# Effect of whey protein against fluvastatin and carbon tetrachloride-induced hepatotoxicity in rats

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## ABSTRACT

*The purpose of this study was to evaluate the hepatotoxicity induced by co- administration of fluvastatin (F) and carbon tetrachloride (CCl₄); and to investigate the hepatoprotective effect of Whey protein isolate (WPI) in F+CCl₄-induced liver injury in animal model. Hepatotoxicity was induced by F (4 or 8 mg/kg, p.o.) and CCl₄ (0.8 mg/kg, i.p, twice weekly) for 30 days in rats. Silymarin (50mg/kg, p.o.) or WPI (100, 200 mg/kg, p.o.) were administered for 30 days. Hepatotoxicity was assessed by alteration of serum alanine aminotransferase (ALT) aspartate aminotransferase (AST), total triglycerides (TGs) and total cholesterol (TC) levels as well as alteration of liver malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH) contents, total antioxidant capacity (TAC), superoxide dismutase (SOD) activity and hydroxyproline (HYP) content and histoarchitecture alterations. Co-administration of fluvastatin two dose levels and CCl₄ significantly elevated serum ALT, AST, TGs, TC levels, NO and MDA contents in liver homogenate. Moreover, they reduced HYP, GSH , TAC and SOD activity. Microscopic examination showed severe vacuolar degeneration of hepatocytes, focal cellular infiltration, complete distortion of liver tissue architecture, DNA abberation and fibrosis. WPI administration reversed the deleterious effect induced by F+CCl₄. In conclusion, WPI improved the antioxidant status of hepatocytes and it had promising antifibrotic effect in this model.*

Key words: hepatotoxicity, fluvastatin, carbon tetrachloride, whey protein isolate, hydroxyproline.
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## INTRODUCTION

Drug-induced hepatotoxicities are the common cause of acute liver failure, include antibiotics, lipid lowering agents, oral hypoglycemics, psychotropics, antiretrovirals and anti-inflammatory [1]. Uncommon, drug-induced liver injury (DILI) is a major health concern that challenges pharmaceutical industry and drug regulatory agencies alike [2]. The resultant effects of toxins, infectious agents, medications, and serum inflammatory mediators are the main causative agent of disease processes, leading to loss of normal histological architecture, reduced cell mass and loss of blood flow. Consequently, functional liver capacity will be lost [3].

Oxidative stress has been implicated in the mechanisms of drug and chemical induced toxicity [4, 5]. It plays an important role in various liver diseases [6]. It is a common pathogenic mechanism to initiate and progress the hepatic damage [7].

Statins, 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA reductase inhibitors), are the most efficacious hypolipidemic drugs [8]. Organic anion-transporting polypeptide (OATP 2) plays a role in the hepatic uptake of statins such as pravastatin, pitavastatin, atorvastatin, and fluvastatin [9, 10]. OATP2- inhibitors decrease statins

hepatic uptake and enhance the irsystemic exposure to the drugs. Moreover, statins are oxidized mainly by CYP3A4 [11].

Carbon tetrachloride ($CCL_4$) is widely used as hepatotoxic model to investigate the hepatoprotective agents in experimental animals [12]**.** $CCL_4$ activated by hepatic cytochrome P450 (CYP2E1, CYP2B1, CYP2B2, and possibly CYP3A4) to form the trichloromethyl radical [13]. This radical binds to cellular molecules (nucleic acid, protein and lipid), impairing crucial cellular processes such as lipid metabolism, with the potential outcome of fatty degeneration (steatosis) [14]**.** This radical can also react with oxygen to form the trichloromethylperoxy ($CCl_3OO^\bullet$) which is a highly reactive species, that initiates the chain reaction of lipid peroxidation, which attacks, destroys polyunsaturated fatty acids and inhibit antioxidant enzyme system [15].

Silymarin, an extract of milk thistle (Silybummarianum), which is the most commonly, used for liver disorders, owing to its purported hepatoprotective properties [16]**.** Silymarin has antioxidant effect which reduces free radical production and lipid peroxidation caused by $CCL_4$ [17].

In spite of tremendous scientific advances in the field of hepatology in recent years, liver diseases are on the rise and remain a serious health problem. Presently, a few hepatoprotective drugs and that too from natural sources, are recommended for the treatment of liver disorders. Hence, people are looking at the traditional systems of medicine for remedies to hepatic disorders [18]. Whey protein, a by-product of the cheese-making process;which is typically a mixture of beta-lactoglobulin (~65%), alpha-lactalbumin (~25%), andserum albumin (~8%), which are soluble in their native culture forms [[19, 20] and constitutes ~20% of the total bovine milkprotein. Whey proteins are a cystine-rich protein source. Consumption of cystine-rich whey protein can increase plasma GSH concentrations in humans [21], protect against ROS-induced cell damage [22]**,** and inhibit MDA production [23, 24]. It has been suggested that WP has an antioxidant activity probably depending on the abundance of cysteineandglutamylcysteine groups which are in other food proteins.

An interest in the use of antioxidant nutritional supplements has been sparked by epidemiologic evidence suggesting that dietary antioxidants in food constituents may protect or prevent the incidence of many diseases [25], therefore, WP may be considered as a possible therapeutical tool in oxidative stress correlated diseases as hepatotoxicity

## MATERIALS AND METHODS

### 2.1. Animals:
Albino Wistar male rats, weighing 150-160g were used throughout the experiments. They were purchased from Animal House Lab., National Research Centre, and Giza, Egypt. Animals received human care in compliance with the guidelines of the animal care and use committee of National Research Centre, and Giza, Egypt, experiments were performed according to the National Regulation of Animal Welfare and Institutional Animal Ethical Committee.

The animals were kept in a quiet place and were allowed free access water and standard food pellets throughout the period of investigation.

### 2.2. Chemicals:
$CCL_4$was purchased from El-Gomhouria Company for drug and chemicals, Cairo, Egypt.

### 2.3. Drugs:
Whey protein isolate was purchased from DAVISCO Company, USA. Silymarin was purchased from Novartis Pharma, Cairo; Egypt. Fluvastatin was purchased from Sigma-Aldrich, Germany.

### 2.4. Experimental design:
Rats were randomly allocated in to 12 groups (6 rat each ) and treated for 30 successive days as follows : Group 1was given distilled water ( 10 ml/kg ) and served as normal control, Groups 2 and 3  were orally administered fluvastatin 4 and 8 mg/kg, respectively(26) **;** Groups 4 injected with $CCL_4$ (0.8 mg/kg, i.p., twice weekly for 4 weeks) (27),Groups 5 and 6 were given fluvastatin 4 and 8 mg/kg, p.o., respectively, in concomitant with $CCL_4$ ; Groups 7 and 8 were given silymarin (50 mg/ kg,  p.o.) (28)  in concomitant with each dose of fluvastatin and $CCL_4$ administration, Groups 9 - 12 were given WPI (100 and 200 mg/kg, p.o.) (29) concomitant with each dose of fluvastatin and $CCL_4$ administration.

**2.5 Methods:**
**2.5.1. Preparation of blood sample and tissue homogenate:**
Blood samples were withdrawn from the retro-orbital vein of each animal, under light anesthesia by diethyl ether, according to the method of Cocchetto and Bjoronsson [30]. Blood was allowed to coagulate and then centrifuged at 3000 rpm for 15min. immediately after blood sampling, animals were sacrificed by cervical dislocation and the liver tissues were rapidly removed, washed in ice-cooled saline, plotted dry and weighed. A weighed part of each liver was homogenized, using a homogenizer (Medical instruments, MPW-120, Poland), with ice-cooled saline to prepare 20% w/v homogenate. The homogenate was then centrifuged at 4000 rpm for 5 min. at 4°C in a cooling centrifuge to remove cell debris (Laborzentrifugen, 2k15, Sigma, Germany).

**2.5.2 Biochemical markers:**
The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to Reitman and Frankel [31], serum levels of total triglycerides (TGs) and total cholesterol (TC) levels were determined according to Fassati et al. [32] and Richmond [33] respectively, nitric oxide (NO), malondialdehyde (MDA) contents were determined according to Miranda et al. (34) and Uchiyama and Mihara [35] , respectively. Liver reduced glutathione (GSH), superoxide dismutase (SOD) and total antioxidant capacity (TAC) activities were measured, according to Beutler et al. [36], Marklund [37] and Koracevic et al. [38], respectively, using Biodiagnostic kits, Egypt. Liver hydroxy proline (HYP) content was measured by ELISA according to Tian et al. [39] using Koma Biotechnology KIT, Korea.

**2.6 Histopathological studies:**
The left lobe of each liver was dissected and fixed in 10% formalin, dehydrated in gradual ethanol (50-100%), cleared in xylene and embedded in paraffin. 4-5μm thick sections prepared and stained with hematoxilen and Eosin (H&E) for photomicroscopic observation [40].  Images were captured and processed using Adobe Photoshop version 8.0.

Sections were put on positively charged slides and stained immune histochemically for COX-2 antibody using a streptavidin– biotin immunoenzymatic method.

Other sections were stained for DNA [41]and counterstained with Light Green. DNA analysis was performed by leica Qwin 500 image cytomery in Pathology department, National Research Centre, Cairo, Egypt. For each section 100-120 cells were randomly measured. The threshold values were defined by measuring control cells. The results are presented as histograms and tables which demonstrate the percentage of the diploid cells (2C). The proliferating cells (3C), the tetraploid cells (4C) and the aneuploid cells (>5C). The proliferating cells were further classified according to Lee et al. (1999) into; (<10%) low proliferation index, (10-20%) medium proliferation index and high proliferation index is >20%.  The DNA histogram classified according to Danque et al. [42].

**2.7 Statistical analysis:**
Data were expressed as mean ± S.E. Analysis was done using ANOVA followed by the LSD test for multiple comparisons. Difference was considered significant at 0.05 level of probability using Graph pad prism program.

**RESULTS AND DISCUSSION**

**Effects of silymarin and whey protein isolate on serum liver enzymes:**
The present biochemical results revealed that the two dose levels of fluvastatin (4 or 8 mg/kg) elevated serum ALT activity by 73 % and 79 % respectively, as well as serum AST activity by 10% and 17% respectively, as compared to normal control group (table 1& 2).

Argo et al. (43) reported that mild-to-moderate elevations in liver transaminases are the most commonly observed side effect of statin- treatment in clinical practice, followed in frequency by muscular symptoms. Although these elevations in liver enzymes usually remain asymptomatic, they affect between 0.5 and 5% of all patients treated with statins in clinical studies [44]**.**

In the current work, $CCL_4$ caused significant elevation in serum ALT and AST by 65% and 24% respectively. Moreover, the combination of fluvastatin two dose levels with $CCL_4$ (0.8 ml/kg) significantly  elevated serum ALT activity by 103% and110% respectively, as well as serum AST activity by 78% and 91% respectively, in dose dependent manner as compared to normal control group and more than fluvastatin alone (table 1& 2).

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Attaby et al. [45] showed that rats treated with $CCL_4$ (2 ml/kg) activities significantly elevated ALT and AST to 108% and 100%, respectively. This elevation was lower than our data when fluvastatin (8 mg/kg) was administered with $CCL_4$ (0.8 ml/kg) for 30 days of treatment.

While silymarin treatment significantly reduced liver enzymes activity by ALT: 13% &12% and AST: 35 % & 40 %, respectively, as compared with F4 + $CCL_4$ or F8 + $CCL_4$ - toxicated groups (table 1& 2). These findings are consistent with Pradeep et al. [46] which suggested the protection of the structural integrity of the hepatocytes membrane or regeneration of damaged liver cells by silymarin.

WPI (100 mg/kg) treatment significantly reduced serum ALT and AST activities by (ALT: 13% &15% and AST: 42 % & 40 %, respectively), as compared with toxicated groups (table 1& 2). Moreover, WPI ( 200 mg/kg) significantly decreased serum ALT and AST activities by (ALT:15 % & 18 % and AST: 43% & 42% respectively), as compared with fluvastatin two dose levels and $CCL_4$-toxicated groups as shown in tables 1& 2.

Ashoush et al. [47] reported that WPI decreased serum liver enzymes activity in carbon tetrachloride-induced hepatotoxicity in rat.

### Effects of silymarin and whey protein isolate on serum lipid profile:
In the current study, treatment with $CCL_4$ alone elevated serum TG level by 18%, and the combined treatment with F4 + $CCL_4$ or F8 + $CCL_4$ elevated serum TG level by 63% and 34 % respectively. As well as the higher dose of fluvastatin in combination with $CCL_4$ elevated serum TC level by 3% as compared to normal control group and fluvastatins alone (F4 and F8) as shown in tables 1& 2.  Nasir et al. [48] found that rats treated with $CCl_4$ significantly increased TC and TG levels as compared with normal control rats. This due to $CCl_4$ interferes with triglyceride secretion and causes steatosis, fibrosis, and necrosis in mice [49, 50]. Moreover, the hepatotoxic effect of fluvastatin due to disruption of hepatic CYP3A activity and organic transporter expression (OATP1a) which are responsible for statins metabolism [51].

Our data revealed that silymarin significantly decreased serum TG by 41 % and 32 %, respectively, as compared to fluvastatin two dose levels and $CCL_4$-toxicated groups (tables 1& 2). **Sobolova et al.** [52] reported that silymarin significantly reduced TC and TG absorption in rats fed on high cholesterol diet.

However, WPI (100 mg/kg) treatment significantly decreased serum TG and TC levels by (TG: 52% &42 %and TC:2% &3% respectively), in addition, WPI (200 mg/kg) decreased serum TG and TC level by (TG: 33% &15 % and TC:4% &2% respectively), as compared with fluvastatin two dose levels and $CCL_4$-toxicated groups ( table 1& 2).

### Effects of silymarin and whey protein isolate on liver malondialdehyde (MDA) and nitric oxide (NO) contents:
The present study revealed that $CCl_4$ – treatment significantly elevated MDA and NO contents in liver homogenate by 14% & 48%, respectively as compared to normal control.

$CCl_4$ metabolites induced oxidative stress in the liver, which propagate inflammatory response [53)]**.** $CCl_4$ is one of the xenobiotic induced acute and chronic tissue injuries [54]; its exposure increased lipid peroxidation and free radical formation resulting tissue necrosis [55]**.** $CCL_4$ induces DNA damage and fragmentation as well as depletes CYP2E1 activity inducing apoptosis [56].

The combination of fluvastatin two dose levels with $CCL_4$-induced hepatotoxicity, liver- MDA content elevated by 24 % and 37 %, respectively (Fig 1A & 1B) and NO in liver content by 97 % and 203 %, respectively (Fig 1C& 1D) comparing with normal control, and more than F4 and F8 treatment alone**.**

Silymarin co-treatment reduced oxidative stress, it decreased MDA- liver content by 18% and 17% respectively (Fig 1A& 1B), and NO in liver content by 34% and 54 % respectively (Fig 1C& 1D), as compared to fluvastatin two dose levels and $CCL_4$-toxicated groups, these results are in accordance with those of Shaker et al. [(57] which reported that silymarin have hepatoprotective and antioxidant effects on $CCl_4$ - poisoned rats.

Our results revealed that WPI (100 mg/kg) decreased liver MDA content by 17% and 12% respectively, while WPI (200 mg/kg) significantly decreased liver MDA content by 28% and 27%, respectively (Fig 1A& 1B), as compared to fluvastatin two dose levels with $CCL_4$- toxicated groups. Many reports found that whey protein decreased MDA content and had potential effect in preventing further accumulation of free radicals and the oxidative stress; this may be due to its powerful antioxidant capacity and/or its anti-inflammatory effect [58, 59]. Moreover, WPI (100, 200

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mg/kg) significantly decreased NO in liver content by 47 % and 54 %, respectively and by 51 % and 64 %, respectively as compared to their corresponding toxicated groups (Fig 1C& 1D).

**Effects of silymarin and whey protein isolate on reduced glutathione (GSH), total antioxidant (TAC) and superoxide dismutase (SOD) activities:**

 Our data showed that $CCL_4$ treatment reduced TAC and SOD activities by 22 % and 51% respectively. In addition the combination of fluvastatin two dose levels with $CCL_4$ significantly decreased GSH content by 15 % and 25%, respectively (Fig 1E&1F), TAC activity by 30 % and 20 % (Fig 1G&1H) and SOD activity by 79 % and 72 % respectively (Fig 1I&1J), as compared to normal control and mre than fluvastatin alone. Shaker et al. [60] showed that GSH content significantly decreased by 36% in CCL4 (2 ml/kg) treated rats.

Treatment with silymarin increased liver GSH content by 47 % and 95 % (Fig 1E& F) as compared to their corresponding toxicated groups and increased  SOD activity by 117% as compared with F4+ $CCL_4$-toxicated groups (Fig 1I). Silymarin coadministration significantly increased TAC activity by 21% and 16%, respectively as compared to fluvastatin two dose levels + $CCL_4$-toxicated groups (Fig 1G &1H). Nema et al. [61] reported that silybummmarianum has high safety and act as hepatoprotective and antioxidant agent against $CCl_4$ poisoning in rats.

Our data revealed that  WPI (100 mg/kg ) significantly elevated liver GSH activity by 39 % and  44%, respectively (Fig 1E&1F), TAC activity by 23% and 17%, respectively (Fig 1G&1H) and SOD activity by  181% and  114%, respectively (Fig 1I&1J) and  WPI (200 mg/kg) significantly elevated liver GSH activity by  44 % and  54%, respectively (Fig 1E&1F), TAC activity by 29 % and  18% respectively (Fig 1G&1H) and SOD activity by  333% and  214%  respectively (Fig 1I&1J), as compared to fluvastatin two dose levels with $CCL_4$-toxicated groups, these results are in agreement  with Nada [62] who showed that whey protein increases glutathione levels by supplying the precursors required for intracellular glutathione synthesis, and  exerts its effect due to their antioxidant activity through increased the level of SOD.

**Effects of silymarin and whey protein isolate on liver hydroxyproline (HYP) content and histopathological changes:**

The present biochemical results revealed that $CCL_4$ elevated liver HYP content by 54% (Fig. 1K &1L) as compared to normal control group. $CCL_4$ –treatment resulted the formation of lipid peroxidation and free radicals production [63] which causes necrosis of hepatocytes, induces inflammation, and promotes the progression of hepatic fibrogenesis [64]**.** These results are confirmed by histopathological investigations which revealed that the normal structure of liver tissue (Fig 2A) was affected by $CCl_4$ as vacuolar degeneration of many hepatocytes, karrhyolysis formation , some acidophilic cells and aggregations of inflammatory cells (Fig 2B).

Fluvastatin two dose levels elevated HYP content in liver homogenate by 47 % and 60%, respectively (Fig 1k&1L) as compared to normal control group, these results are confirmed by histopathological study. Microscopical examination showed that rat received the lower dose of fluvastatin had normal structure of the tissue except for mild dilatation and congestion of some blood sinusoids , slight cellular infiltration is observed near the central vein. While, rat received F4 showed deformation, vacuolar degeneration of many hepatocytes and small dark nuclei in some hepatocytes (Fig 2C & 2D).

The combination of fluvastatin two dose levels with $CCL_4$ increased tissue HYP by 61% and 78 %, respectively as compared with normal control, and more than fluvastatin alone (Fig 1k& 1L). Our results showed that F4+$CCl_4$ had thickening of the central vein's wall (arrow), marked vacuolar degeneration of some hepatocytes (arrowhead) and dilatation of blood vessels (Fig 2E). While rat received F8+ $CCl_4$ showed severe vacuolar degeneration of many hepatocytes (V), multiple small areas of hemorrhage (arrowhead), focal cellular infiltration (arrow) and complete distortion of liver tissue architecture in the damaged area (Fig 2F).

Silymarin treatment decreased liver HYP content by 40% and 17%, respectively (Fig 1K&1L) as compared with fluvastatin two doses levels and $CCL_4$-toxicated groups. These finding proved that silymarin had slight vacuolar degeneration of some hepatocytes, dilatation and congestion of blood vessels (Fig 3A, 3B). These results are in a accordance with many investigators who reported that silymarin treatment arrest hepatic fibrosis whether in acute or chronic infection, and it has anti-fibrotic properties due to inhibition of transforming growth factor-beta (TGF-β) induced de novo synthesis of collagen type I [65,66].

Our result showed that the two doses level of WPI has a powerful effect in decreasing liver HYP content; WPI(100 mg/kg) by 43 % and 42 %, respectively as compared with fluvastatin two doses levels and $CCL_4$-toxicated groups (Fig 1K&1L). Histopathological examination of livers from group treated with WPI 100+ F4+ $CCl_4$ showed very slight vacuolar degeneration of some hepatocytes at the periphery of lobules (arrowhead), mild thickening of central

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veins wall (arrow) (Fig 3C). While, rat received WPI 200+F8+ CCl$_4$ showed vacuolar degeneration of many hepatocytes (V), dilatation with congestion of blood sinusoids (arrowhead), fibrosis and cellular infiltration with distortion of architecture at the same area (arrow) (Fig 3E). However, WPI (200 mg/kg) significantly decreased liver HYP content by 49% and 50%, respectively (Fig 1K&1L) as compared to fluvastatin two dose levels with CCL$_4$-toxicated groups. These results were confirmed by histopathological study which proved WPI 200 mg/ Kg normalized the hepatic tissue except for a little fibrous tissue at the central vein (Fig3D&3F).

**Effects of silymarin and whey protein isolate on COX-2 enzyme (immune-histochemical study):**
Staining sections with COX-2 antibody revealed that fluvastatin caused stimulation of inflammatory process in normal hepatic tissue (Fig. 4 B). This role is markedly increased when it is used along with CCl$_4$ in a dose dependent manner (Figure 4 C & 4 D).

Silymarin has a COX-2 inhibitory effect caused by fluvastatin+ CCl$_4$. This effect was clear with low dose of fluvastatin (Fig. 4E), and less clear with high dose of fluvastatin (Figure 4F).

High dose of WPI normalized hepatic tissue (negative stain with COX-2 antibody) that observed in group treated with F4 + CCl$_4$ as shown in Fig. 4G & 4 F. Less result was obtained from group treated with F8 + CCl$_4$ , as shown in Fig. 4 H.

**Effects of silymarin and whey protein isolate on DNA content:**
Normal distribution of DNA content in the liver cells of the control group showed that 3.57 % of the examined cells contained DNA (<1.5C), 89.28% contained diploid DNA value (2C), 5.35% contained (3C) DNA value (low Proliferation Index) and 1.78% of the examined cells at (4C) area (Histogram 1A). Examination of cells from group of rats received CCl4 only showed that 1.0% of examined cells contained DNA content (<1.5C), while those contained diploid DNA value (2C) were (20.0%). Cells contained (3C) DNA value were 40% (high proliferation index), while 35% of examined cells contained tetraploid (4C) value of DNA (Histogram 1B). Examination of cells from groups treated with high dose of fluvastatin (Histogram 1C) and high dose of fluvastatin with CCl4 (Histogram 1D) showed that the cells contained DNA (<1.5C) were 0.94% and 0.0 %, and those contained DNA value (2C) were 37.73% and 8.41% respectively, which means the decrease in DNA content (hypoploidy) compared to the control. While those contained DNA value (3C) were 37.73% and 36.44% (high proliferating index), respectively and (4C) were 20.75% and 39.25% respectively.

Examination of cells from groups treated with silymarin and  high dose of fluvastatin in concomitant with CCl4 along (Histogram1E), with high dose of WPI along with low dose of fluvastatin and CCl4 (Histogram 1F) and high dose of WPI along with high dose of fluvastatin and CCl4 (Histogram 1G) revealed that 89.74%, 33.64% and 4.63% of the examined cells contained DNA (< 1.5C) respectively. Percentage of cells contained diploid DNA value, were 10.25%, 58.87% and 26.85%, respectively. (3 C) were 0.0% and 7.47% (low proliferating index)  as well as 55.55% (high proliferating index) respectively, (4C) were 0.0%, 0.0% and 8.33% respectively.

These results indicated that treatment with WPI along with fluvastatin and CCl4 showed DNA values comparable to the control values especially with low dose of fluvastatin, while, groups treated with high dose of fluvastatin with or without CCl4 showed decreased DNA values (hypoploidy) (Table 3).

**Table (1): Effects of silymarin (50 mg/kg, orally) and whey protein isolate (100 and 200 mg/kg) on the liver enzymes and lipid profile in fluvastatin (4mg/kg) and ccl4- induced hepatotoxicity**

| Parameters | control | F4 | CCL4 | F4+ ccl4 | S + F4+ CCL4 | WP100+F4+ CCL4 | WP200+F4+ CCL4 |
|---|---|---|---|---|---|---|---|
| **Alanine aminotransferase (ALT)** | 148 ± 1.85$^a$ | 255.45 ± 2.09$^{bd}$ | 244.6± 1.7$^b$ | 300.40± 2.29 $^c$ | 262.30 ± 2.04$^d$ | 261.40 ± 2.04$^d$ | 255.25 ± 1.08$^d$ |
| **Aspartate aminotransferase (AST)** | 308.90 ± 2.32$^a$ | 338.60 ± 2.83$^b$ | 383.80 ± 2.69$^c$ | 549.80 ± 2.40$^d$ | 354.80 ± 2.80$^e$ | 319.40 ± 2.99$^a$ | 314.70 ± 2.54 $^a$ |
| **Triglyceride (TG)** | 39.42 ± 2.04$^{ad}$ | 38.74 ± 1.06 $^{ad}$ | 46.70 ± 1.26$^b$ | 64.37 ± 1.40$^c$ | 37.88 ± 1.12$^d$ | 31.15 ± 0.95$^e$ | 43.39 ± 1.15$^{ab}$ |
| **Total cholesterol (TC)** | 423.84 ± 1.18$^{ac}$ | 408.59± 1.53$^b$ | 425.52 ± 2.13 $^{ac}$ | 430.57 ± 2.47$^a$ | 428.28 ± 2.12$^{ac}$ | 421.48± 1.28$^c$ | 412.04 ± 1.55$^b$ |

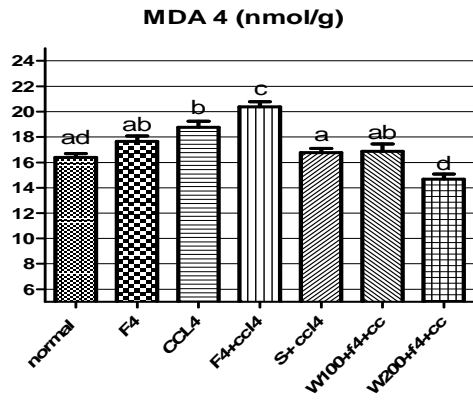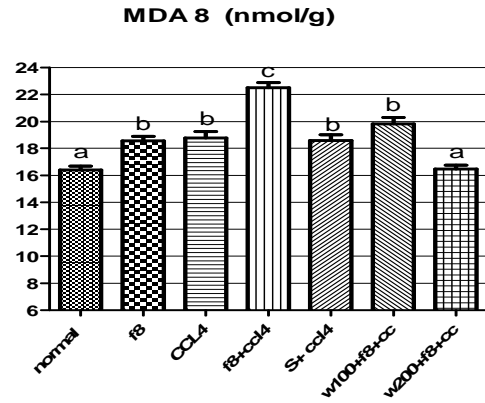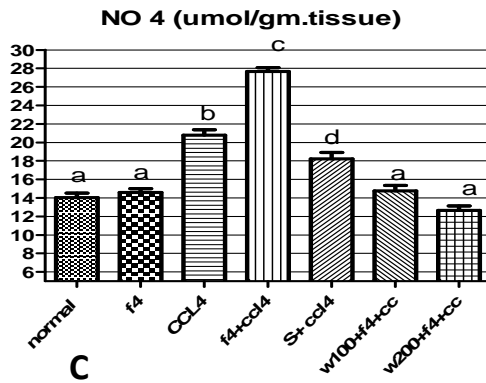*CCl4: carbon tetrachloride, F4: fluvastatin, S: silymarin, WP:  whey protein*
*DATA were expressed as mean ± SE (n= 6). Data were analyzed by ANOVA- one way; p≤0.05.The different alphabetical superscript is significantly different between groups.*

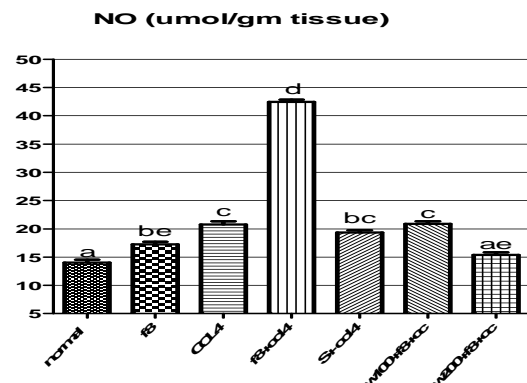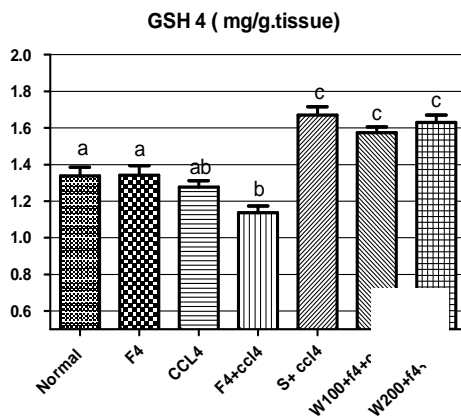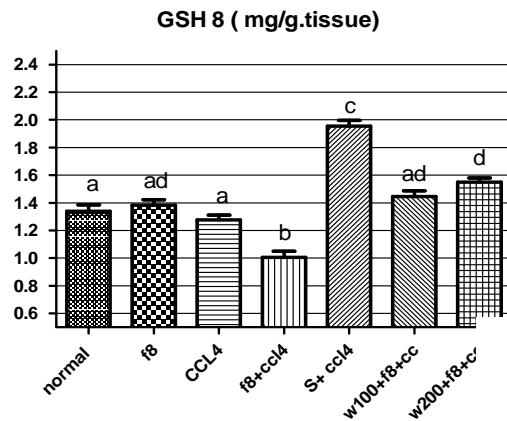**Table (2): Effects of silymarin (50 mg/kg, orally) and whey protein isolate (100 and 200 mg/kg) on the liver enzymes and lipid profile in fluvastatin (8mg/kg) and ccl4- induced hepatotoxicity**

| Parameters | Control | F8 | CCL4 | F8+ CCL4 | S + F8+ CCL4 | WPI 100+F8+ CCL4 | WPI 200+F8+ CCL4 |
|---|---|---|---|---|---|---|---|
| Alanine aminotransferase (ALT) | 148 ± 1.85 [a] | 264.75 ± 1.43 [be] | 244.6 ± 1.37 [c] | 310.25 ± 2.47[d] | 273.95 ± 2.28 [b] | 263.40 ± 2.14[be] | 255.25 ± 2.53[ce] |
| Aspartate aminotransferase (AST) | 308.90 ± 2.32 [a] | 361.20 ± 2.34 [b] | 383.8 ± 2.69[c] | 589.40 ± 2.79 [d] | 356.40 ± 2.28[b] | 352± 2.70 [be] | 341.10 ± 2.97[e] |
| Triglyceride (TG) | 39.71 ± 1.17 [a] | 35.74 ±0.96[ad] | 46.70 ± 1.26[b] | 53.05 ± 1.37[c] | 35.84 ± 1.11[ad] | 30.97 ± 1.49[d] | 45.02± 1.29 [b] |
| Total cholesterol (TC) | 423.24 ± 1.18[a] | 403.8 ± 1.21[b] | 425.52 ± 2.13[a] | 437.2 ± 0.96[c] | 428.3 ± 1.62[a] | 422.4 ± 1.15[a] | 428.1 ± 1.18[a] |

*CCl4: carbon tetrachloride, **F4**: fluvastatin, **S**: silymarin, **WPI**: whey protein isolate*

*DATA were expressed as mean ± SE (n= 6). Data were analyzed by ANOVA- one way; p≤0.05.The different alphabetical superscript is significantly different between groups.*

**MDA 4 (nmol/g)**

**MDA 8  (nmol/g)**

**A**

**B**

**NO 4 (umol/gm.tissue)**

**NO (umol/gm tissue)**

**C**

**D**

**GSH 4 ( mg/g.tissue)**

**GSH 8 ( mg/g.tissue)**

**E**
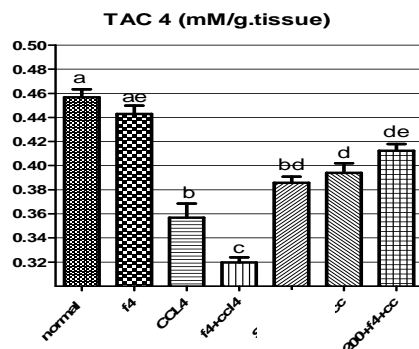
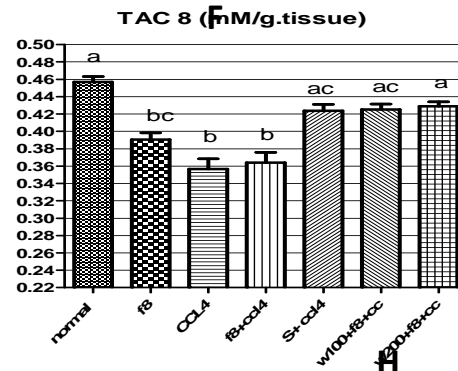**TAC 4 (mM/g.tissue)**

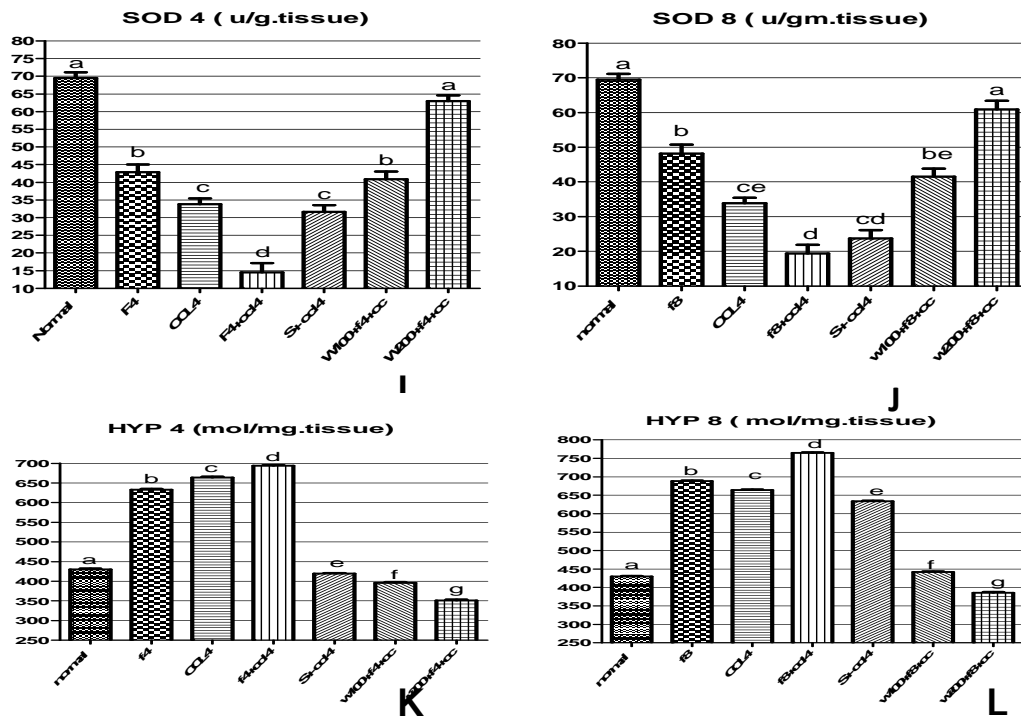**TAC 8 (mM/g.tissue)**

**G**

**H**

**Fig1: Effects of silymarin (50 mg/kg) or whey protein isolate (WPI 100 and WPI 200 mg/kg) on the hepatic nitric oxide (NO) and (MDA) contents, reduced glutathione (GSH), total antioxidant capacity (TAC) and superoxide dismutase (SOD) activities as well as content of hydroxy proline ( HYP)**

*CCl4: carbon tetrachloride, F4: fluvastatin, S: silymarin, WPI: whey protein isolate*
*DATA were expressed as mean ± SE (n= 6). Data were analyzed by ANOVA- one way; p≤0.05.The different alphabetical superscript is significantly different between groups.*
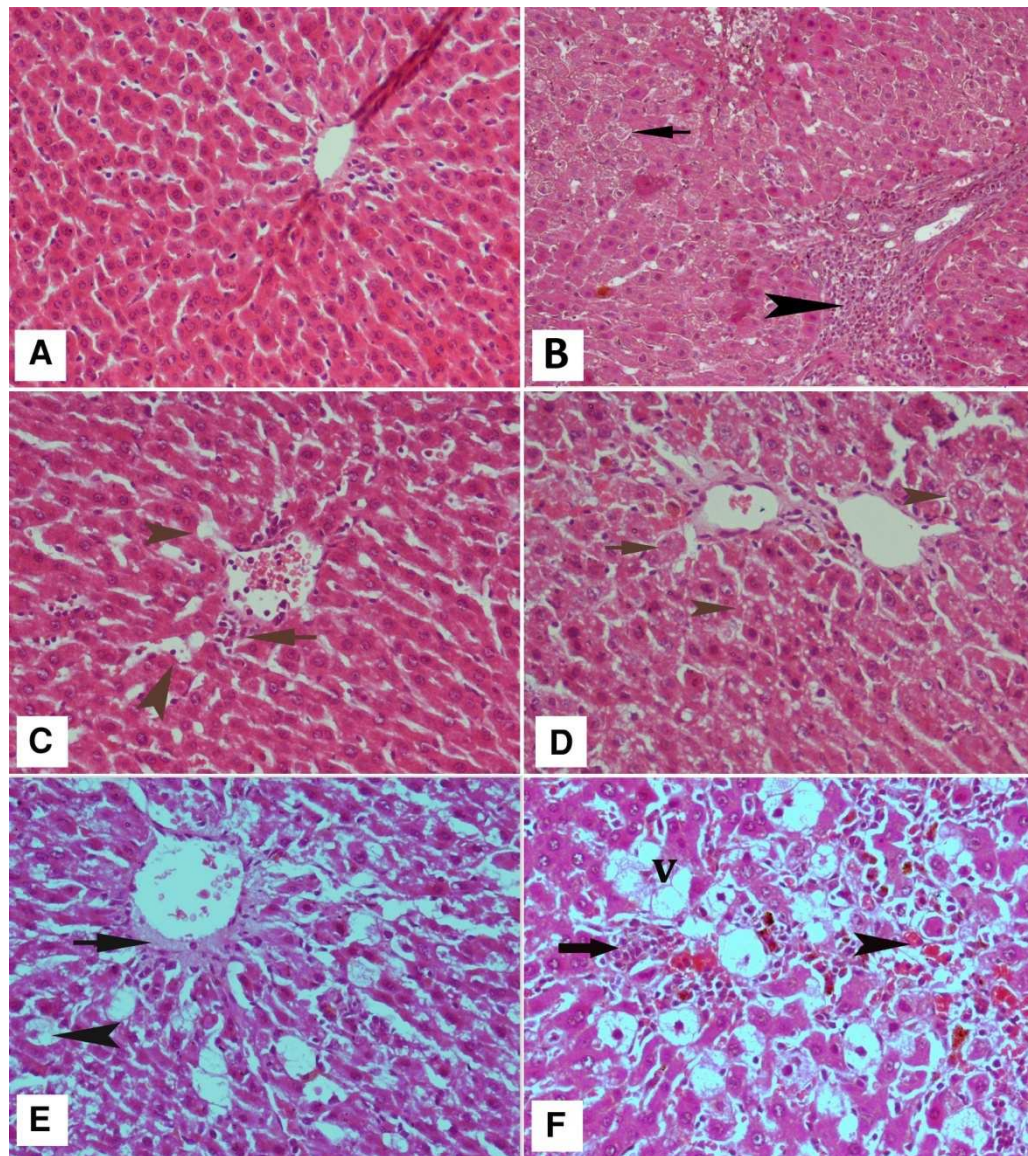
**Figure 2: a photomicrograph of a section of liver tissue of (A) : a normal rat, (B): a rat received CCl₄, (C): a normal rat received flouvastatin drug in a low dose,(D) a normal rat received fluvastatin in a high dose,(E) a rat received CCl₄ and fluvastatin in a low dose,(F) a rat received CCl₄ and fluvastatin in a high dose.(Hx. & E. X 200)**
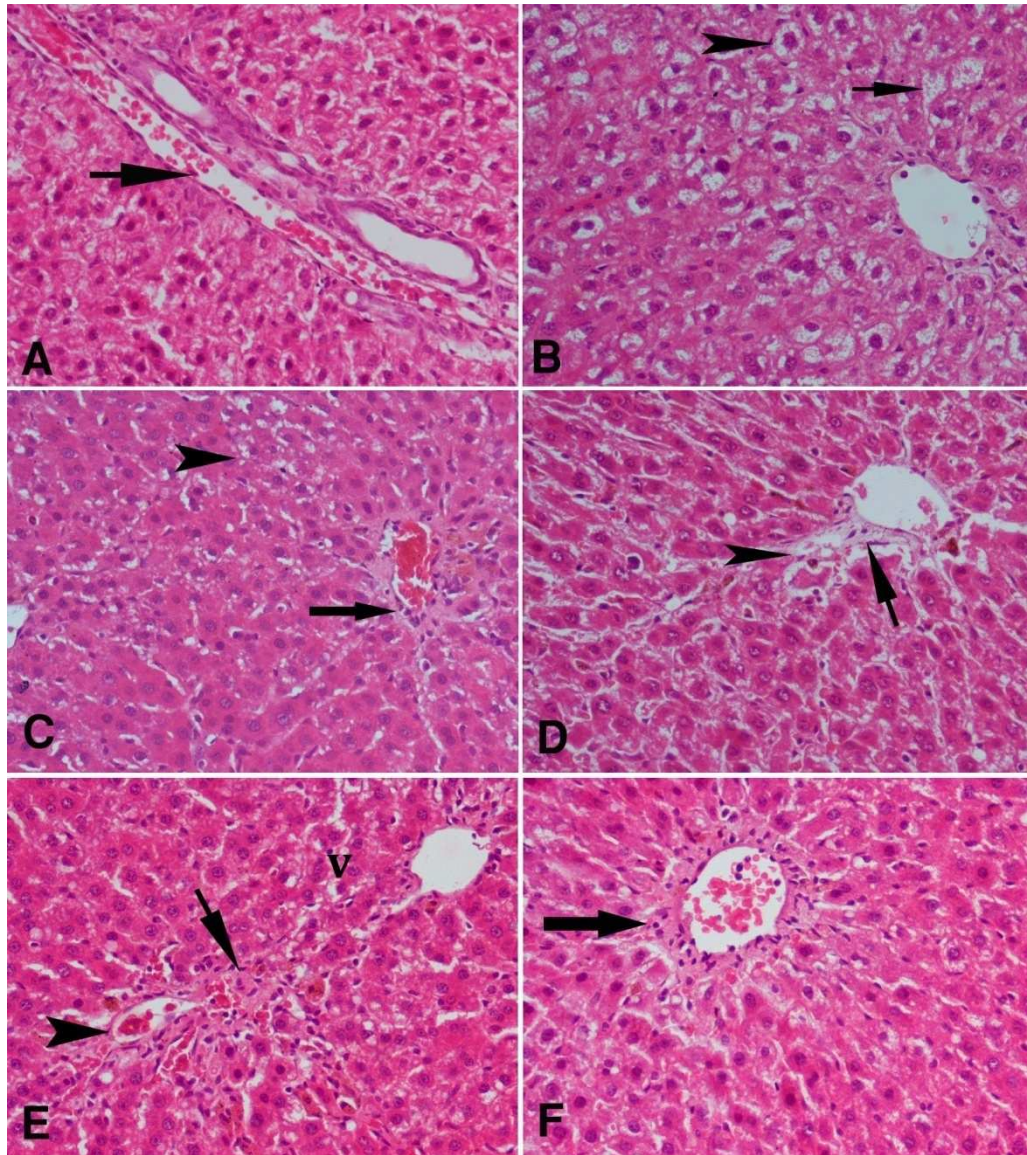
**Fig 3: a photomicrograph of sections of liver tissue from (A) a rat received CCl₄ and fluvastatin in a low dose and treated with silymarin, (B) a rat received CCl₄ and fluvastatin in a high dose and treated with silymarin (C) a rat received CCl₄ and fluvastatin in a low dose and treated with whey protein in a low dose,(D) a rat received CCl₄ and fluvastatin i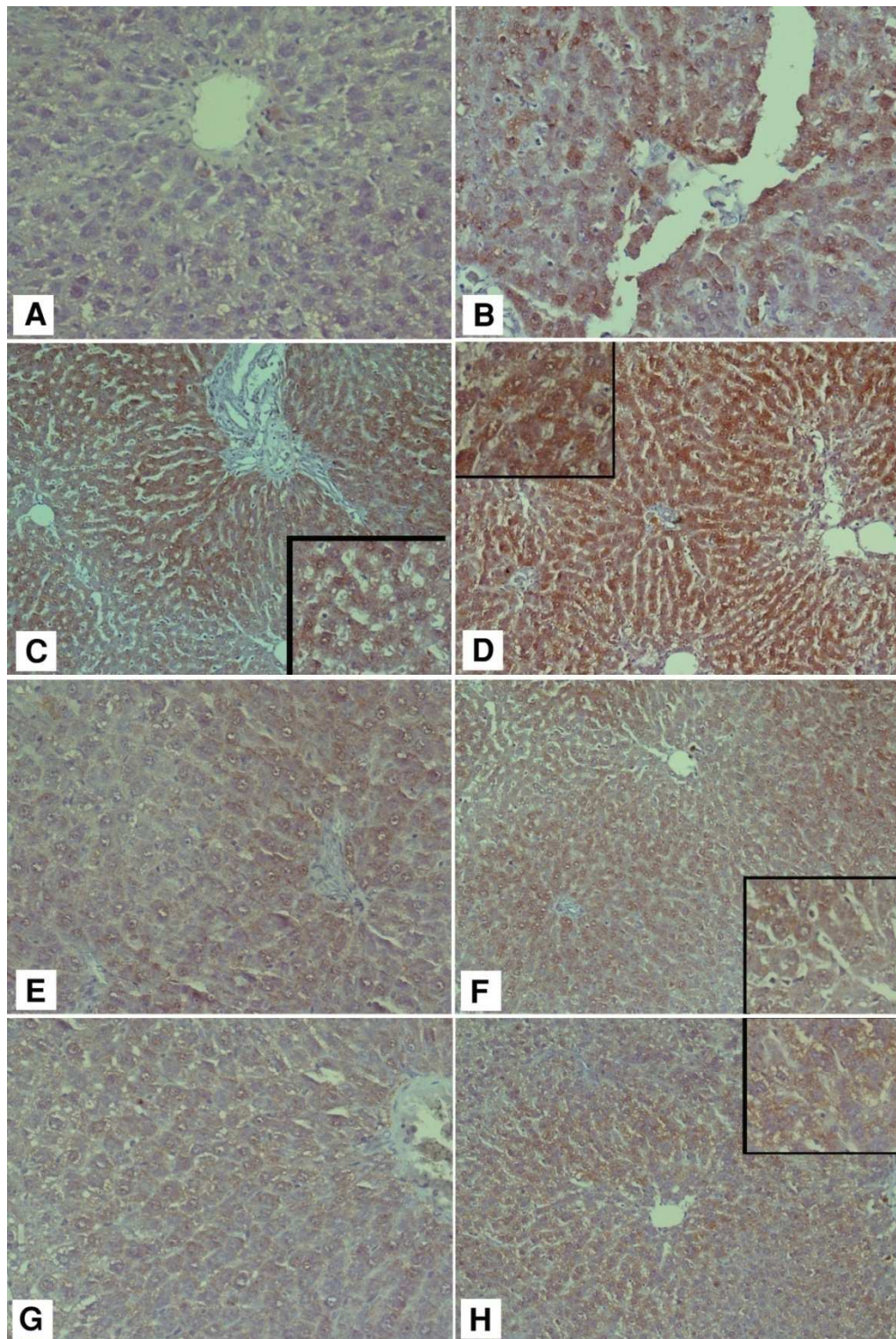n a low dose and treated with wheyprotein in a high dose,(E) a rat received CCl₄ and fluvastatin in a high dose and treated with whey protein in a low dose, (F) a rat received CCl₄ and fluvastatin in a high dose and treated with whey protein in a high dose.(Hx. & E. X 200)**

**Figure 4: A photomicrograph of sections of liver tissue from: (A) a normal control rat shows negative result for the stain. (B) a normal rat received fluvastatin in a high dose,(C) a rat received low dose of fluvastatin along with CCl₄ ,(D) a rat received high dose of fluvastatain along with CCl₄ ,(E)a ratreceived low dose of fluvastatin, CCl₄ and silymarine,(F) a rat received high dose of fluvastatin, , CCl₄ and silymarine ,(G) a ratreceived low dose of fluvastatin, CCl₄ and high dose of WPI,(H) a ratreceived high dose of fluvastatin, CCl₄ and high dose of whey protein**
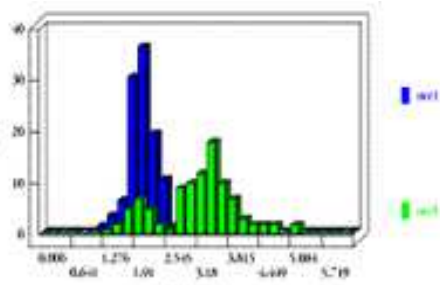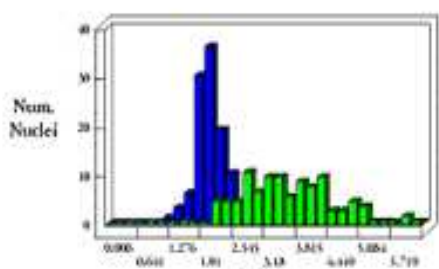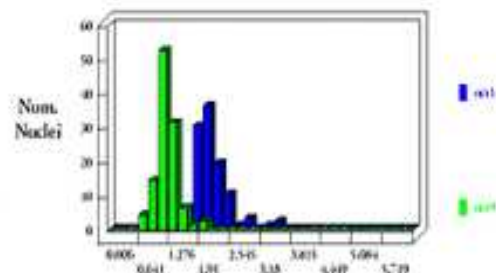*(immunohistochemical stain with Cox2 antibody X 200 (A, B, E & G)& 100 (C, D, F & H)*
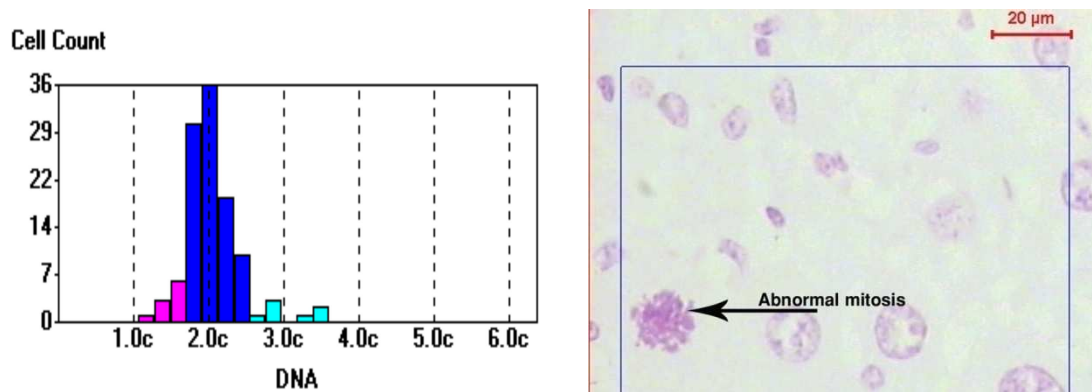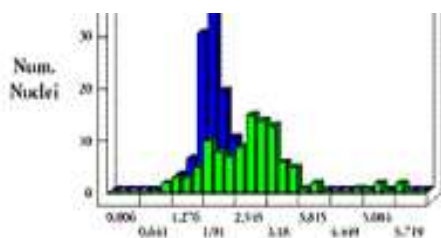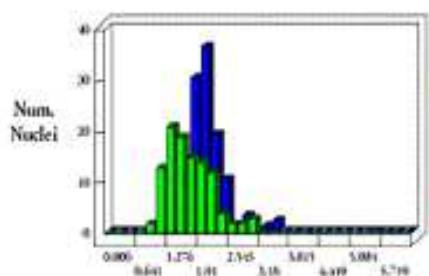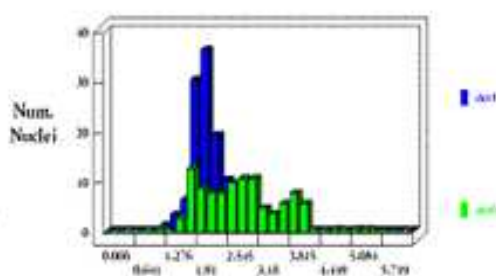
**Histogram1: Effects of silymarin and whey protein isolate on DNA content**

*(A) Normal rat, (B) CCL₄, (C) high dose of fluvastatin , (D) high dose of fluvastatin +CCL₄, (E) high dose of fluvastatin +CCL₄ + silymarin, (F) low dose of fluvastatin +CCL₄WPI(200 mg/ kg), (G) high dose of fluvastatin +CCL₄+ WPI(200 mg/ kg)*

**Table (3): Effects of silymarin and whey protein isolate on DNA content**

| | DNA index (total) | < 1.5 C | DNA index | 1.5 – 2.5 C | DNA index | 2.5 – 3.5 C | DNA index | 3.5 – 4.5 C | DNA index | > 4.5 C | DNA index |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Normal Control** | 1.000 | 3.571% | 0.665 | 89.286% | 0.976 | 5.357% | 1.377 | 1.786% | 1.742 | 0.0% | - |
| **CCl₄** | 1.556 | 1.0% | 0.722 | 20.0% | 0.976 | 40.0% | 1.523 | 35.0% | 1.853 | 4.0% | 2.403 |
| **F8** | 1.378 | 0.943% | 0.536 | 37.736% | 0.988 | 37.736% | 1.427 | 20.755% | 1.883 | 2.83% | 2.492 |
| **F8 CCl₄** | 1.823 | 0.0% | - | 8.411% | 1.075 | 36.449% | 1.476 | 39.252% | 1.954 | 15.888% | 2.691 |
| **Silym+F8+CCl₄** | 0.604 | 89.744% | 0.576 | 10.256% | 0.844 | 0.0% | - | 0.0% | - | 0.0% | - |
| **WPI 200+F4+CCl₄** | 0.855 | 33.645% | 0.620 | 58.879% | 0.924 | 7.477% | 1.374 | 0.0% | - | 0.0% | - |
| **WPI 200+F8+CCl₄** | 1.383 | 4.63% | 0.641 | 26.852% | 0.997 | 55.556% | 1.456 | 8.333% | 1.846 | 4.63% | 2.659 |

*CCl4: carbon tetrachloride, **F**: fluvastatin, **S**: silymarin, **WPI**: whey protein isolate*
*DATA were expressed as mean ± SE (n= 6) and represent % distribution of DNA content. Data were analyzed by ANOVA- one way; p≤0.05.The different alphabetical superscript is significantly different between groups.(Total numbers of cells = 100).*

## CONCLUSION

The present study suggests that this is the first experimental model in which oral administration of F+ CCL₄-induced hepatotoxicity and showed evidence of hepatic fibrosis in rats. WPI has a potent hepatoprotective activity in F+CCl₄- induced liver injury in rats. This preventive effect of WPI is due to its free radical scavenging, ant oxidative and anti-fibrotic properties.

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