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The Suppressive Role of Proanthocyanidins Against Experimentally Induced Hepatic Fibrosis in Male Rats.

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Abstract The protective effects of proanthocyanidins (PAs) have been investigated against hepatic fibrosis progression prompted by carbon tetrachloride (CCl_4) in male rats. The experimental design was divided into two periods of four and eight weeks. The experimental rats of each period were divided into five groups. The rats of the first groups in two periods were served as controls did not receive any treatments. Second group received only olive oil at a dose (2 mL/kg bw), orally. Third group of rats were orally administered PAs (500mg/kg bw) daily. Fourth group of rats were orally administered CCl_4 dissolved in olive oil (V/V) at a dose of (2 ml/kg bw) day after day. Fifth groups were orally administered PAs for one week alone, then concomitant with CCl_4 at the same doses as the 3rd and 4th groups. After the experimental duration, measurements of selective indicative parameters of liver function and oxidative stress were performed. It could be concluded that administration of PAs showed a remarkable improvement in the serum levels of hepatic function biomarkers of CCl_4-intoxication rats as (AlP, GGT, and LDH), lipid profiles, glucose, albumin, and globulin. Proanthocyanidins also reduced the increase in oxidative stress caused by NO, while rised the activities of hepatic catalase and superoxide dismutase that were reduced by CCl_4-intoxication, signifying their antioxidant capacity. Moreover, PAs have an ameliorative effect on haematological parameters (RBCs, WBCs, Hb, platelets). As a result of these findings, PAs administration showed a suppressive role against hepatic fibrosis progression induced by CCl_4 in male rats and is recommended for use as a complementary supplement for the treatment of a variety of hepatic diseases.

keywords: Proanthocyanidins, carbon tetrachloride, hepatic fibrosis, antioxidant capacity, oxidative stress

1.Introduction

1.1 General background

Liver is a dynamic organ that performs a variety of functions for life. Hepatocytes are the key cell type in the liver parenchyma, making up to 80% of the hepatocellular mass and acting abundant of its energy metabolism and detoxification functions. Because most liver damages lead to hepatocyte death, it's have distinctive regenerative capability such as a marked capacity to respond to increase in metabolic demands of organism (3). Numerous factors have been related to liver injury. Auto-immune disorders are prominent among them (14). Therapeutic medications and toxicants (44), alcohol abuse, and toxins are all examples of xenobiotics (40). liver injury connected to altered liver function induced by exposure to a drug or another noninfectious agent is known as hepatotoxicity.Carbon tetrachloride (CCl_4) is a powerful toxin often utilized in scientific study to provide an experimental model that simulates oxidative stress in a variety of pathophysiological circumstances (52). Where, The unstable free radicals trichloro-methyl radical (CCl3), proxyl trichloromethyl (OOCCl3), and reactive oxygen species are

produced in the liver by cytochrome P4502E1 (CYP2E1) (29). These free radicals be able to cause lipid peroxidation, release inflammatory mediators and trigger cell death and necrosis (21). Among varied sources of natural products found plants, while medicinal plants play an significant role in human health care for decades (36). Recent progress in the fields of bioactive compounds a lead to the discovery of potent drugs that can be developed for many health problems (20). Proanthocyanidins (PAs) consist of a mixture of flavan-3-ol units and flavan-3,4-diols (leucoanthocyanidins) in complicated methods. As, (+)-catechins(−)-epicatechins, and their derivatives(+)catechingallate,

(+)gallocatechingallate, (−)-epicatechin gallate, and (−)-epigallocatechin gallate are all flavan-3-ol monomeric units.Both (+)-catechins and (−)-epicatechins are thought to be starter units for PA polymerization, with flavan-3-ols and flavan-3,4-diols acting as extension units for additional PA polymerization (67). Proanthocyanidins are among the most powerful natural chemicals in terms of antioxidant ability (33) ,Immunomodulatory (56), and anti-inflammatory (33).This study was conducted to explore the potential hepatoprotective effects of PAs with the goal of improving liver function.

2. Materials and methods

2.1 Experimental animals:

Adult Male Wistar rats weighing 100–120 g (VACSERA, Cairo, Egypt) were used for the current study. Rats were under typical laboratory settings, in stainless steel cages with a 12 h light/dark cycle. Rats were acclimatized to the place for 7 days' prior the beginning of the experiments. The experimental methodology followed the National Institutes of Health's guide for the care and use of laboratory animals (NIH Publication No. 8523, revised 1996) and the local experimental animal ethics committee's guidelines. Throughout the study, all rats were fed a regular chow diet and water *ad libitum*.

2.2 Chemicals

Carbon tetrachloride (CCl_4) from BDH Laboratory supplies Poole, BH15 1TD England, Proanthocyanidins (PAs) was supplied by Sigma Chemical Co. (St. Louis, MO, USA). All additional chemicals, including olive oil, were of the highest grade available

2.3 Experimental design

Rats were divided into five groups of 12 rats each as follows.

1- Control groups (Cont): rats did not administer any treatment.

2- Olive oil groups (O Oil): rats were orally administered olive oil alone (2ml /kg bw) day after day for 4 and 8 weeks.

3- PAs groups (PAs): rats were orally administered (PAs) at dose (500mg/kg bw) daily for 5 and 9 weeks.

4- Carbon tetrachloride treated groups (CCl_4): Rats were orally administered CCl_4 dissolved in olive oil day after day for 4 and 8 weeks**.**

5- PAs + CCl_4 treated group (PAs+CCl_4): rats were orally administered (PAs) (500mg/kg bw) daily for one-week prior CCl_4 treatment, then PAs and CCl_4 were administered concomitantly every other day at the same doses administered to the third and fourth groups for 4 and 8weeks.

2.4 Sample collection:

After 4 and 8 weeks of CCl_4 intoxication, rats were fasted overnight and sacrificed 24hrs following the final treatment. To get hold of sera, blood samples were obtained in clean centrifuge tubes, allowed to clot, and then centrifuged at 800 rpm for 15 minutes at 4°C.

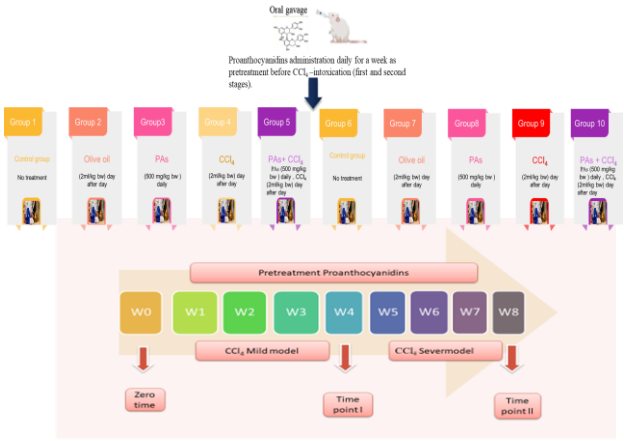


Fig. 1. illustrated Experimental design.

For the biochemical study, the sera were frozen at -20°C. Rat liver was removed, and portion was weighed and homogenized. The homogenates were centrifuged at 1500rpm, and

supernatant was stored at -20°C until biochemical analysis performed.

2.5. Biochemical studies:

Liver index = (liver weight / body weight × 100). **ALP, γ-GT and LDH activities** in serum were estimated in accordance with, (5) (47) and (Vanderlinde, 1985), respectively. **Alb, T. Chol and TG** were estimated by a colorimetric method of (10),(28) , (15) respectively. The concentration of (HDL-C) in serum was estimated by the method of (19), (LDL-C) in serum the method described by (Wieland and Seidel, 1983).Serum very low-density lipoprotein (VLDL-C) level was calculated according to the following equation: VLDL-C = TG/5, as described by (Warnick et al., 1990)Where, 5 is a calculation factor. Glucose by spinreact kit (26). **NO,TAC, SOD and CAT** were estimated by methods of (55),(30), (42)and (1) ; (16) respectively.

2.6. Haematological studies:

Haematological parameters including the count of red blood cells (RBCs), white blood cells (WBCs), haemoglobin (Hb) content, and platelets count were carried out by haematological analyzer (Sysnex Ts-21) Japan (Dacie and Lewis, 1999).

2.7. Statistical analysis

mean and standard error of the mean (SEM) (n = 6) is used to represent the data. One-way ANOVA was used for statistical comparisons, followed by Tukey's post-hoc test.When the P value was <0.05, a significant difference was evaluated.Graph Pad Prism 8.0 was used for all statistical analyses (Graph Pad Software Inc., San Diego, CA, USA).

3. Results

3.1. PAs ameliorated body weight, liver weight and liver index in CCl_4-treated rats.

The induction of liver fibrosis by CCl_4 caused a significant (P<0.05) reduction in body weights while a significant (P<0.05) increase was observed in liver weight and liver index after 4 and 8 weeks of intoxication. On the other hand, administration of PAs into CCl_4 treated rats caused a significant (P<0.05) rise in body weights, and decline in liver weight with liver index. Treatment with PAs or olive oil only displayed insignificant changes in liver weights, liver index and body weights in control rats. (Figure 2, A-C).

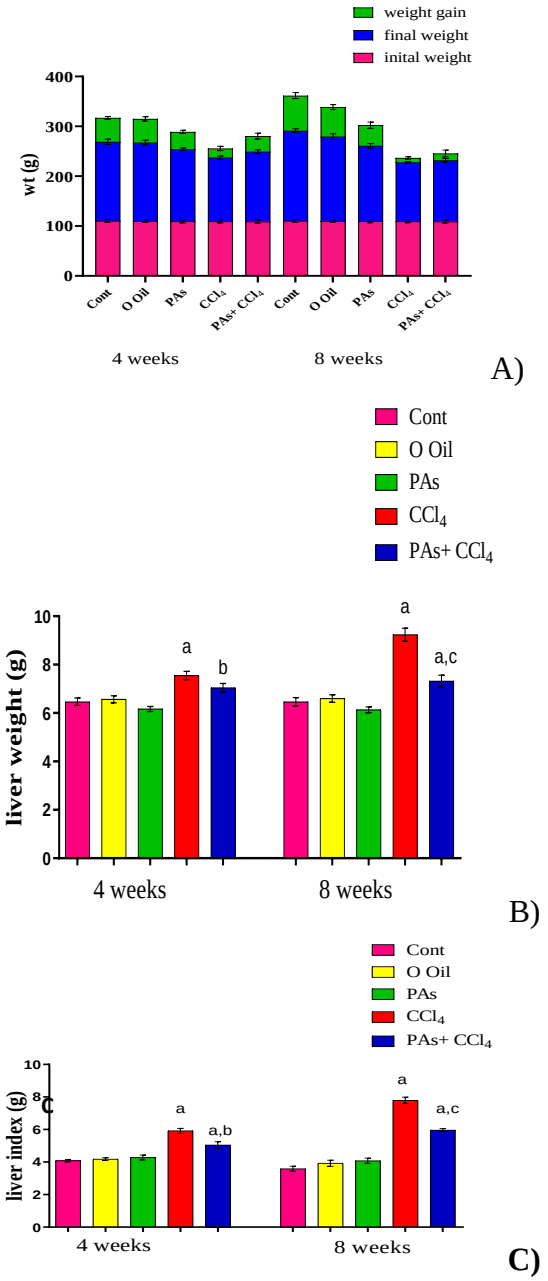


Fig. 2. illustrated body weight, liver weight and liver index.

Data are expressed as mean ± SEM (n=6 for each group). a, b, c significant change at P < 0.05. a: significance as compared with control, b: significance as compared with CCl_4 group in 4weeks, c: significance as compared with CCl_4 group in 8weeks. Cont: control, O Oil: olive oil, PAs: proanthocyanidins, CCl_4: carbon tetrachloride.

3.2. PAs improved Hematological parameters in fibrosis rat model:

The induction of liver fibrosis by CCl_4 affected a significant (P<0.05) reduced in RBCs, Hb, platelets in addition to a significant (P<0.05) increase was observed in WBCs count

after 4 and 8 weeks of intoxication. On the other hand, administration of PAs into CCl4 treated rats caused a significant (P<0.05) amelioration for the hematological parameters. No variations were detected in RBCs, WBCs, Hb and platelets in PAs or olive oil oral administration in control rats after period of treatment (Figure 3, A-D).

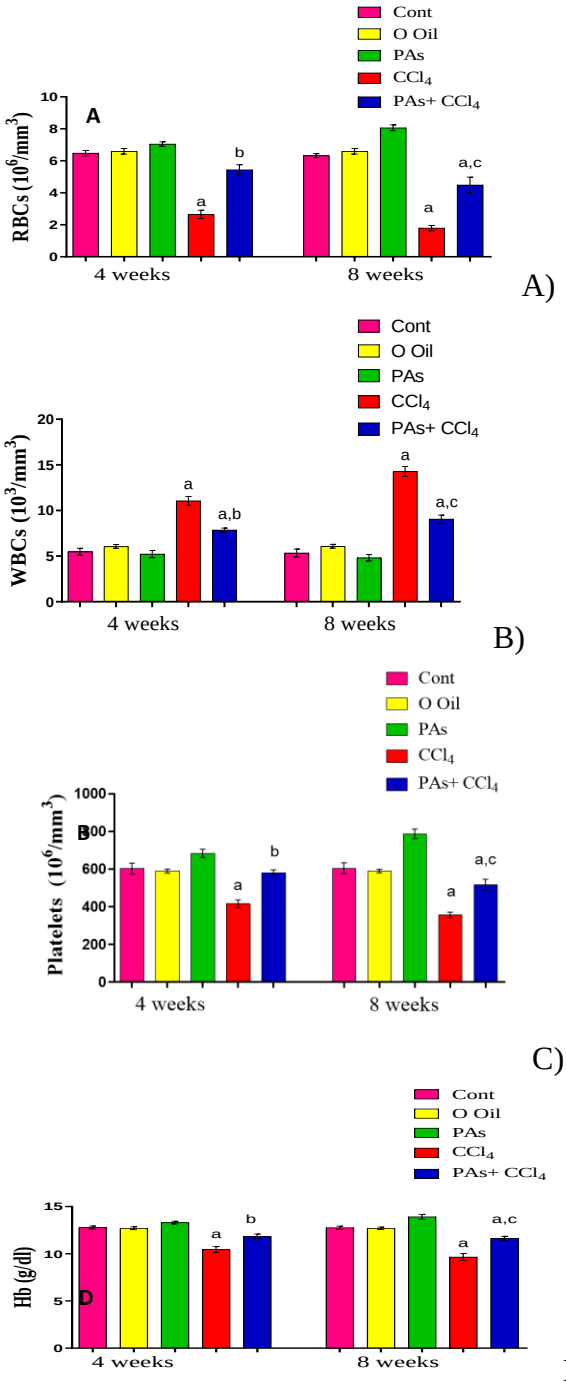
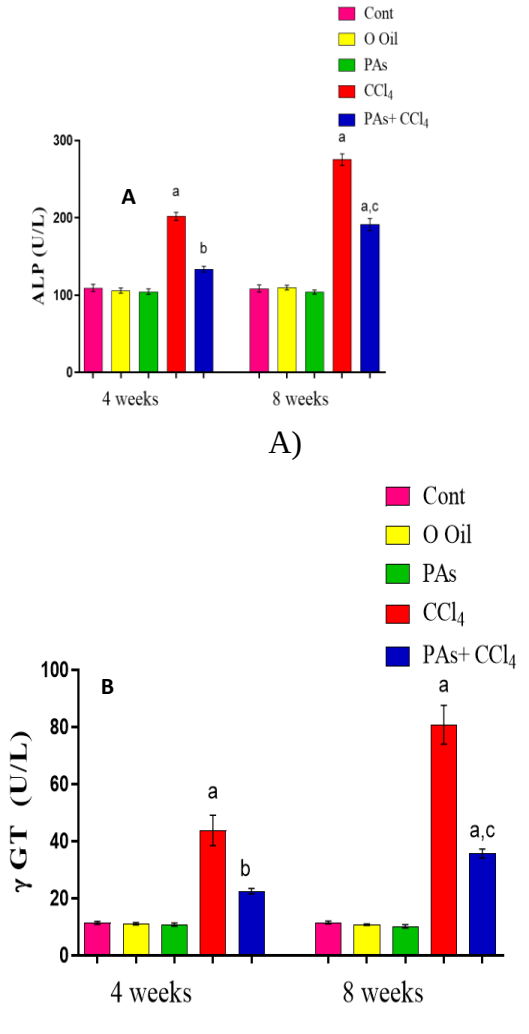


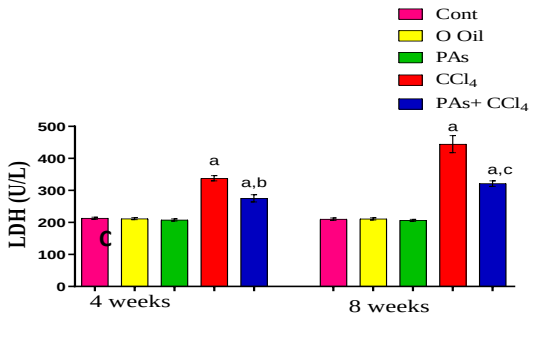
Fig. 3. Assessment of hematological parameters (RBCs, WBCs, Platelets and Hemoglobin).

Statistics are expressed as mean ± SEM (n=6 for each group). a, b, c significant change at P < 0.05. a: significance as compared with control, b: significance as compared with CCl_4 group in 4weeks, c: significance as compared with CCl_4 group in 8weeks. Cont: control, O Oil: olive oil, PAs: proanthocyanidins, CCl_4: carbon tetrachloride.

3.3. PAs improved liver function parameters in CCl_4-treated rats.

The promotion of liver fibrosis by CCl_4 resulted in a significant (P<0.05) elevation of serum levels of liver function enzymes, including ALP, γ-GT, LDH, while albumin and globulin was significantly (P<0.05) decreased in serum after 4 and 8 weeks of intoxication. The severity of changes in these clinical biomarkers was increased with time following CCl_4 treatment. On the other hand, administration of PAs into CCl_4 treated rats caused a significant (P<0.05) improvement in the levels of these biomarkers in serum. Treatment with PAs or olive oil only displayed insignificant changes in these biomarkers in control rats after 4 and 8 weeks of treatment (Figure 4, A-E).





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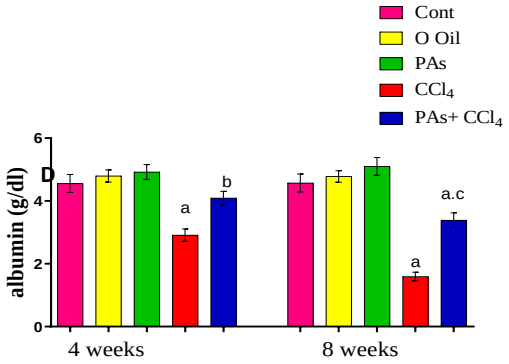

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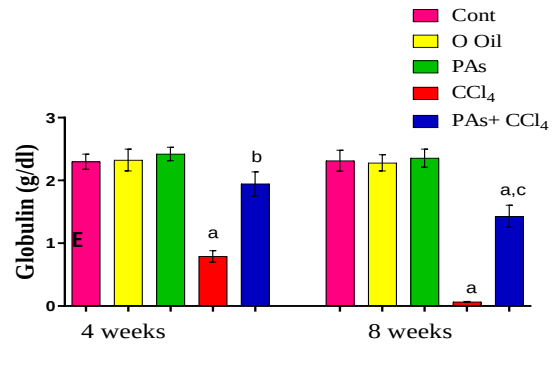


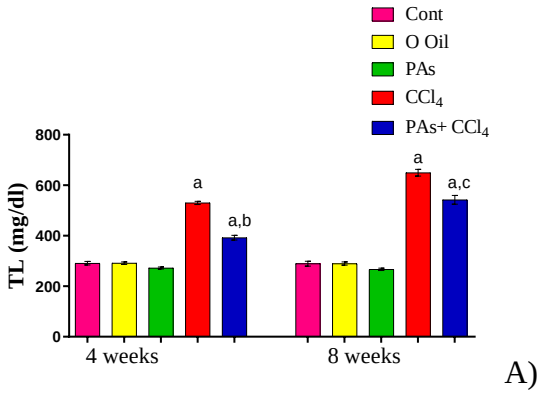
E)

Fig. 4. Showed activities of ALP, γ-GT, LDH (U/L) and albumin, globulin levels (g/dl) in serum of the control and different animal groups.

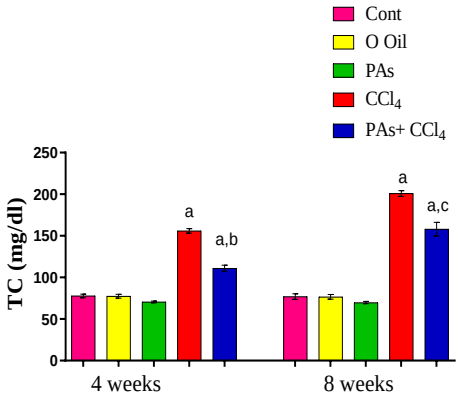
Statistics are expressed as mean ± SEM (n=6 for each group). a, b, c significant change at P < 0.05. a: significance as compared with control, b: significance as compared with CCl_4 group in 4weeks, c: significance as compared with CCl_4 group in 8weeks. Cont: control, O Oil: olive oil, PAs: proanthocyanidins, CCl_4: carbon tetrachloride.

3.4. PAs enhanced Serum lipid profile and glucose in CCl_4-treated rats.

The data presented in Figure (5, A-G) Showed the levels of lipid profile and glucose in serum of the control and different animal groups. induction of liver fibrosis by CCl_4 led to a significant (P<0.05) elevation in serum level of total lipids, TC, TG, LDL-C, VLDL and glucose with a significant (P<0.05) decline in serum levels of HDL-C after 4 and 8 weeks of CCl_4 intoxication. Administration of PAs in rats with CCl_4-induced liver fibrosis resulted in a significant (P<0.05) amelioration of these alterations in lipid fractions and glucose level. With PAs, and olive oil in normal rats, there's no alteration in the serum lipid profile or glucose levels.



A)



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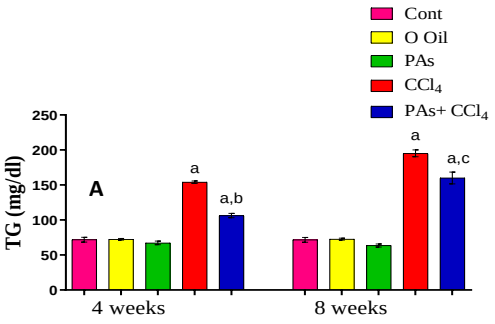

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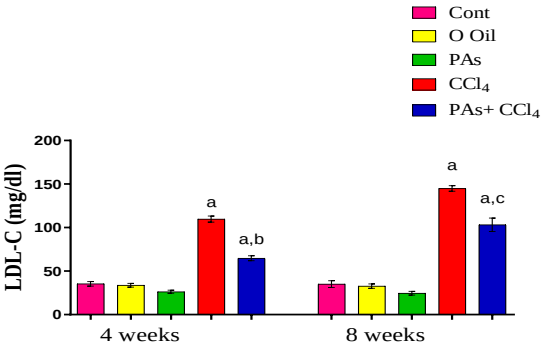

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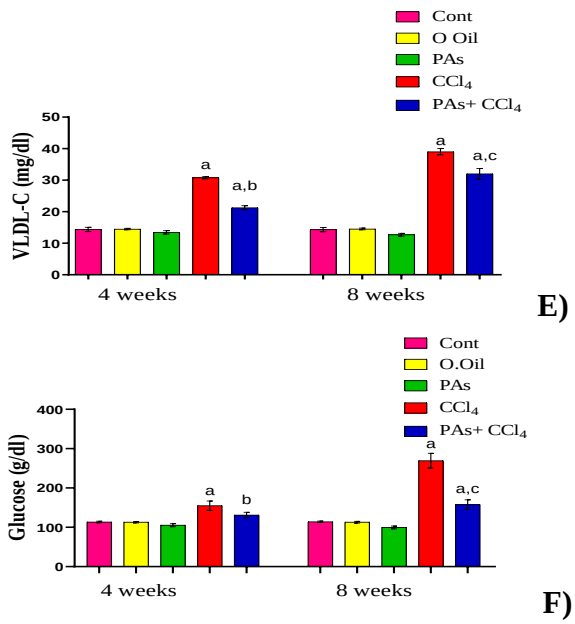

Fig.5. Showed lip id profile (g/dl) in serum and glucose level of the and different animal groups.

ata are represented as mean ± SEM (n=6 for each group). a, b, c significant change at P < 0.05. a: significance as compared with control, b: significance as compared with CCl_4 group in 4weeks, c: significance as compared with CCl_4 group in 8weeks. Cont: control, O Oil: olive oil, PAs: proanthocyanidins, CCl_4: carbon tetrachloride.

3.5 PAs suppressor oxidative stress in CCl_4-treated rats.

The progression of hepatic fibrosis due to CCl4 manifested in a significant (P<0.05) rise in hepatic Nitric oxide (NO) level after 4 and 8 weeks of CCl_4 intoxication. However, administration of PAs in rats intoxicated with CCl_4 resulted in a significant (P<0.05) lessening in hepatic (NO). Treatment with PAs or olive oil alone had no alteration NO in livers in control rats during period of administration (Figure 6, A).

3.6. PAs ameliorated hepatic antioxidant of fibrosis rat model

Hepatic antioxidant (SOD, CAT and TAC) levels showed a significant (P<0.05) decrease after 4 and 8 weeks of hepatic fibrosis induction in rats compared to normal healthy rats. Conversely, PAs with administration for CCl_4-intoxicated rats, hepatic SOD, CAT and TAC levels were significantly (P<0.05) elevated. The treatment with PAs or olive oil alone showed an insignificant difference in SOD, CAT and TAC levels compared with control rats (Figure 5, B-D).

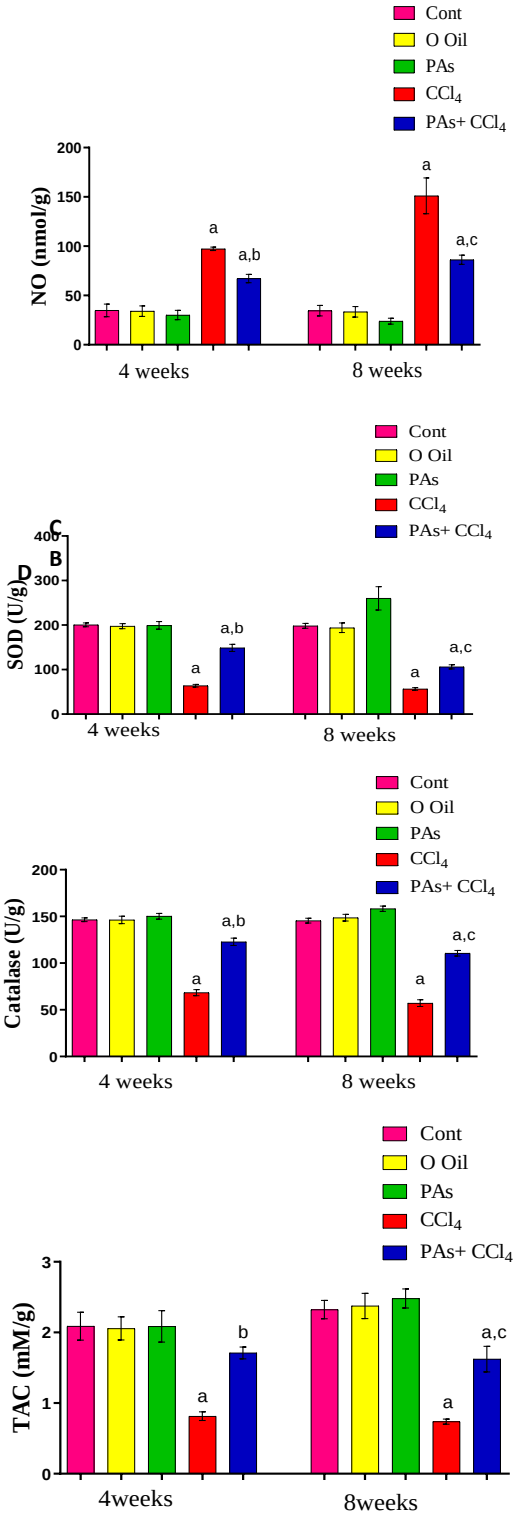

Fig .6. Presented oxidative stress (NO) and antioxidants in liver of control and different animal groups.

Statistics are represented as mean ± SEM (n=6 for each group). a, b, c significant change at P < 0.05. a: significance as compared with control, b: significance as compared with CCl_4 group in 4weeks, c: significance as compared

with CCl_4 group in 8weeks. Cont: control, O Oil: olive oil, PAs: proanthocyanidins, CCl_4: carbon tetrachloride.

4. Discussion

The present study showed that the oral administration of proanthocyanidins at a dose 500mg/kg b.wt. demonstrated significant protection against CCl_4-induced liver fibrosis as manifested by the reduction in toxin mediated rise in serum ALP, ɤGT, and hepatic NO in rats. Hepatocellular injury in CCl_4-induced animals is nearly identical to the toxicity of chemical medicines in the clinic. Therefore, the CCl_4 is reorganized as a typical hepatotoxic chemical commonly used in an experimental animal model of acute liver injury (35). Oxidative stress is a serious factor in the pathophysiology of liver disease. Excessive formation of free radicals in living organisms, radicals which including hydroxyl, hydrogen peroxide, nitric oxide, and superoxide, produces homeostatic imbalance and oxidative stress, resulting in damage to biological components such as proteins, nucleic acids, and membrane lipids (34). In rat hepatocytes, proanthocyanidins can defend the cellular membrane from oxidative damage, preventing protein and lipid oxidation. Furthermore, studies also demonstrated that PAs have forceful actions similar restricting levels of NO, and ROS (18).

Liver fibrosis stimulation in this study, executed regular observation of signs of rat groups. During the experimental duration, control rats were in good condition. As for the rats in the CCl_4-intoxicted groups during four weeks, their body weight kept on reduced. While during eight weeks, their weight decreased with declined appetite and change general behavior of the rats. Significantly decrease in body weight and increase in liver weight was induced by administration of CCl_4 as compared to healthy control rats. so it is clear that, the liver weight ratios significantly increased in rats treated with CCl_4 (9).

Change in body weight are associated with the inflammatory disease conditions and it might be due to less intake of food. CCl_4 administration reduced the body weight, the CCl_4-induced group revealed marked increase in liver weight (57). Because of the infiltration of fatty acids and glycerol into the hepatocytes caused by CCl4 intoxication, the liver rats grew in size and weight (65). The significant reduction in percentage weight change associated with CCl_4 treatment can be attributed to a decrease in food consumption observed during the study period. A possible contributing element could be the anaesthetic mimicking effects of CCl_4, which resulted in sluggishness of all physical responses as well as a decreased reaction to stimuli (46). On the other hand administrated PAs for CCl_4-intoxicated rats significantly restored the abnormal body weight alterations (41). These ameliorative effects can be attributed to the presence of tannins as reported by(43).

In the present study, oral administration of CCl_4 for (4 and 8 weeks) greatly affected all hematological parameters, as decrease in RBCs count, Hb content, platelets count and a notable increase of WBCs our findings are in agreement with that reported by (48), (Elshater et al., 2013).This could be linked to the toxicity of CCl_4, which has been shown to cause liver damage when metabolised by cytochrome P450 by producing highly reactive trichloromethyl (CCl3) and trichloromethylperoxy (CCl3OO) radicals. (62). Besides, the ability of free radical to increase WBCs count indicates that these radicals, to an extent, affected the defense mechanism of treated rats (13). However, the decline in RBC count and Hb level caused by CCl_4 treatment could be attributed to disrupted hematopoiesis, erythrocyte destruction, a decrease in the pace of their creation, and/or an increase in their removal circulation. Furthermore, a reduction in RBC and Platelets was seen in the current study, which can be explained by the inhibition of both erythropoietic and thrombopoietic activities of the bone marrow. (48). Moreover, administration of CCl_4 induced macrocytic, hypochromic anemia as CCl_4 caused a significant increase lipid peroxidation, degradation of membrane proteins, alteration of membrane-bound enzymes as well as erythrocyte osmotic fragility (38).

Otherwise, the effect of PAs administered to CCl_4-intoxicated groups could be attributed to the fact that it had a protective effect against lipid peroxidation, the production of thiobarbituric acid reactive products, and

oxidative hemolysis generated H_2O_2. It can minimise lipid peroxidation and oxidative hemolysis by lowering free radicals, chelating metal ions, or both.

The authors concluded that may modify membrane-dependent processes not just chemically, but also by interacting directly with cell membranes and/or crossing the membrane, causing modification of the lipid bilayer and lipid protein interactions (59). Furthermore, rats demonstrated a significant renewal of these haematological parameters by restoring them to near normal levels due to the presence of 3- and 5-hydroxyl groups with 4-oxo group functions as electron donors to form bonds with electrophilic ions, thereby aiding in the recoupment of the antioxidant defense system and protecting heme from CCl_4-induced oxidative stress. (41).Also, the abnormal hematologic parameters caused by CCl_4 were ameliorated by PAs administration. may be due to the role of flavonoids, which are known to be vasculoprotectors , powerful antioxidants, and possibility of reducing the accumulation of toxic CCl_4 resultant metabolites (50) . Damaged hepatocytes are rich in reactive oxygen intermediates, and these chemicals stimulate stellate cells (HSCs). As a result, the hepatoprotective benefits of PAs may be due to reduced paracrine impulses that cause hepatic fibrosis via activated HSCs (Shin et al., 2010).

In the present study, it was found that CCl_4-intoxication at 4 weeks and 8 weeks has a significant effect on liver function since the activities of serum enzymes were significantly increased compared to those of normal value (60). Through CCl_4 treatment, elevated levels of ALP, LDH, and ɤ -GT are obtained, confirming liver pathophysiology. This elevation in serum hepatic enzymes indicated deterioration in hepatic function due to parenchymal injury after CCl_4-intoxication. Elevation may be explained by the basis of increase in hepatic cell membrane fluidity and permeability that lead to enzymes release into circulation (12). Preceding research discovered that cytosolic liver marker enzymes could leak out of these inflated and necrotic hepatocytes into the blood stream where they were plainly elevated, i.e., correlated with extensive centrilobular necrosis, degeneration, and cellular infiltration of the liver.

According to the reports, CCl_4 generates free radicals that alter hepatocyte cellular permeability, resulting in higher levels of serum biochemical markers such as ALP (31). Accordingly, we found significant elevation of serum ALP, LDH and ɤGT, while albumin levels in the CCl_4-treated group were considerably lower. As previously published, these findings are markers of hepatocyte malfunction, cellular leakage, and impaired functioning integrity of the cell membrane in the liver (27). The above mentioned enzymes turned back to their normal levels in treatment with PAs for CCl_4 intoxicated groups .This may be due to prohibition of intracellular enzyme permeation created by cell membrane stability or cellular regeneration (11). Effective albumin readjustment suggests that liver cells are quickly regaining their functional and secretory functions (23). Intoxication by CCl_4 leads to damage of Golgi apparatus, this in turn adversely affects packaging and release of protein from the hepatocytes. In the present study, Oral administration of PAs protected the intoxicated rats against hepatotoxic effect of CCl_4 as proved remarkably restored serum albumin by PAs (7).

The results of the current study showed that administration of CCl_4 for 4 weeks and 8weeks to the normal rats induced a significant disturbance in the various lipid components. This is characterized by a significant increase in TC, TG, and LDL-C values and a considerable decrease in HDL-C.The increased esterification of fatty acids, suppression of fatty acid β-oxidation, and reduced excretion of cellular lipids may all contribute to the rise in cholesterol levels (6). Moreover, CCl_4-intoxication causes a significant decrease in HDL associated cholesterol (HDL-cholesterol), while, a significant increase in low-density lipoprotein (LDL-cholesterol) observed after hepatocellular damage using a nonlethal dose of CCl_4, this may be attributed to the covalent binding of CCl_4 metabolites, $CCl_3^{\bullet}$ and $CCl_3OO^{\bullet}$, to cell constituents. In the present experimental conditions, administered PAs exhibited significant amelioration of lipid profile these results are parallel with (4). PAs had a substantial reducing effect on lipids. , this is mostly attributed to the regulation of genes encoding hepatic lipid droplets proteins,

SREBP1c, HMG CoA Reductase and peroxisome proliferator-activated receptor-a , that are connected to lipid metabolism (66).

The blocking of VLDL secretion by the liver as a result of alterations in the expression of genes linked to lipid metabolism is one of the explanations of PAs hypotriglyceridemic effect (7). It has been shown that PAs decrease TGs by activation of FXR (Farnesoid X receptor (FXR), transient upregulation of SHP expression and subsequent repression of SREBP1 in liver (7).

The current study showed glucose rise which could be related to hepatocyte damage caused by CCl_4 intoxication (8) or decreasing of glycogen contents in hepatocytes (25). Also, indicating of severe pancreatic toxicity (37). The generation of ROS in response to the high concentrations of glucose may also cause mitochondrial dysfunction and trigger β cells apoptosis (33). PAs is a natural sources antioxidant that may be significant in the activity of various mitochondrial enzymes involved in glucose oxidation and ATP production (2). Results demonstrated that glucose was significantly lower in rats treated with PAs. It could be because PAs have the ability to increase insulin levels in the blood, hence decreasing glucose levels (39).

Oxidative stress, which is caused by an imbalance in the generation of reactive oxygen species (ROS) and antioxidant defence, can pose serious physiological challenges (49). This study found that CCl_4 treatment for 4 and 8 weeks dramatically lowered levels of SOD, CAT, and TAC while drastically increased the levels of NO in hepatic tissue. This could be due to an increase in the production of ROS, which inhibits the creation of antioxidant enzymes and generated toxic radicals by CCl_4(32).

Moreover, in the pathogenesis of liver injury, Nitric oxide (NO) release (17). Its generation could be attributed to the catalysis of inducible Nitric oxide synthase (iNOS), which mediates nitrosative stress and consequent cellular dysfunction, which implicates iNOS in various inflammatory disorders as a detrimental mediator (53). A significant increase in NO that might be responsible for the inflammatory alterations caused by ROS, inhibits NO synthetase (eNOS), resulting in excessive NO synthesis. Because of its antioxidative activity, PAs, on the other hand, scavenges NO generation. PAs activates the PI3-kinase/Akt pathway and phosphorylates eNOS in endothelial cells, which restores NO production (41). Diminution SOD activity by CCl_4 intoxication, indicating a buildup of superoxide anion radicals, which combine with NO to generate peroxinitrite (ONOO) anion, a more potent and damaging free radical. Its actions have the potential to cause macromolecule damage (DNA, RNA, lipids, and proteins) (45). On the other hand, the present data showed that PAs administered caused a significant protection observed by reduction in NO when compared with the high significant elevation of these in CCl_4-intoxicated rats. Proanthocyanidins could protect against oxidative damage by reduce free radical concentration, block their propagation, is significant in preventing diseases (36) and chelate metals with their o-diphenol groups(Rojas and Brewer, 2007). Besides, the existence of electron donating groups connected to the aromatic ring, such as CH3 and OH, should make hydrogen atom calculation easier, allow them to undertake redox reactions, scavenge free radicals more quickly, and protect them from oxidative stress (41).

5. Conclusion

In conclusion, PAs reduces fibrosis in the liver of CCl_4-intoxicated rats. The improvement of hepatic function biochemical, hematological parameters and lipid profile also, glucose. It is recommended that PAs exerted the anti-fibrotic effect by restoring redox stability, suppressing oxidative stress. These conclusions care the therapeutic effect of PAs for hepatic diseases.

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