Apocynin prevented inflammation and oxidative stress in carbon tetra chloride induced hepatic dysfunction in rats

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Abstract

Background:

Liver fibrosis is a leading pathway to cirrhosis and a global clinical issue. Oxidative stress mediated tissue damage is one of the prime causes of hepatic dysfunction and fibrosis. Apocynin is one of many strong antioxidants.

Objective:

To evaluate the effect of apocynin in the carbon tetra chloride (CCl_4) administered hepatic dysfunction in rats.

Methods:

Female Long Evans rats were administered with CCl_4 orally (0.5 ml/kg) twice a week for 2 weeks and were treated with apocynin (100 mg/kg). Both plasma and liver tissues were analyzed for AST, ALT and ALP activities. Oxidative stress parameters were also measured by determining malondialdehyde (MDA), nitric oxide (NO), myeloperoxidase (MPO), advanced protein oxidation product (APOP). In addition, antioxidant enzyme activities such as superoxide dismutase (SOD) and catalase activities in plasma and liver tissues were analyzed. Moreover, inflammation and tissue fibrosis were confirmed by histological staining of liver tissue sections.

Results:

Apocynin significantly reduced serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) activities in carbon tetrachloride treated rats. It also exhibited a considerable reduction of the oxidative stress markers (such as malondialdehyde (MDA), myeloperoxidase (MPO), nitric oxide (NO), and advanced protein oxidation product (APOP) level) which were elevated due to CCl_4 administration in rats. Apocynin treatment also restored the catalase and superoxide dismutase activity in CCl_4 treated rats. Histological analysis of liver sections revealed that apocynin prevented inflammatory cells infiltration and fibrosis in CCl_4 administered rats.

Conclusion: These results suggest that apocynin protects liver damage induced by CCl_4 by inhibiting lipid peroxidation and stimulating the cellular antioxidant system.

Key words: apocynin, malondialdehyde, myeloperoxidase, superoxide dismutase.

Introduction

Liver fibrosis is characterized by increased deposition and altered composition of extracellular matrix, such that there is an excess of collagens I, III, and IV. This might lead to liver cirrhosis, liver failure, hepatocellular cancer along with fibrogenic development. Advanced fibrosis and cirrhosis are generally considered to be irreversible conditions even after removal of the injurious agent (Iredale et al., 1998). Cirrhosis which is the end stage of liver fibrosis is the leading cause of liver disease related morbidity and mortality (Zhao et al., 2014). Hepatic cirrhosis or fibrosis is known to be an irreversible distortion and alteration of the normal tissue architecture; this kind of alteration develops during chronic liver damage. In This phenomenon, multiple processes are involved in the progress of this lesion including: oxidative stress by free radicals (FR); chronic inflammation mediated in part by the release of pro-inflammatory cytokines from Kupffer cells; and fibrosis induced by the paracrine action of pro-inflammatory and profibrogenic cytokines produced by Kupffer cells and hepatocytes on hepatic stellate cells (López-Reyes et al., 2008).

Recently, the possible effect of free radical mediated oxidative injury in the pathogenesis of alcohol-induced liver diseases has received increasing attention. Clinical studies have shown that products of lipid peroxidation can be detected in the liver and in the blood of heavy drinkers and that oxidative damage increases proportionally with the amount of ethanol consumed (Albano et al., 1996). Harmful oxidative reaction with strong oxidizing compounds is believed to damage cells and tissues, therefore leading to chronic diseases and senescence. Free radicals usually contain unpaired electrons in their atoms. These unpaired electrons usually give a considerable degree of reactivity to free radicals. Free radicals, such as superoxide anion radical, hydroxyl radicals, lipid free radicals, nitrogen dioxide and nitric oxide free radicals, together with hydrogen peroxide, singlet oxygen and ozone, are major forms of reactive oxygen species (ROS) *in vivo* (Zhu et al., 2012). Many hepatotoxicants including CCl_4, nitrosamines, and polycyclic aromatic hydrocarbons require metabolic activation, especially by liver cytochrome P450 (P450) enzymes to form reactive, toxic metabolites that in turn produce liver injury in experimental animals and humans. Carbon tetrachloride, a well-known model compound for the production of chemical hepatic injury, requires biotransformation by hepatic microsomal P450 to produce hepatotoxic metabolites, namely trichloromethyl free radicals (CCl_3 and/or CCl_3OO. Trichloromethyl free radicals can react with sulfhydryl groups, such as glutathione (GSH) and protein thiols, and the covalent binding of trichloromethyl free

radicals to cell proteins is considered the initial step in a chain of events that eventually lead to membrane lipid peroxidation and finally to cell necrosis (Jeong et al., 2002).

Currently, there has been increased interest in using natural antioxidants to prevent the free radical induced hepatic toxicity. Apocynin, also known as acetovanillone, is a natural compound structurally related to vanillin(Fan et al., 1999). Apocynin was discovered from *Picrorhizakurroa* plant during an attempt on activity-guided isolation of immunomodulatory constituents from plant extract. Ithas been used as an efficient inhibitor of the complex NADPH-oxidase in many experimental models involving phagocytic and nonphagocytic cells (Petronio et al., 2013),and to increase the synthesis of glutathione in alveolar epithelial cells by increasing gamma-glutamylcysteine synthesis through activation of the transcription factor AP-1 (Ben-Shaul et al., 2001). Apocynin administration in HFD-fed animals has been shown to improve insulin sensitivity, reduce the diverse plasma inflammatory cytokines, suppress gene expression of inflammation-related molecules in both liver and adipose tissue, and decrease the activity of transcription factor NF-κB in liver tissue(Lu et al., 2007b; Meng et al., 2011b). Apocynin treatment could remarkably reduce systemic oxidative stress and suppress hepatic lipid peroxidation and increased antioxidant capacity (Meng et al., 2011a). However, beneficial role of apocynin in liver fibrosis was not investigated. Thus, the current study was undertaken to evaluate the role of apocynin in CCl_4 induced liver damage in rats.

Materials and Methods

Experimental Animals and Treatment:

Ten- to twelve-weeks-old, 24 Long-Evans female rats (150–180 g) were obtained from Animal Production Unit of Animal House at the Department of Pharmaceutical Sciences, North South University, and were kept in individual cages at room temperature of 25±3∘C with a 12 h dark/light cycles. They have free access to standard laboratory feed (pellet food crushed to coarse powder) and water, according to the study protocol approved by Ethical Committee of Department of Pharmaceutical Sciences, North South University, for animal care and experimentation.

To study the hepatoprotective effects of apocynin, experimental rats were equally divided into four groups (seven rats in each group): Group I (Control), Group II (Control + apocynin), Group III (CCl_4) and Group IV (CCl_4+apocynin). Rats of group I were treated with 1mL/kg of saline (0.85%) and olive oil (1 mL/kg) intragastrically twice a week for two weeks. Rats of

groups III and IV were treated with CCl_4 (1: 3 in olive oil) at a dose of 1 mL/kg intragastrically twice a week for two weeks. However, animals of groups II and IV were treated with apocynin 100 mg/kg orally every day for two weeks. The weighed quantity of apocynin was dissolved in olive oil. Olive oil was chosen as the vehicle because apocynin is soluble in olive oil. Animals were checked for the body weight and water intake on a daily basis. After two weeks, all animals were anesthetized using ketamine, and then all the animals were weighted, sacrificed, collected the blood and organs like heart, kidney, spleen and liver. Immediately after collection of the organs, they are weighted and stored at −20°C for further studies. Blood was drawn via syringe and centrifuged at 8000 rpm for 15 min at 4°C. Then serum was transferred using a micropipette into micro centrifuge tubes and stored at 4°C until analyzed.

Chemicals

CCl_4 were obtained from Merck (Germany) and apocynin from the Kuri& Company, office: 78, Motijheel C/A Dhaka-1000, Bangladesh. Thiobarbituric acid (TBA) was purchased from Sigma Chemical Company (USA) and trichloroacetic acid (TCA) from J.I. Baker (USA). Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase assay kits were obtained from DCI diagnostics (Budapest, Hungary), 50, 50-dithiobis-2 - nitrobenzoate (Ellman's reagent) from Sigma (USA) and sodium hydroxide from Merck (Germany). All other chemicals and reagents used were of analytical grade.

Assessment of Hepatotoxicity

Liver marker enzymes (alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were estimated in plasma by using Diatech diagnostic kits (Hungary) according to the manufacturer's protocol.

Preparation of Tissue Sample for the Assessment of Oxidative Stress Markers

For determination of oxidative stress markers, liver tissue was homogenized in 10 volumes of phosphate buffer containing pH 7.4 and centrifuged at 8000 rpm for 15 min at 4∘C. The supernatant was collected and used for the determination of protein and enzymatic studies as described below.

Estimation of lipid peroxidation as malondialdehyde (MDA)

Plasma concentrations of thiobarbituric acid reactive substances (TBARS) are the index of lipid peroxidation and oxidative stress. Lipid peroxidation in liver was estimated calorimetrically measuring MDA using thiobarbituric acid reactive substances (TBARS) followed by

previously described method (Niehaus and Samuelsson, 1968). The absorbance of clear supernatant was measured in Elisa reader against reference blank at 535 nm.

Estimation of nitric oxide (NO):

NO was determined according to the method described by Tracy et al.(1995) as nitrate and nitrite In this study, Griess-Illosvoy reagent was modified by using naphthyl ethylene diaminedihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). NO level was measured by using standard curve and expressed as nmol/gm of tissue.

Determination of advanced protein oxidation products assay (APOP)

Determination of AOPP levels was performed by modification of the method of Witko-Sarsat et al. and Tiwari et al.,[(Hussein et al., 2005; Schwartz and Vanoli, 1981)]. The chloramines - T absorbance at 340 nm being linear within the range of 0 to 100 mmol/L, APOP concentrations were expressed as μmol/L chloramine -T equivalents.

Determination of Catalase level (CAT)

CAT activities were determined by the method of Chance and Maehly with some modifications [(Chance and Maehly, 1955; Khan, 2012)]. Changes in absorbance of the reaction solution at 240 nm were determined after one minute. One unit of CAT activity was defined as an absorbance change of 0.01 as units/min.

Statistical analysis

All values are expressed as mean ± standard error of mean (SEM). The results were evaluated by using One-way ANOVA followed by Newman-Keuls post hoc test using Graph Pad Prism Software, version 6. Statistical significance was considered $p < 0.05$ in all cases.

Results

Effect of Apocynin on bodyweight, food and water intake of carbon tetra chloride induced rats

Body weight of each rat was recorded every day during the experiment, and change in weight was noted for all groups as shown in Table 1. No significant change in body weight, food weight and water weight between the groups was observed.

Effect of apocynin on liver wet weight of carbon tetra chloride induced rats

There was no significant change in weight of wet liver between CCl_4 induced rats and control group, and treatment group as shown in Table 1.

Effect of apocynin on AST, ALT and ALP activity in liver tissue of carbon tetra chloride induced rat

There was significant elevation in the activities of ALT, AST, and ALP in the liver assessment with CCl_4 treatment when compared to the control rats. Treatment with apocynin decreased these elevations of parameters (Figure 1).

Effect of apocynin on oxidative stress parameters MDA, NO and APOP level in plasma and liver tissue of carbon tetra chloride induced rats.

In plasma, the MDA level of CCl_4 group was significantly higher than the control. However, after treatment with apocynin, the MDA level in the plasma decreased considerably (Figure 2A). Similarly, the MDA level in the liver of CCl4 group was found initially to be much higher than the control. But after the treatment with apocynin, as Figure 2D shows, it was observed the decrease in the MDA level in the liver.

There is a significant increase in the plasma level of NO in CCl_4 induced rats compared to the control (Figure 2B). Apocynin normalizes the level of NO in the treatment group. Similarly, there was a significant elevation of NO level in the liver assessment in the CCl_4 induced rats when compared with the control. The NO level decreased after the consumption of apocynin (Figure 2E).

The level of APOP in the plasma increased significantly in the CCl_4 rats when it was compared to that of the control (Figure 2C). Apocynin treatment normalized the APOP level. In similar way in the liver assessment, as the Figure 2F shows, the significant increase of APOP level in the liver was observed. After apocynin treatment, the APOP level decreased considerably.

Effect of apocynin on MPO activity in liver tissue of carbon tetra chloride induced rats.

The MPO activity level was measured in liver. In CCl_4 induced rats, the MPO level was significantly high and elevated than the control rats. The apocynin decreased the MPO level after consumption (Figure 3).

Effect of apocynin on antioxidant enzyme catalase and SOD activity in plasma and liver tissue of carbon tetra chloride induced rats

Catalase activity in plasma and liver was measured. There was a significant decrease in the catalase activity level both in plasma and liver in CCl_4 induced rats. The catalase level increased after the treatment with apocynin (Figure 4A and 4B).

SOD activity was assessed in both liver and plasma. In both plasma and liver assessment, the SOD activity decreased significantly in CCl_4 induced rats compared to the control rats. Apocynin treatment elevated the SOD activity after treatment (Figure 4C and 4D).

Effect of apocynin on hepatic inflammatory cells infiltration and fibrosis in liver tissue of carbon tetra chloride induced rats

H and E staining analysis of the liver showed marked vacuolar degeneration in carbon tetra chloride induced rats (Figure 5). The carbon tetra chloride induced rats also showed massive infiltration by inflammatory cells (Figure 5C) as well as increase interstitial collagen deposition in liver (Figure 5G) compared to Control (Figure 5A & 5E) rats. Apocynin markedly reduced inflammation (Figure 5D) and collagen deposition (Figure 5H) in carbon tetra chloride induced rats.

Discussions

The onset and development of liver cirrhosis is related to an increase in hepatic free radical generation (Camps et al., 2001). Oxidative stress derived from NADPH oxidase has been implicated to many liver diseases, including cirrhosis, ischemia reperfusion injury, hepatocellular carcinoma, hemorrhagic shock, and acetaminophen-induced liver toxicity (Lu et al., 2007a). In the last decade, apocynin, a constituent of root extracts of *Picrorhizakurroa*, has been used in many laboratories as a potent and selective inhibitor of the O_2^- anion-generating NADPH oxidase of activated neutrophils. There has been a growing interest in apocynin as an antioxidant and anti-inflammatory agent(Riganti et al., 2006).

It was reported that apocynin could reverse high cholesterol-induced liver injury, including abnormal liver function tests, microvascular leakage, and inflammatory infiltrates of macrophages, reduce expression of gp91 phox, decrease TBARS, and replenish cellular GSH and NADPH, all of which alleviated hepatic oxidative burden during hypercholesterolemia (Lu et al., 2007a).In this study, we demonstrated the Hepatoprotective action of apocynin in CCl_4induced liver fibrosis in rats. Present results are supportive of previous literature. Oxidative stress induced by NADPH oxidase as a result of CCl_4 treatment has been reported to play a critical role in CCl_4-mediated ROS generation and liver toxicity (El-Sawalhi and Ahmed, 2014), leading to elevations of AST, ALT and ALP activity. In the current study, it was observed that the treatment with apocynin decreased the elevations of AST, ALT and ALP activity in liver tissue of carbon tetra chloride induced rats as shown in Figure 1.

Apocynin also decreased the level of MDA, a marker of oxidative stress, in both plasma and liver after the level was found to have been elevated by the CCl_4 induction in CCl_4 induced rats. Similarly, the apocynin treatment decreased and normalized the NO level, which was initially elevated by CCl_4 induction as shown in Figure 2. NO is readily upregulated in the liver under a number of conditions, including endotoxemia, hemorrhagic shock, ischemia-reperfusion, sepsis, infection, hepatitis, ozone exposure, and liver regeneration (Li and Billiar, 1999). In the present study, a significant decrease of catalase activity level in both plasma and liver for the CCl_4 induced rats was observed. Treatment with apocynin increased levels of catalase, an enzyme that protects cells from oxidative damage by Reactive Oxygen Species (ROS) (Dashboard).

The progression from steatosis to steatohepatitis involves a further decrease in the antioxidant capacity of the liver, as shown by the significant decrease in the activity of CAT and SOD, the two major enzymes affording antioxidant protection. These changes are accompanied by an increased CYP2E1 activity, which involves a high production of free radicals and reactive intermediates, thus rendering the liver more susceptible to oxidative stress. Enhancement in hepatic CYP2E1 activity could lead to SOD and CAT inactivation upon progression of the disease (VIDELA et al., 2004). In our result we have observed that, in CCl_4 induced rats, the SOD level in both plasma and liver was significantly decreased, but the effect was overturned in apocynin treated rats. This further indicates that the hepatoprotective effect of apocynin is through limiting of free radicals.

One of the principal molecules released after recruitment and activation of phagocytes is myeloperoxidase (MPO), an important enzyme that is involved in the generation of reactive oxygen species. MPO is highly expressed by neutrophils and as such is widely used as a neutrophil marker. These oxidants may contribute to host tissue damage at sites of inflammation through reactions with a wide range of biological substrates, including DNA, lipids, and protein amino groups (Rensen et al., 2009). In our result, it was found that in CCl_4 induced rats, the MPO level was significantly high and elevated when it was compared to the control rats; apocynin treatment decreased the MPO level.

Our study demonstrates for the first time that apocynin has hepatoprotective role in CCl4 induced hepatic dysfunction that can be attributed to its powerful antioxidant and anti-inflammatory activities as several common markers of oxidative stress were down regulated and markers of antioxidant activity were upregulated.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Table 1: Effect of apocynin on body weight, food and water intake and organ weight of CCl_4 treated rats.

Parameters	Control	CCl_4	Control+APC	CCl_4 +APC
Initial Body weight (g)	181.29±3.25	180.09±1.94	160.59±0.72	175.21±4.07
Final Body weight (g)	178.67±7.42	183.31±4.62	175.49±2.80	194.21±5.49
Food Intake (g/day)	20.96±3.55	16.62±2.40	19.05±0.84	16.38±1.11
Water Intake (mL/day)	19.06±2.53	19.72±3.65	20.26±1.06	18.53±1.19
Kidneys wet weight (g/ 100 g of body weight)	0.65± 0.03	0.53±0.01	0.76±0.017	0.68±0.02
Liver wet weight (g/ 100 g of body weight)	3.69±0.13	3.39±0.07	3.27±0.084	3.15±0.126
Spleen wet weight (g/ 100 g of body weight)	0.31±0.03	0.46±0.04	0.40±0.0139	0.34±0.034

Data are presented as mean±SEM, n=6-7 or otherwise stated. Statistical analysis was conducted by One Way ANOVA followed by Newman-Keuls post hoc test, significance was considered as $p<0.05$ in all cases.

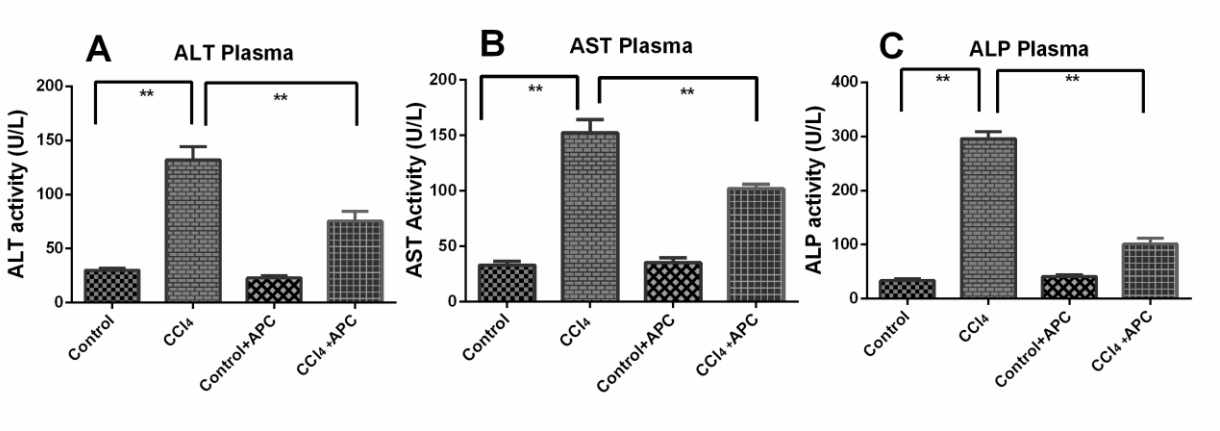

Figure 1: Effect of apocynin on AST, ALT and ALP activity in liver tissue of carbon tetra chloride induced rats. Data are presented as mean±SEM, n=6-7 or otherwise stated. Statistical analysis was performed by One Way ANOVA with Newman-Keul's post hoc test. Statistical significance is considered as $p<0.05$.

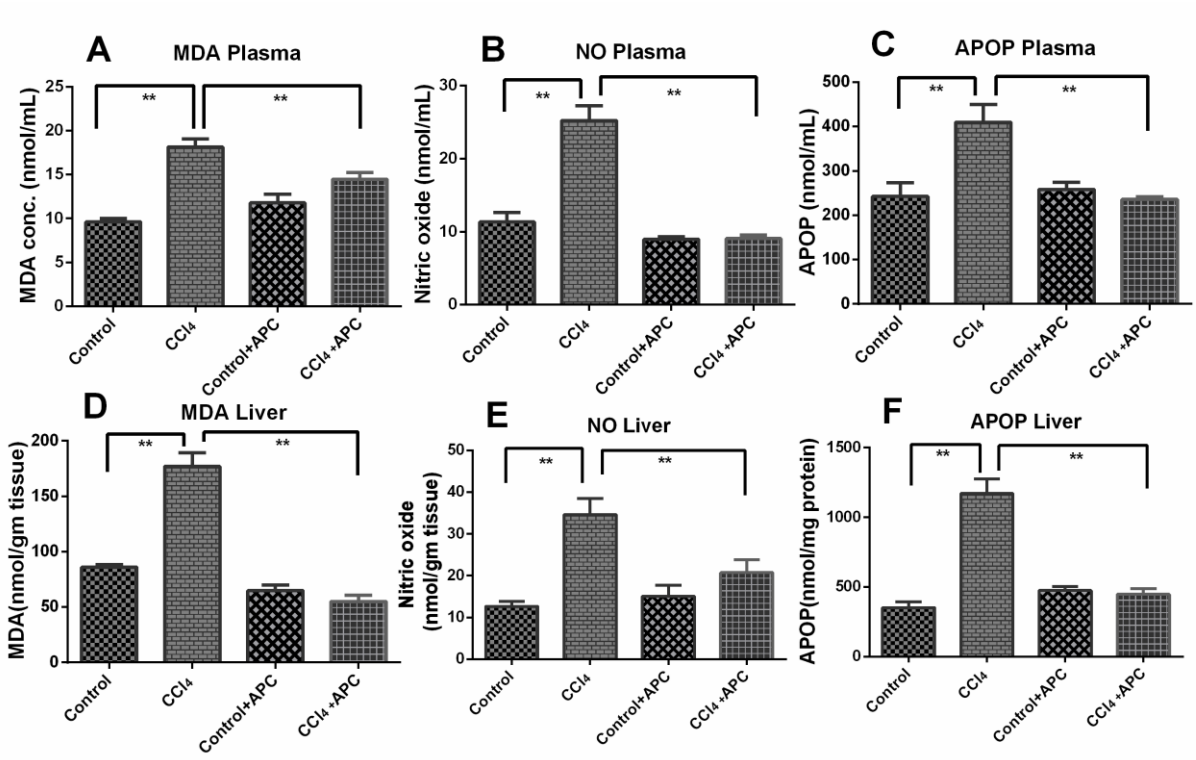

Figure 2: Effect of apocynin on oxidative stress parameters MDA, NO and APOP level in plasma and liver tissue of carbon tetra chloride induced rats. Data are presented as mean±SEM, n=6-7 or otherwise stated. Statistical analysis was performed by One Way ANOVA with Newman-Keul's post hoc test. Statistical significance is considered as $p<0.05$.

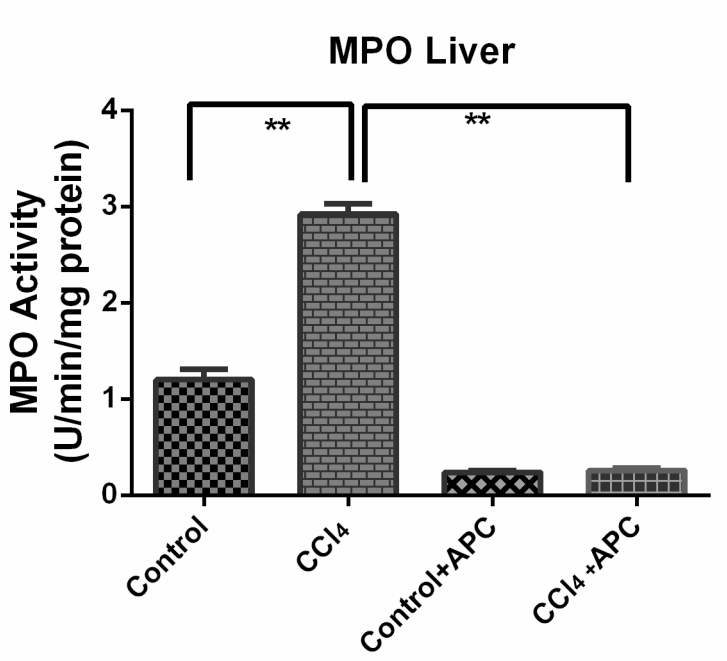

Figure 3: Effect of apocynin on MPO activity in liver tissue of carbon tetra chloride induced rats. Data are presented as mean±SEM, n=6-7 or otherwise stated. Statistical analysis was performed by One Way ANOVA with Newman-Keul's post hoc test. Statistical significance is considered as $p<0.05$.

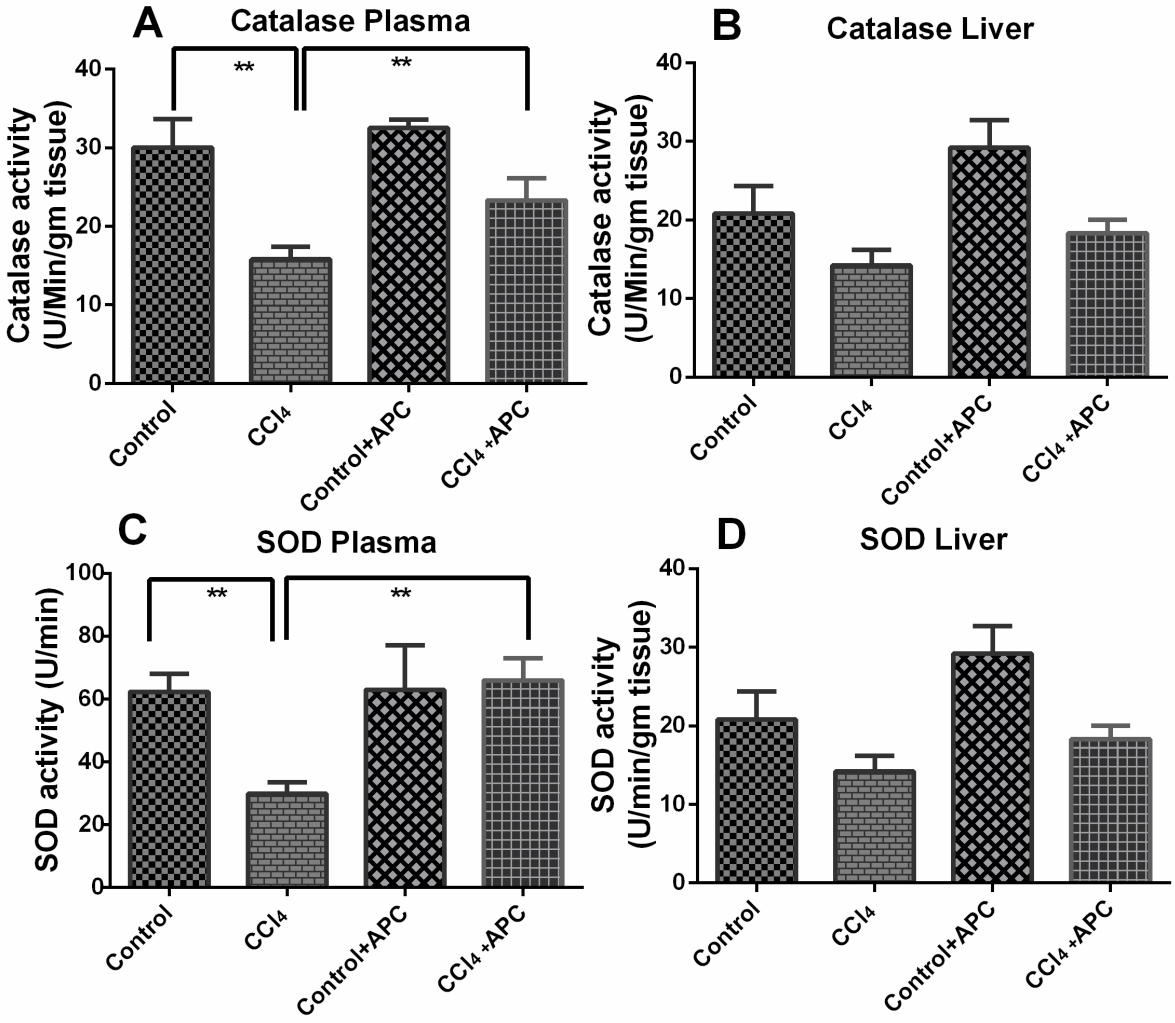

Figure 4: Effect of apocynin on antioxidant enzyme catalase and SOD activity in plasma and liver tissue of carbon tetra chloride induced rats. Data are presented as mean±SEM, n=6-7 or otherwise stated. Statistical analysis was carried out by One Way ANOVA with Newman-Keul's post hoc test. Statistical significance is considered as $p<0.05$.

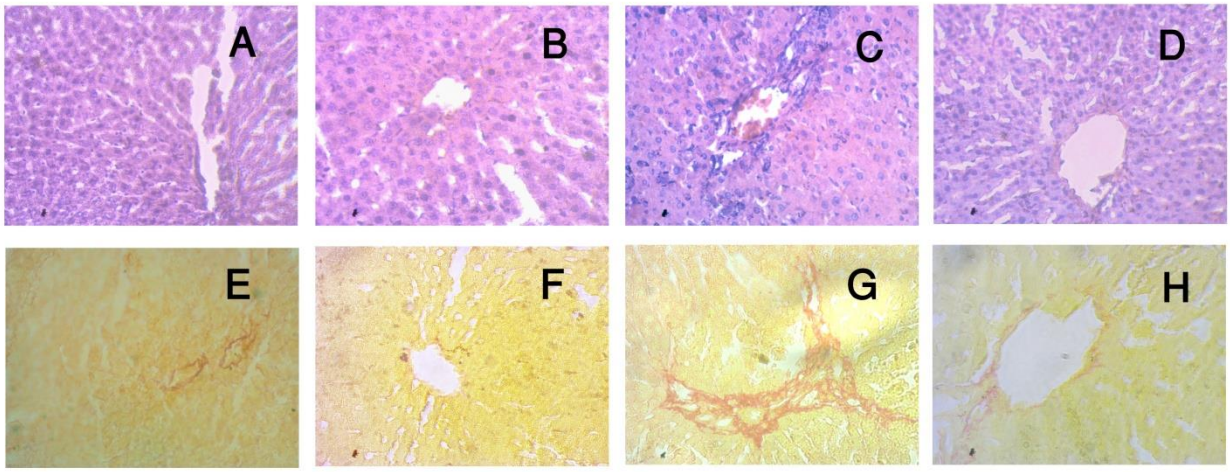

Figure 5: Effect of apocynin on inflammatory cells infiltration and fibrosis in liver tissue of carbon tetra chloride induced rats. A, E-Control; B, F-Control+apocynin; C, G- CCl_4 and D, H- CCl_4+ apocynin. Magnifications 40X.