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Hepato-Renal Protection of Silymarin in Comparison with Vitamin E in Rats

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Abstract: Liver and kidney are exposed to a lot of oxidant substances that are both from exogenous and endogenous sources. The aim of this study was to compare between the antioxidant effects of silymarin and vitamin E against carbon tetrachloride (CCl_4) induced oxidative stress in rats. Male albino rats were divided into four groups. The first group received corn oil s.c. twice weekly and distilled water daily by gavage and served as a control. The second group was treated s.c. with CCl_4 (320mg/100g b.wt.) diluted in corn oil twice per week. The third group was treated with CCl_4 together with daily oral administration of silymarin (10mg/100g b.wt.). The fourth group was treated with CCl_4 simultaneously with s.c. administration of vitamin E.(20mg/100g b.wt.) diluted in corn oil twice weekly. The treatment regimen in all groups last for six weeks. The obtained results showed a highly significant increase in ALT, AST, ALP activities, urea and creatinine levels in serum and malondialdehyde level (MDA) in liver and kidney homogenates in CCl_4 treated group (2^{nd} group) as compared with control. Significant reductions in all parameters were recorded in silymarin and vitamin E treated groups in comparison with CCl_4 treated one. The reduction was more pronounced in silymarin treated group followed by vitamin E treated one. Subcutaneous administration of CCl_4 also induced a significant reduction in Hb g%, PCV%, RBCs and WBCs counts. A significant reduction in reduced glutathione levels and catalase enzyme activities in liver and kidney homogenates and index weights of these organs in comparison with control group were recorded. These parameters were markedly improved with silymarin treatment compared to vitamin E treatment . In summary, administration of silymarin or vitamin E ameliorated CCl_4 induced oxidative stress in rats, with silymarin as the most efficient antioxidant followed by vitamin E.

Key words: Silymarin · Vitamin E · Antioxidant · Free Radicals · Oxidative Stress · CCl_4 · Liver · Kidney · Functions

INTRODUCTION

Drug exposure, ionizing radiations and environmental pro-oxidant pollutants induce free radical formation. Lipid peroxidation initiated by free radicals is considered to be deleterious for cell membranes and has been implicated in a number of pathological situations [1, 2]. The generation of small amounts of free radicals appears to have an important biological junction, but oxidative stress is caused by excess production of reactive oxygen species. Oxidative stress can produce alteration of protein and nucleic acid structure, increase in intracellular free calcium, damage to membrane ion transport and permeability and destruction of the cells by lipid peroxidation [1].

The valuable medicinal properties of different plants are due to presence of several constituents such as saponines, tannins, alkaloids, alkenyl phenols, glycoalkaloids, flavonoids sesquiterpenes lactones, terpenoids and phorbol esters. Among them some act as synergistic and enhance the bioactivity of others [3].

Silymarin is known as milk thistle or *Silybum marianum* and is a member of the aster family that has been used as a medicinal plant since ancient times [4, 5]. Silymarin is a mixture of flavonolignanes in which silibinin is the main compound. Although, silymarin performs its properties in different ways, a range of ones has been related to its antioxidant activities [6-9]. Silibinin and silymarin have important and protective activities against oxidative stress on various organs, such as liver, pancreas and gastrointestinal tract [10-13].

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According to several early studies, silymarin has hepatoprotective properties. Silymarin causes prevention of free radical damage, stabilization of plasma membranes and stimulation of new liver cell production [14].

Previous *in vitro* studies reported a protective effect of silymarin on kidney cells against oxidative damage induced by paracetamol, cisplatin [15], aflatoxin B1 [16], fumonisin B1 [17] and ischemia/ reperfusion injury(I/R) [18]. That protection occurs through decreasing the increased risk of oxidative stress damage and a restoration of the thiol status (GSH) in the kidney [19].

Antioxidant vitamins as vitamin E and C are important in protecting against many oxidant-mediated inflammation and inflammatory tissue damage [20].

Vitamin E can protect against liver damage and prevent the fibrosis and cirrhosis progression in many toxic exposure substances [21].

It is well established that carbon tetrachloride (CCl_4) an industrial solvent, has been widely used in experimental animal models to investigate chemical toxin-induced liver injury [1]. The CCl_4 produced damage to liver cells and was followed by the significant increase in serum alanine aminotransferase ALT activity and hepatic lipid peroxidation after 24 hrs. In addition to carbon tetrachloride (CCl_4), is a well established hepatotoxin. It was demonstrated that liver is not the only target organ of CCl_4 and it causes free radical generation in other tissues also such as kidney, heart, lung, testis, brain and blood [1]. It has also been reported that exposure to CCl_4 induces acute and chronic renal injuries. There are little studies concerning the protective effect of silymarin on kidney functions so the present study aimed to evaluate the protective efficiency of silymarin in comparison with vitamin E on hepatic and renal functions after CCl4 experimental induction of oxidative stress in wister rats.

MATERIALS AND METHODS

Chemicals: CCl_4: Wt. per ml 20°C. 1.592-1.595g, was obtained from (EL-Naser Chemical Co. Egypt). Vitamin E:Wt per ml.1000 mg Di-alpha tocopherol acetate, was obtained from pharmaceutical company (Pharco, Alexandria - Egypt). Silymarin was friendly provided by Medizen Pharmaceutical plant Co. (Borg El-Arab, Egypt). All the diagnostic kits assaying hepatic and renal function tests and the levels of the antioxidants and MDA in liver and kidney homogenates were obtained from Bio-diagnostic Company, Egypt.

Animals and Experimental Design: Twenty adult male Wister rats (weighing 150-180 g, 18 weeks age) were obtained from a closed random bred colony at the Medical Research Institute of Alexandria University, Egypt. Animals were housed in plastic cages with free access to the commercial basal food and water. The standard laboratory diet was purchased from Damanhur Feed Co. (Behera, Egypt). The animals were acclimatized 2 weeks prior to the experiments. Rats received humane care in compliance with the guidelines of the National Institutes of Health (NIH) of Animal Care and the local committee approved this study.

Rats Were Divided into 4 Groups (5 Rats Each)

Group 1: (Control rats) was injected s.c. with 0.2 ml/ 100 g b.wt. of corn oil (vehicle of CCl_4 and vit E) twice weekly and was daily administered 0.2 ml of distilled water/ 100 g b.wt. (vehicle of silymarin) intra-gastrically using a stomach tube.

Group 2: (CCl_4 treated rats) received CCl4 diluted in corn oil (1:1 ratio) in a dose of 0.2ml/100g b.wt. (320 mg/100g b.wt.) s.c. twice weekly according to Lodhi *et al*. [22].

Group 3: (CCl_4 and silymarin treated rats received CCL_4 diluted in corn oil (1:1 ratio) in a dose of 0.2ml/100g b.wt. (320 mg/100g b.wt.) s.c. twice weekly together with daily oral administration of silymarin at a dose of 10 mg/100 g b.wt. in 0.2 ml distilled water/ 100 g b.wt. intra-gastrically using a stomach tube [22].

Group 4: (CCL_4 and vit E treated rats) was injected s.c. with CCL_4 in corn oil (0.2ml/100g b.wt.) concurrently with administration of vit E at a dose of 20 mg/100g b.wt. diluted in 0.2 ml corn oil/ 100g b.wt. twice weekly according to Halim *et al*. [23].

Animals received vit E or silymarin 2hrs. prior to CCL_4 administration. All treatment regimens were conducted for 6 weeks.

Blood Sampling: At the end of the experiment, individual blood samples were drawn from their retro-orbital plexus under light ether anesthesia before being killed by decapitation. Two blood samples were collected from each animal. The first sample was collected on Disodium salt of ethylene diamine tetra-acetic acid (EDTA) for hematological studies. The second one was collected without anticoagulant for obtaining serum and kept frozen at –20°C until used for biochemical analysis.

Blood Analysis: Hemoglobin concentration was determined according to the method described by Drabkin and Austin [24] using commercially available diagnostic kits (Bio Diagnostic Co., Egypt). Packed cell volume percent was determined by microhematocrite technique [25]. Erythrocytic and total leukocytic counts were performed using Double improved Neubauer haemocytometer [25].

Organs Weights: All rats were euthanized at the end of the experiment. After animal dissection, the liver and kidney were rapidly removed, grossly examined and weighed. The index weight (I.W.) of each organ was calculated according to Matousek [26] where, I.W. = organ weight (g) / body weight (g) x 100.

Serum Biochemical Parameters: Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured colourimetrically according to the method described by Reitman and Frankel [27]. Alkaline phosphatase (ALP) was measured colourimetrically according to the method described by Kind and King [28]. Serum urea was measured by enzymatic colourimetric method as described by Coulomb and Farreau [29]. Serum creatinine was measured by colourimetric kinetic method according to Husdan and Rapoport [30].

Antioxidant Status and Oxidative Stress Assays: liver and kidney of each rat was kept frozen at – 70°C for assessment of reduced glutathione (GSH) and lipid peroxidation (LPO) contents and catalase activity.GSH level was assessed spectrophotometrically according to Sedlak and Lindsay [31]. The method is based on the reductive cleavage of 5, 5-dithiobis- (2-nitrobenzoic acid) by sulfhydryl (-SH) group to yield a yellow colour with maximum absorbance at 412 nm. LPO was quantified as malondialdehyde (MDA) according to the method described by Ohkawa *et al*. [32]. MDA was measured after the reaction with thiobarbituric acid in acidic medium at 95°C for 30 minutes to form thiobarbituric acid reactive product, the absorbance of the resultant pink product can be measured spectrophotometrically at 534 nm. Catalase enzyme (CAT) activity was assayed according to Aebi [33]. Catalase activity was estimated based on the reaction of the enzyme with a known quantity of H2O2. the reaction is stopped after exactly one minute with catalase inhibitor, in the presence of peroxidase (HRP) the remaining H_2O_2 reacts with 3,5- Dichloro-2-hydroxybenzene sulforic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with a color intensity (measured spectrophotometrically at 500-520 nm) inversely proportional to the amount of the catalase in the original sample.

Statistical Analysis: Results were statistically analyzed by one-way analysis of variance followed by Duncan's multiple range test [34]. Data are presented as means plus or minus the standard error. The minimum level of significance was set at $p < 0.05$.

RESULTS

Antioxidant Status and Oxidative Stress Assays: Compared to the control group, LPO in liver and kidney tissues was markedly increased in all treated groups as represented by a significant increase (P <0.05) in MDA level. CCL_4 + vit E treated rats and CCL_4 + silymarin treated rats expressed a significant reduction in levels of MDA in relation to CCL_4 treated rats, the reduction was more pronounced in CCL_4+ silymarin group (Table 1). Moreover, Table 1 shows that GSH levels and catalase activities were significantly decreased (P <0.05) in the CCl4 treated rats compared to control group. While there was a significant elevation in their values in CCL_4 + vit E and CCL_4 + silymarin treated rats as compared with CCL4-treated rats. The elevation in their values in liver tissues was more pronounced in CCL4 + silymarin treated group.

Table 1: Effect of CCL_4 alone or in combination with either Silymarin or vitamin E on liver and kidney lipid peroxidation(MDA) level, GSH content and catalase activity of rats

Parameter	Liver LP (nmol MDA/g wet tissue)	Renal LP (nmol MDA/g wet tissue)	liver GSH (umol /g wet tissue)	Renal GSH (umol/g wet tissue)	Liver catalase (unit/g wet tissue)	Renal catalase (unit/g wet tissue)
Group						
Control	4.88± 0.12 ^{d}	4.14±0.11^{d}	14.96±0.28^{a}	10.47±0.24^{a}	8.02±0.20^{a}	6.46±0.11 ^{a}
CCL_4	10.60± 0.19 ^{a}	7.81±0.08 ^{a}	9.46±0.26 ^{d}	7.73±0.13 ^{c}	5.80±0.12 ^{c}	3.82±0.09 ^{c}
CCL_4+Silymarin	6.12± 0.06 ^{c}	6.35±0.14 ^{c}	13.36±0.34 ^{b}	8.48±0.15 ^{b}	7.77±0.09 ^{a}	5.33±0.08 ^{b}
CCL_4+ Vitamin E	7.91± 0.17 ^{b}	6.97±0.10 ^{b}	11.86±0.18 ^{c}	8.54±0.33 ^{b}	6.99±0.13 ^{b}	5.09±0.05 ^{b}

Values are expressed as mean±S.E. N= 5. Values with different letters at the same column are significantly different at $P \leq 0.05$.

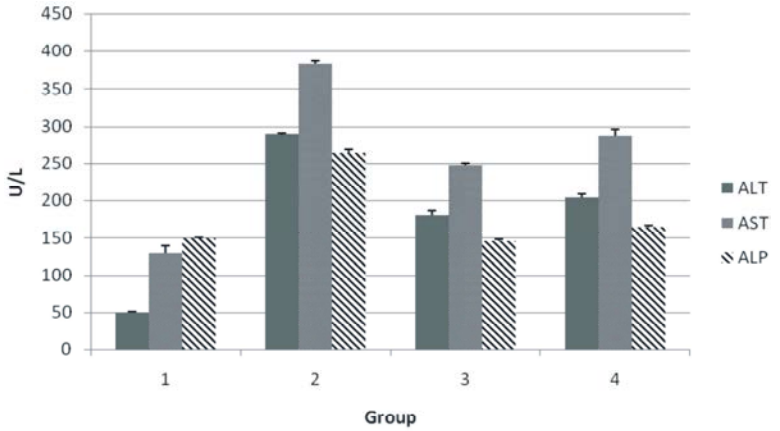

Fig. 1: Effect of CCL_4 alone and in combination with either Silymarin or Vitamin E on serum ALT, AST and ALP activities of rats.
Group:1=Control. 2:CCL_4. 3:CCL_4+ Silymarin. 4:CCL_4+ Vitamin E Values are expressed as mean±S.E. N= 5.

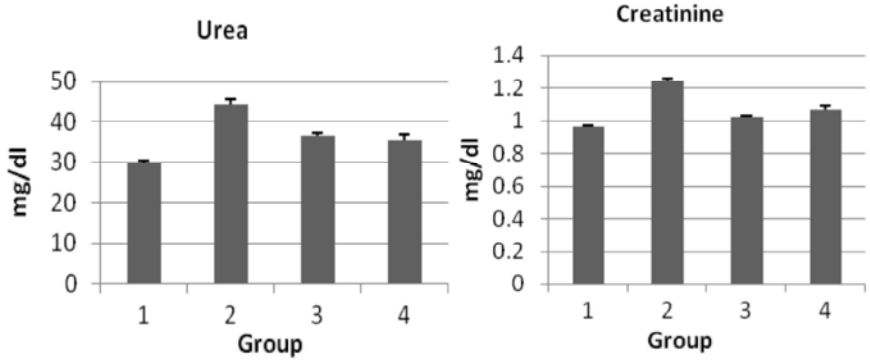

Fig. 2: Effect of CCL_4 alone and in combination with either Silymarin or Vitamin E on serum urea and creatinine activities of rats.
Group:1=Control. 2:CCL_4. 3: CCL_4+Silymarin. 4: CCL_4+ Vitamin E Values are expressed as mean±S.E. N= 5.

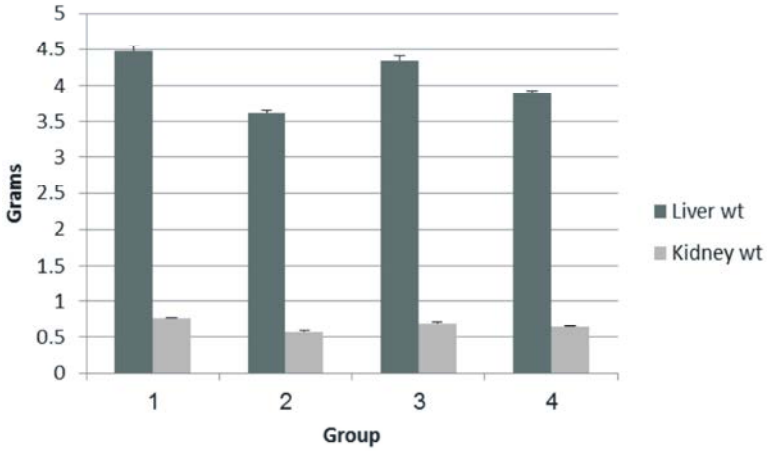

Fig. 3: Effect of CCL_4 alone and in combination with either Silymarin or Vitamin E on index weight of liver and kidney of rats.
Group:1=Control. 2:CCL_4. 3:CCL4+Silymarin. 4:CCL_4+ Vitamin E Values are expressed as mean±S.E. N= 5.

Table 2: Effect of CCL_4 alone or in combination with either Silymarin or Vitamin E on Hb g%, PCV%, RBCs and WBCs counts of rats

	Parameter			
Group	Hb g (%)	PCV%	RBCs (x10^6/cmm)	WBCs (x10^3/cmm)
Control	12.86±0.26 [a]	39.00±1.00 [a]	6.31±0.07 [a]	11.43±0.17 [a]
CCL4	10.71±0.05 [c]	29.00±0.45 [c]	5.28±0.09 [d]	9.83±0.06 [c]
CCL4+Silymarin	12.05±0.17 [b]	38.60±0.75 [a]	6.07±0.08 [b]	11.10±0.08 [b]
CCL4+ Vitamin E	11.94±0.27 [b]	34.80±1.20 [b]	5.74±0.04 [c]	10.89±0.06 [b]

Values are expressed as mean±S.E. N= 5. Values with different letters at the same column are significantly different at $P \leq 0.05$.

Serum Biochemical Parameters: The obtained results showed that there was a significant increase (P <0.05) in serum ALT, AST, ALP activities (Fig. 1), urea and creatinine (Fig. 2) levels in all treated groups except ALP activity in CCL_4 + vit E treated group compared to the control. While there was a significant reduction in their levels in CCL_4+ vit E and CCL_4 + silymarin treated rats as compared to CCL_4 treated rats. The reduction was more pronounced in CCL_4+ silymarin - group.

Organs Weights: Regarding to liver and kidney index weight there was a significant decrease (P <0.05) in their values in CCL_4 treated rats and CCL_4 + vit E treated group compared to the control, The reduction was less evident in CCL_4 + vit E treated rats compared to the control (Fig. 3).

Hematological Parameters: There was a significant reduction in Hb g%, PCV%, RBCs and WBCs counts in all treated groups except for PCV% in CCL_4 + silymarin treated group as compared to the control (Table 2). However, there was a significant elevation in their values in CCL_4+ vit E and CCL_4 + silymarin treated rats as compared with CCL_4-treated rats**.** The elevation in PCV and RBCs values was more pronounced in CCL_4+ silymarin treated group (Table 2).

DISCUSSION

The generation of small amounts of free radicals appears to have an important biological junction, but oxidative stress is caused by excess production of reactive oxygen species. Oxidative stress can produce alteration of liver and kidney functions.

The findings of the present investigation showed that CCL_4 treatment (group 2) caused a significant impairment in liver and kidney functions. CCL_4 caused a significant increase in serum ALT, AST and ALP activities compared to their corresponding values in control group (group 1) and the other two groups (groups3&4). Serum or plasma enzyme levels have been used as markers for monitoring chemically induced tissue damages. The damage or death of hepatocytes usually results in the leakage of the enzymes in the affected tissue after rupture of the plasma membrane and cellular damage and their release into the blood stream [35, 36]. Elevation of serum AST, ALT and ALP is a known effect of CCL_4 toxicity which specifically affect the liver [37].

Serum creatinine and urea concentrations were significantly higher in CCL_4- treated rats compared to control group and the other two groups, these findings agree with the previous findings of Churchill *et al.* and Perez *et al*., [38, 39] which might be contributed to lower creatinine and urea clearance. It is believed that CCL_4 treatment causes glomerular hypercellularity, moderate to severe necrosis and tubule interstitial alterations which causes alteration in the capacity for tubular absorption thus bringing about functional overload of nephrons with subsequent renal dysfunction [40].

The impairment in the liver and kidney function markers was coincident with a significant increase in the kidney and liver lipid peroxidation product, malondialdehyde (MDA) and a decrease in their enzymatic and non-enzymatic antioxidant defense system, where there was a significant decrease in the level of reduced glutathione (GSH) and in the activity of catalase enzyme in liver and kidney tissues indicating a state of oxidative stress and lipid peroxidation in these tissues. The hepatotoxic effect of CCL_4 has been attributed to its metabolism by Cytochrome P450 to yield toxic trichloromethyl (CCl 3) radicals that act as free radical initiators [41]. That free radical reacts rapidly with oxygen to form a trichloromethyl peroxy radical ($.CCl_3 O_2$). This metabolite may attack membrane polyunsaturated fatty acids and causes lipid peroxidation which plays a main role in the induction of liver injury [42] and further causes impairment of membrane function.

The findings of the present investigation agree with the previous findings of Balahoroğlu *et al*. [43] who reported induction of liver and kidney damage by CCL_4.

The status of lipid peroxidation as well as altered levels of certain endogenous radical scavengers is taken as direct evidence for oxidative stress [44].

The pathogenesis of carbon tetrachloride (CCL_4) induced renal dysfunction is not completely known. It may be due to the functional state of liver [45], or renal injury may develop independently to hepatic events, or can be attributed to CCL_4 induction of oxidative stress in many settings [1, 2, 46]; therefore, it might be expected to contribute to renal damage.

In vitro and *in vivo* studies indicated that CCl4 enhances lipid peroxidation and reduced the renal reduced/oxidized glutathione ratio in kidney cortex as well as renal microsomes and mitochondria [41, 47].

Administration of silymarin to CCL_4 treated rats (group3) was significantly able to reduce the activities of liver enzymes, serum levels of urea and creatinine, liver and kidney MDA levels and increased significantly their GSH levels and catalase activities compared to the CCL_4 treated (group 2). The improvement and protective effects on liver and kidney functions markers and in the antioxidant defense system induced by silymarin administration was comparable to that of vitamin E + CCL_4 (group 4). The present findings agree with the previous findings indicating the protective effect of silymarin or vitamin E against oxidative stress induced liver damage [48, 49]. Non-enzymic antioxidants like vitamin- E, GSH and vitamin-C act synergistically to scavenge the free radicals formed in the biological system. GSH acts synergistically with vitamin-E in inhibiting oxidative stress and acts against lipid peroxidation [50]. The depletion of GSH promotes generation of reactive oxygen species and oxidative stress affecting functional as well as structural integrity of cell and organelles membrane [51].

In the present investigation the significant reduction of LPO observed in silymarin and vitamin E treated groups may be related to their antioxidant and free radical scavenging activities [48]. Vitamin E protect cells against CCl4 induced toxicity by detoxifying electrophiles, preventing oxidation of –SH groups of proteins and by scavenging free radicals [41, 52].

The elevated level of GSH in liver and kidney with silymarin protects cellular proteins against oxidation through glutathione redox cycle and also directly detoxifies reactive oxygen species and/or neutralizes reactive intermediate species generated from exposure to CCl4 [49, 46].

Free radical scavenging enzyme, Catalase functions in detoxifying H2O2 into H_2O and O_2 [53, 54]. The decrease in the activity of this enzyme in the present study after CCL_4 induced oxidative stress could be attributed to its excessive utilization in inactivating the free radicals generated during the CCL_4 induced oxidative damage in the liver and kidney. Our results are in line with previous studies by Ramakrishnan *et al*. and Pradeep *et al*. [55, 56] who have shown that silymarin exhibits excellent antioxidant property. Therefore this property of silymarin might have resulted in the recoupment in the activities of the antioxidant enzyme catalase compared to CCL_4 treated rats.

The protective properties of silymarin against CCL_4 induced oxidative stress in rats might be attributed to their antioxidant flavonoids [7], which can prevent lipid peroxidation, changes in composition of the membrane phospholipids, hepatic glutathione depletion and improve the functional markers of liver damage. Silymarin can protect the liver against xenobiotic injury by controlling liver secretion and uptake of plasma lipoprotein and increase the interacellular glutathione content with scavenging of free radicals [57, 9] added that silymarin, play a role as anti-inflammatory agent, through its ability to inhibit neutrophil infiltration and regulate the release of inflammatory mediators. Lettéron *et al*. [58] reported that silymarin prevents carbon tetrachloride-induced lipid peroxidation and hepatotoxicity in mice, firstly, by decreasing the metabolic activation of CCL_4 and secondly, by acting as a chain-breaking antioxidant. In addition to its ability to stimulate protein synthesis resulting in production of new liver cells to replace older and damaged ones [5, 59].

The significant decrease in Hb g%, PCV%, RBCs and WBCs counts in CCL_4 treated group could be attributed to CCL_4 toxicity and its direct effect on hematopoietic system. These results are compatible with those recorded by Abd El-Aziz *et al*. [60]. Treatment with silymarin or vit E improved these hematological parameters. Also, the toxic effect of CCL_4 in the present study was observed by a decrease in the weights of liver and kidney. The metabolic dysfunction induced by CCL_4 could be the cause for weight loss, this results agree with those recorded by Cordeiro and Kaliwal [61]. This reduction was ameliorated in silymarin or vit E treated groups which may be related to their anti-oxidant activities enabling regeneration of cells, thus improving the weight.

In conclusion, our present work shows that the administration of silymarin resulted in a significant hepato–renal-protective property comparable to vitamin-E during CCL_4 induced oxidative stress in rats by restoring the liver and kidney function markers and antioxidant property through reestablishment oxidant–antioxidant balance in liver and kidney tissues.

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