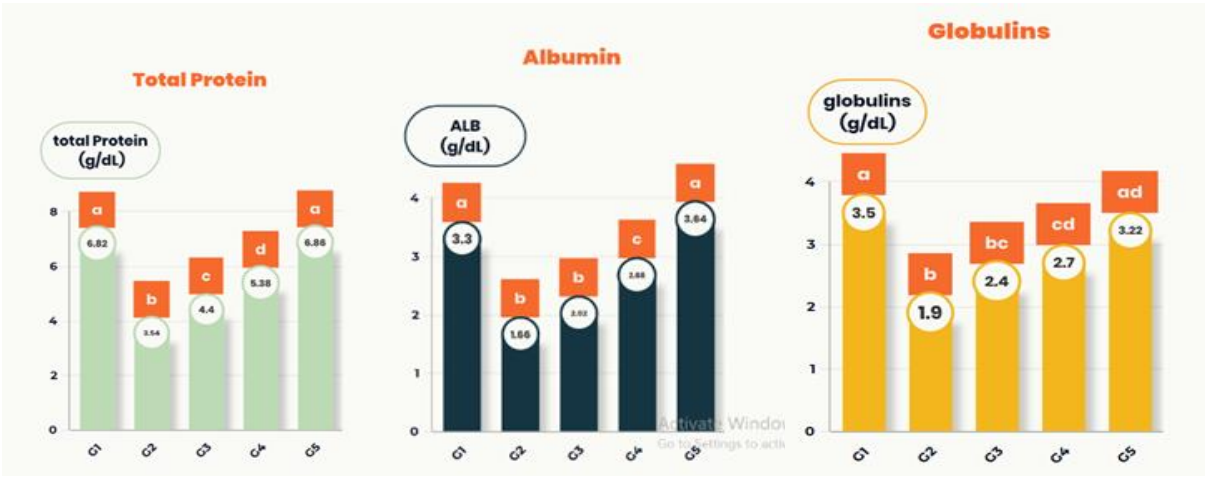

Fig 2. Effects of different treatments on protein profiles (total protein, ALB and globulins) of experimental CCl_4-injured liver rats. Control (G1): rats were solely fed basal diet and drinking water; while groups G2-G5 were with 400 mg/kg BW CCl_4 two times weekly for 6 weeks. G2 did not receive any further treatment and served as a positive control while groups (G3-G5) were treated with saponins (50, 100, and 200 mg/kg, respectively) every twenty-four hours for 6 weeks. The data are presented as means ± SD calculated from five replicates. Different letters refer to significant differences at ($P<0.05$).

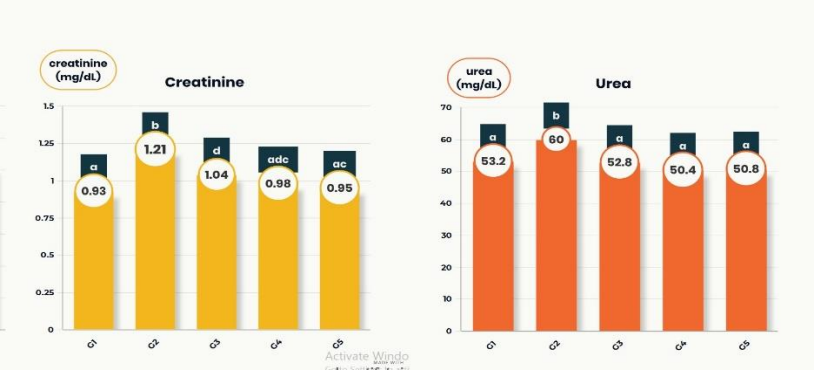

Fig 3. Effects of different treatments on serum levels of urea and creatinine of experimental CCl_4-injured liver rats. Control (G1): rats were solely fed basal diet and drinking water; while groups G2-G5 were with 400 mg/kg BW CCl_4 two times weekly for 6 weeks. G2 did not receive any further treatment and served as a positive control while groups (G3-G5) were treated with saponins (50, 100, and 200 mg/kg, respectively) every twenty-four hours for 6 weeks. The data are presented as means ± SD calculated from five replicates. Different letters refer to significant differences at ($P<0.05$)

3.4 Effects of Steroidal saponins on serum antioxidant enzyme activity

Activities of catalase, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd) enzymes were drastically decreased ($p<0.05$) in serum of rats treated with $CC1_4$ (G2) as presented in **(Fig 4)**. These results revealed that $CC1_4$ induces an extreme oxidative stress and consequently cause depletion of antioxidant enzymes leading to sharp decrease in their activities. Conversely, a meaningful increase ($p < 0.05$) in activities of antioxidant enzymes were noticed after administration of saponins (G3, G4, and G5). When comparing treated rats with CCl_4 only to the normal control group (G1), it can be noticed that rats treated with CCl_4 and saponins in two higher doses, showed substantial improvement in terms of returning to normal levels. Furthermore, Saponins had little effect on these parameters at the lower dose of saponins (50 mg/kg) as recorded in G3. Antioxidant activity and free radical suppression are crucial in preventing the hepatocytes from $CC1_4$ toxicity (Manibusan et al 2007). Obviously, $CC1_4$ induces liver damage. This damage leads to the formation of free radicals and peroxides (Anandan et al 1999). These reactive species can be scavenged by endogenous antioxidant enzymes, which constitute a defense against reactive oxygen species (ROS) (El-Demerdash et al 2016). The accumulation of ROS causes depletion of antioxidant defenses in mitochondria, the critical organelle for energy production through oxidative phosphorylation. Disruption of mitochondrial function might lead to intracellular oxidative stress (Pohjoismäki and Goffart 2017). Further, treatment with pure saponins may protect hepatocytes against hydroperoxides induced by $CC1_4$ and maintain free radical scavenging capability.

3.5 Molecular docking of steroidal saponins (e.g. diosgenin) in glutathione peroxidase

The docking was performed between the catalytic site of GSH-PX4 including the amino acid residues (Sec46, Gln81, Trp136, and Asn137) as shown in fig. 5 and saponins. The results of the molecular docking clearly indicated that the binding energy of steroidal saponins to the catalytic site of GSH-PX4 was a positive value of 469.21 kcal/mol which illustrates that there is no polar or hydrophobic interactions between saponins and any amino acid residue in the catalytic site of GSH-PX. Carbon tetrachloride ($CC1_4$) caused a reduction in glutathione peroxidase activity in serum. The treatment with CCl_4 produced peroxide radicals, which inactivated antioxidant enzymes including SOD, catalase, and GSH-PX (Tirkey et al 2005). Diosgenin is a steroidal saponins that has been reported to have an anti-inflammatory and antioxidant activities (Son et al 2007). To understand the ability of steroidal saponins to counteract the inhibitory effect of CCl_4 on GSH-PX molecular docking was carried out. The docking results suggest that the ability of the steroidal saponins (diosgenin) to activate GSH-PX couldn't be through binding to the catalytic site. Molecular interaction between diosgenin and the binding site of GSH-PX couldn't be performed due to the lack of the information about amino acid residues of the binding

Fig 4. Effect of different treatments on antioxidant enzyme activities of experimental CCl_4-injured liver rats. Control (G1): rats were solely fed basal diet and drinking water; while groups G2-G5 were with 400 mg/kg BW CCl_4 two times weekly for 6 weeks. G2 did not receive any further treatment and served as a positive control while groups (G3-G5) were treated with saponins (50, 100, and 200 mg/kg, respectively) every twenty-four hours for 6 weeks. The data are presented as means ± SD calculated from five replicates. Different letters refer to significant differences at ($P<0.05$).

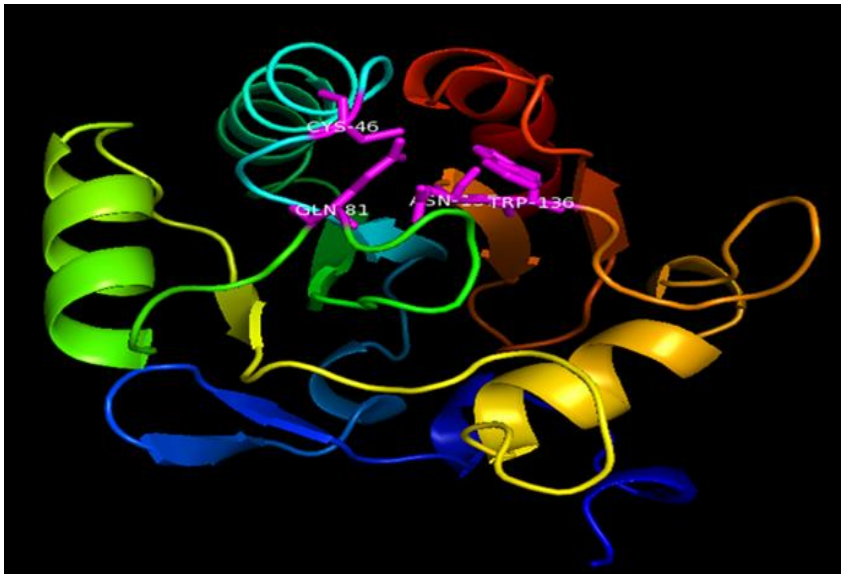

Fig 5. Structure of glutathione peroxidase4 is presented as a cartoon and the catalytic site is shown as magenta sticks

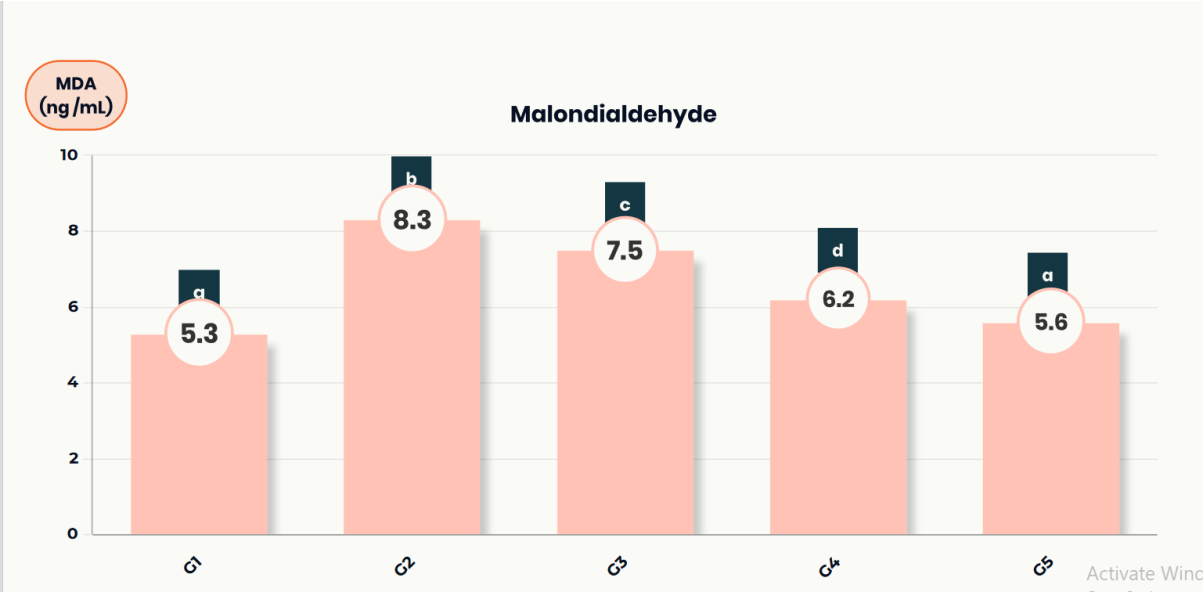

Fig 6. Effect of different treatments on Lipid peroxidation of experimental CCl_4-injured liver rats. Control (G1): rats were solely fed basal diet and drinking water; CCl_4-treated while groups G2-G5 were with 400 mg/kg BW CCl_4 two times weekly for 6 weeks. G2 did not receive any further treatment and served as a positive control while groups (G3-G5) were treated with saponins (50, 100, and 200 mg/kg, respectively) every twenty-four hours for 6 weeks. The data are presented as means ± SD calculated from five replicates. Different letters refer to significant differences at ($P<0.05$).

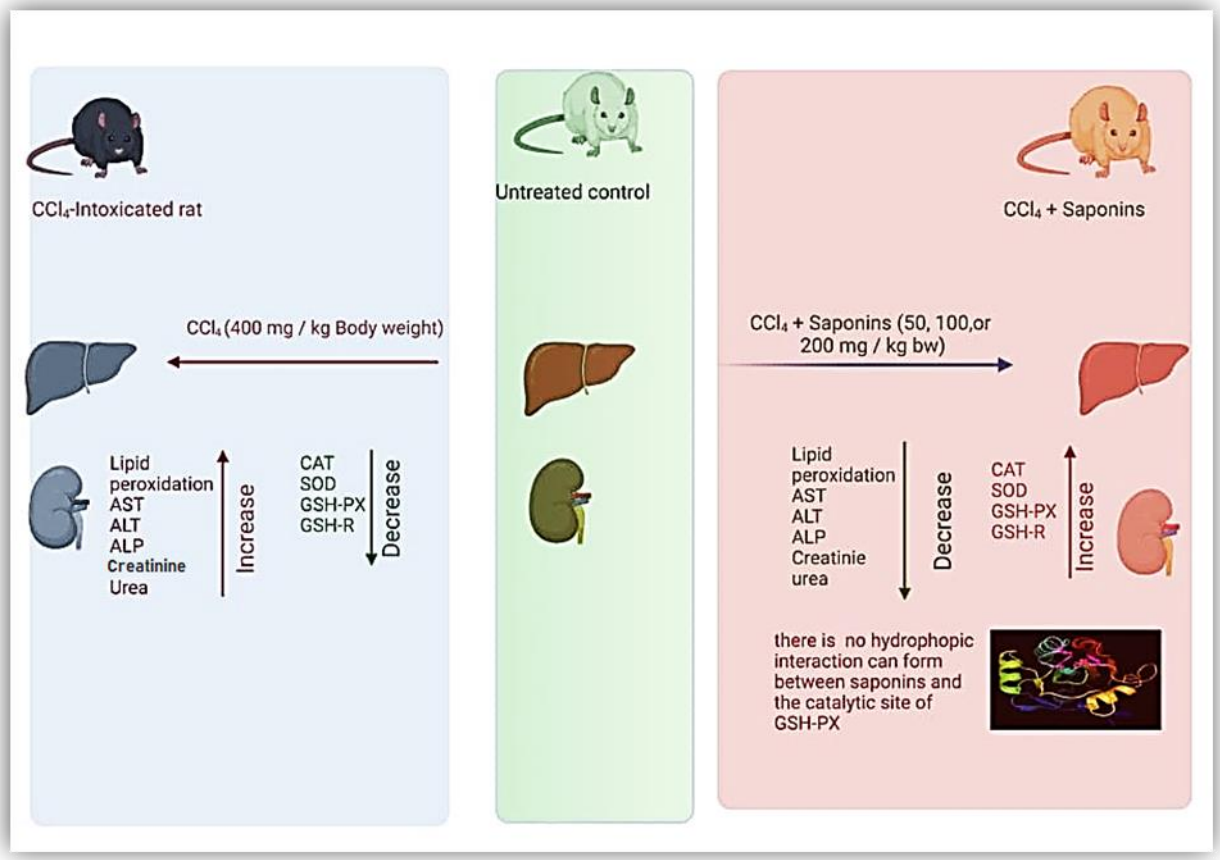

Fig 7. Experimental design to evaluate the effect of steroidal saponins on some biological functions in rats treated with CCl_4.

sites of GSH-PX in the published X- ray crystallography structures of GSH-PX. According to the previous studies (Son et al 2007, Dong et al 2013, Yuan et al 2018, El Aziz et al 2019). The antioxidant activity of saponins is not through an enzymatic pathway but through the scavenging of free radicals which is in agreement with our results.

3.6 Effect of steroidal saponins on lipid peroxidation

Rats treated with CCl_4 400 mg/kg BW (G2) showed significant ($p < 0.05$) increase of malondialdehyde (MDA) levels compared to rats of normal control group (G1) as shown in **(Fig 6)**. Rats of normal control group (G1) and rats treated with different doses of saponins were characterized by low concentrations of MDA. Steroidal saponins, particularly at a dose of 200 mg/kg (G5), reduced MDA to levels close to those obtained in normal control group (G1). Lipid peroxidation, one of the primary reasons for CCl_4-induced liver damage, is generated by free radical derivatives of CCl_4. (Wang et al 2021) indicated that triterpenoid saponins protect against liver injury by toxicants in mice via activation of necrosis and inflammation. An increase in MDA reflects increased lipid peroxidation after $CC1_4$ treatment and indicates oxidative stress (Botsoglou et al 2008). Such stress leads to free radical production. CCl_4 radicals interact with various biological substances, especially fatty acyl residue in membrane phospholipids, resulting in lipid peroxidation (Manibusan et al 2007) Thus, saponins could attenuate lipid oxidation induced by $CC1_4$. Additionally, $CC1_4$ may deactivate CYP2E1 in a lipid peroxidation-independent manner (Kringstein and Cederbaum 1995). Also (Qu et al 2012) suggested that saponins have hepatoprotective activity and saponins could be a source of new natural compound with high potency to alleviate liver dysfunction and fibrosis.

Saponins protected against oxidative stress caused by $CC1_4$ by lowering the amount of generated free radicals, as indicated by the reduced levels of MDA. As illustrated in **(Fig 7)**, saponins reduced the consumption and depletion of liver Superoxide dismutase, catalase, GSH-Px, and GSH-Rd due to free radicals produced by $CC1_4$. The results suggest that saponins, through its free radical scavenging properties, may be able to protect against oxidative hepatocyte membrane injury. Cu/Zn SOD and catalase gene expressions were modestly induced by diosgenin administration, whereas GSH-Px gene expression was considerably induced. This altered antioxidative enzymes balance may be able to eliminate superoxide more effectively. All regulatory cite of all antioxidative enzyme genes, contain NFkB gene expression which is inhibited by the steroidal saponins diosgenin according to previous studies. Although, we still don't know the actual cellular mechanisms responsible for these antioxidative system modifications. As a result, more research into the mechanism of diosgenin-induced changes in antioxidative enzyme gene expression is required. (Son et al 2007).

4 Conclusion

It can be concluded that saponins showed a significant protective effect against $CC1_4$ hepatotoxicity especially at a dose of 200 mg/kg. Also, saponins protected kidney from the renal damage induced by $CC1_4$. In addition, there was no molecular interaction between steroidal saponins and the catalytic site of GSH-PX was recorded.

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