Silymarin Ameliorates Cisplatin-Induced Hepatotoxicity in Male Rabbits

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Abstract: Cisplatin (CDDP) is a widely used anticancer drug, but at high dose, it can produce undesirable side effects such as hepatotoxicity. In this study, the protective activity of silymarin against hepatic tissue damage induced by repeated administration of cisplatin was analyzed morphologically and biochemically. Male Newzeland rabbits were divided into four groups, 6 rabbits in each. Control group, silymarin group (100 mg /kg b.wt./day), Cisplatin treated group (3.5 mg /kg b. wt./day) and Cisplatin plus silymarin treated group. Results revealed that cisplatin caused histopathological effects on the hepatic tissue. These effects includes vacuolation of cells, lymphocytic infiltration, dilation of blood sinusoids and haemorrhage. Histochemical observations revealed a marked depletion of polysaccharide and proteins in the liver cells of cisplatin treated animals. Cisplatin hepatotoxicity was manifested biochemically by elevation of MDA and decrease in GSH and the activities of SOD in the liver tissues. Results showed that administration of cisplatin increased the immunohistochemical expression of Bcl-2 protein. Treatment with silymarin reduced histopathological and biochemical alterations in the liver tissue. These results suggested that silymarin possess protective effects against cisplatin hepatotoxicity in rabbits.
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1. Introduction

Platinum complexes represent an important group of drugs used in the cancer treatment. Cis-diamminedichloroplatinum (II) (cisplatin, CIS) is a member of the group of chemotherapy drugs known as heavy metal alkylating-like agents (Matsusaka *et al*., 2005 ; Iraz, *et al.*, 2006 and park, *et al.,* 2009). Cisplatin is an important chemotherapeutic agent useful in the treatment of several types of cancers. In spite of its significant anticancer activity, the clinical use of cisplatin is often limited because of its unwanted toxic side effects that interfere with its therapeutical efficacy such as nephrotoxicity (Pabla and Dong, 2008), neurotoxicity (Barabas *et al*., 2008), ototoxicity (Rybak *et al.,* 2009) and hepatotoxicity (Liao *et al.,* 2008; El-Sayyad *et al*., 2009). The clinical use of CIS is limited because of its unwanted side effects such as nephrotoxicity (Pabla and Dong, 2008), neurotoxicity (Barabas *et al.,* 2008), ototoxicity (Rybak *et al.,* 2009) and hepatotoxicity (Liao *et al.,* 2008; El-Sayyad *et al*., 2009). Saad *et al.,*((2001; 2002; 2004) reported that, cisplatin administrabbition induced significant increase in serum ALT and AST and significant decrease in serum NO. Side effects includes also; severe nausea and vomiting that may necessitate hospitalization of the patient (D'Olimpio *et al*., 1985 and Kris *et al.,* 1989). Electrolyte disturbances especially hypomagnesemia and to a lesser degree

hypocalcemia, hyponatermia and hypokalemia may also occur (Schilisky & Anderson, 1979 and Craig & Stitzel, 1997). (Pratibha *et al.,* 2006; Hassan *et al.,* 2010; Kart *et al.*, 2010) reported that Cisplatin-induced hepatotoxicity is associated to oxidant damage. Higher doses or low-dose repeated cisplatin therapy that may be required for effective tumor suppression could lead to hepatotoxicity (Lee *et al*., 2008). There is little information about underlying mechanism of cisplatin-induced hepatotoxicity. It has been reported that oxidative stress through the generation of reactive oxygen species (ROS) (Chirino and Pedraza-Chaverri, 2008), decreased antioxidant defense system including antioxidant enzymes (Sadzuka *et al.,* 1992 and Pal, *et al.,* 2008). The Bcl-2 is an anti-apoptotic protein established survival factor whose physiological function is to prevent apoptosis (Hockenberry, *et al.,* 1990).

Flavonoid complex has long been used as a dietary supplement for hepatoprotection (Khadr *et al.*, 2007; Eminzade *et al.,* 2008). These properties seem to be due to their ability to scavenge free radicals and to chelate metal ions. Silymarin,,the root extract from *Silybum marianum*, an antioxidant flavonoid complex is known to have hepatoprotective effect against numerous liver diseases. Kang *et al.,* (2004) reported that Silymarin is known to have hepatoprotective and anticarcinogenic effects. This ability of silymarin leads to a significant increase in

the cellular antioxidant defense machinery by ameliorabbiting the deleterious effects of free radical reaction and by the increase in GSH content, which is important in maintaining the ferrous state (Abu Ghadeer *et al*., 2001; Ramadan *et al.,* 2002). Singh and Agarwal (2002) reported that, silymarin strongly prevents both photocarcinogenesis and skin tumor promotion in mice, by scavenging free radicals and reactive oxygen species and strengthening the antioxidant system. Lee *et al*., (2003) reported that, administrabbition of silymarin (100 mg / kg) by gastrogavage twice a day for two consecutive days resulted in an elevation of hepatic superoxide dismutase levels.

The aim of the present study was to evaluate the possible protective effects of silymarin on cisplatin -induced hepatotoxicity in male rabbits.

2. Materials and Methods

Animals and experimental groups:

Twenty four male New Zealand rabbits (ranging from 2500 to 3000g body weight (b.w.) were obtained from the Animal Farm, Taif City, KSA. The rabbits were kept in individual stainless-steel cages in a room at temperature 20 2 C, humidity 50–60% and light/dark periods 12h/12h. Standard pellet diet and tap water were provided ad libitum. The animals were divided into 4 groups each containing 6 animals:

The first group: control group, rabbits were administered propylene glycol in saline 75/25 (v/v).

The second group: rabbits were i.p. injected once a day with Silymarin (100 mg/kg/day dissolved in 0.2 ml of propylene glycol in saline 75/25 v/v.

The third group: rabbits were injected once a day with Cisplatin (3.5 mg /kg/day I.P.) dissolved in saline solution for 5 days (Yingjun *et al.,* 2008).

The Fourth group rabbits received a daily i.p. injection of silymarin (100 mg/kg/day), 2 hr after silymarin injection, animals were injected with Cisplatin (3.5 mg /kg / day). Cumulative dose of cisplatin, 17.5 mg/kg) Time of the experiment was 5 days for all groups. Silymarin was given continuously for another 5 days after the end of the experiment.

Histopathological Examinations:

At the end of the experiment, animals were sacrificed under ether anaesthesia. Small parts of liver tissue were fixed in formalin, dehydrated in ethanol, cleared and embedded in paraffin by conventional techniques (Barrera *et al.,* 2003). Sections (5 μm) thickness were cut and were stained with hematoxylin and eosin (H&E), then were examined under light microscope.

Histochemical Examinations:

For histochemical investigations, fixed sections were stained with periodic acid Schiff's (PAS) technique (Hotchkiss, 1948) for demonstration of polysaccharides (liver glycogen). Total proteins were demonstrated by the mercury bromophenol blue method (Mazia, 1953).

Oxidative stress and antioxidant enzyme assays

Malondialdehyde (MDA) in the liver

Malondialdehyde (MDA) levels in liver tissue homogenates were determined spectrophotometrically using the method of (Buege and Aust, 1978). 0.5 ml of tissue homogenate was shaken with 2.5 ml of 20% trichloroacetic acid in a 10 ml centrifuge tube, then 1 ml of 0.67% thiobarbituric acid was added, shaken and warmed for 30 minutes in a boiling water bath followed by rapid cooling. After this, 4 ml of n-butyl-alcohol was added and shaken. The mixture was centrifuged at 3,000 rpm for 10 minutes. The resultant n-butyl-alcohol layer was taken and MDA content was determined from the absorbance at 535 nm. The results were expressed as nmol/g tissue.

Superoxidedismutase (SOD):

Superoxidedismutase (SOD): activity in liver homogenate was determined according to the method of Minami and Yoshikawa (1979). This method is based on the generation of superoxide anions by pyrogallol autoxidation, detection of generated superoxide anions by nitro blue tetrazolium. The formazan color developed was determined spectrophotometrically (Spectronic 501, Shimadzu, Japan). Enzymatic activity was expressed as μg/g tissue.

Reduced glutathione (GSH):

The liver homogenate, reduced glutathione (GSH) was determined spectro- photmetrically according to the methods of Ellman (1959) using Ellman'sreagent [5,50-dithio-bs- (2 nitrobenzoic acid)]. The results were expressed as μmol/g tissue.

Immunohistochemical studies

Paraffin sections of the liver were used for detecting Bcl-2 immunoreactivity. Sections were de-waxed and incubated for 1hr at room temperature in 0.3% hydrogen peroxide in phosphate-buffered saline, then were washed three times (10 min. each) in the same buffer. They were incubated for 16 hr at 4°C in PBS containing 2% normal goat serum (NGS) and 0.5% triton X-100, and then washed and followed by overnight incubation at 4°C with the primary monoclonal antibody of (Mouse anti-EMA, ICN, Costa Mesa, CA, USA and Sigma Chem. Comp.) anti-Bcl-2. The primary antibodies were bounded by a rat adsorbed biotinylated anti-mouse secondary antibody in PBS for 1hr at room temperature. Slides were incubated in avidin-biotin complex linked to peroxidas which was seen with 0.03% diaminobenzidine hydrochloride and 0.005% hydrogen peroxide in 0.1 M Tris buffer. All sections

were counter stained with hematoxylin dehydrated, cleared, and mounted in Canada balsam.

Statistical analysis

The obtained data were presented as means ± standard error. One-way ANOVA was carried out, the values were considered significantly at $P<0.05$. The statistical comparisons among the groups were performed with Mann–Whitney Rank Sum Test using a statistical package program (SPSS version 15.0).

3. Results

Histopathology:

The microscopic examinations of sections of liver of control and silymarin treated rabbits showed normal structure of the hepatic lobules. Hepatocytes were normal polygonal with oval shaped and most of their nuclei appear centrally located. The central vein is surrounded by the hepatocytes with eosinophilic cytoplasm and distinct nuclei. The hepatic sinusoids are shown in normal size between the hepatocytes (Figs 1A&B). The histopathological examination of the liver tissue of Cisplatin treated animals revealed remarkable changes versus control animals. The arrangement of the anastomosing plates of hepatocytes were disrupted and there were disorganization of the hepatic cords, degenerative changes in numerous hepatocytes, vacuolation of cells and necrotic changes in few zones. Congested blood sinusoids and activation of Kupffer cells, were observed. Most nuclei appeared significantly reduced in size (Fig. 1C). Dilation of blood sinusoid, swollon cytoplasm and pyknosis of the nuclei were seen in (Fig. 1D). Pretreatment of silymarin 2 h before cisplatin remarkably reduced the pathological changes induced by cisplatin; hepatocytes appeared regular in arrangement and their borders, less cytoplasmic vacuolization, sinusoids are in normal size. General appearance of liver tissue is similar to those in control animals but Kupffer cells are still increased in numbers (Fig. 1D).

Histochemistry

Periodic acid Schiff reaction in the liver of control and silymarin-treated samples exhibited normal carbohydrate content. It is distributed densely in the hepatocytes (Figs 2 A&B). Compared to both control and silymarin treated samples, liver of cisplatin-treated animals exhibited marked reduction of carbohydrate (Fig 2C). In Cisplatin plus silymarin treated group, carbohydrate content of the liver cells was as that of the controls (Fig 2D).

Bromophenol blue in the liver of control and silymarin-treated samples exhibited normal protein content. It is distributed densely in the hepatocytes (Figs 3A&B). Liver of cisplatin-treated animals exhibited marked reduction of proteins compared with control animals (Fig 3C). In Cisplatin plus silymarin treated group, protein content of the liver cells was as that of the controls (Fig 3D).

Immunohistochemical reaction:

In this study, antiapoptotic immune positive reactions in the liver was investigated with Bcl-2 antibodies. Bcl-2 protein immunoreactions of control group showed strong positive immunoreaction for Bcl-2 protein of cytoplasm (Fig.4A). In cisplatin treated group, there was negative immunoreaction for Bcl-2 in the cytoplasm of hepatocytes.(Fig.4B). So the incidence of apoptosis was prominent in the cisplatin treated group than that of the control. In case of cisplatin plus silymarin group, there is moderate immunoreaction for Bcl-2 protein in cytoplasm of most hepatocytes (Fig.4C). Thus, co-administration of silymarin decreased apoptosis.

Lipid peroxidation and antioxidant enzymes:

The hepatic contents of antioxidant parameters, namely, MDA, SOD and GSH, were determined (Table 1). Cisplatin induced remarkable changes in these parameters. The levels of lipid peroxidation products were significantly increased (46%) in the liver tissue of rabbits exposed to cisplatin, while GSH and SOD were decreased (45%) and (33%) respectively when compared with the controls. Treatment with silymarin prior to cisplatin resulted in a significant decrease of MDA (20%) and significant increase of GSH (23%) and SOD (17%).

Table 1. Effect of Silymarin on Cisplatin-induced changes in the lipid peroxidation, Reduced glutathione and Superoxide dismutase of male rabbits. Values represents as mean ± SE.

Parameters \ Groups	Lipid peroxidation (LPO) (nmole /g tissue)	Reduced glutathione (GSH) (μmole /g tissue)	Superoxide dismutase (SOD) (μg/g tissue)
Control	22.35 ± 1.87	54.33 ± 1.66	98.87 ± 1.12
Silymarin	20.95 ± 2.11	57.27 ± 2.07	95.33 ± 1.31
Cisplatin	32.75 ± 2.03*	29.53 ± 2.95*	65.73 ± 1.11*
%	46% higher than control	45% lower than control	33% lower than ontrol
Silymarin&Cisplatin	26.19 ± 1.97 ☻	36.24 ± 1.82 ☻	77.43 ± 1.21 ☻
%	20% lower than cisplatin	23% higher than cisplatin	17% higher than cisplatin

* Significant difference at P<0.05 compared with the control group.

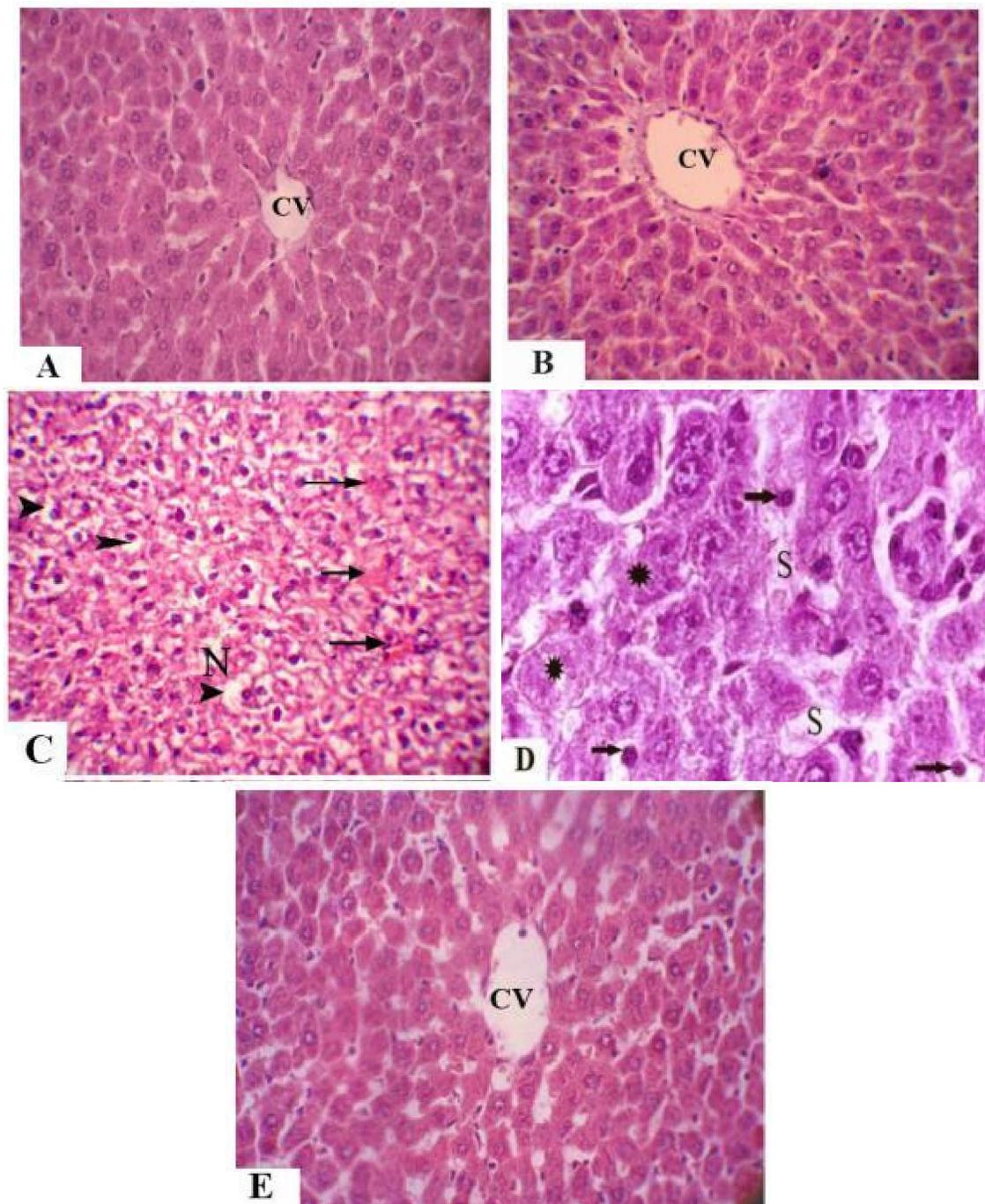☻ Significant difference at $P<0.05$ compared with the cisplatin group.

Figure 1: (A) Photomicrograph of a section of the liver of control rabbit showing normal central vein (CV), blood sinusoid and hepatocytes.   (H & E, X 400)

 (B) Photomicrograph of a section of the liver of rabbit treated with silymarin displaying normal hepatic strand normal blood sinusoids, hepatocytes.   (H & E, X 400)

 (C) Photomicrograph of a section of the liver of rabbit treated with cisplatin showing necrotic area (N), vacuolation (arrow heads), congested blood sinusoid (arrows) and small sized nuclei. (H & E, X400)

(D) Photomicrograph of a section of the liver of rabbit treated with cisplatin illustrating pyknotic nuclei (arrows, swollen cytoplasm (stars) and enlarged sinusoids (S).   (H& E, X 1000)

 (E) Photomicrograph of a section of the liver of rabbit treated with cisplatin followed by silymarin showingg improvement of hepatic cells.   (H & E, X 400)
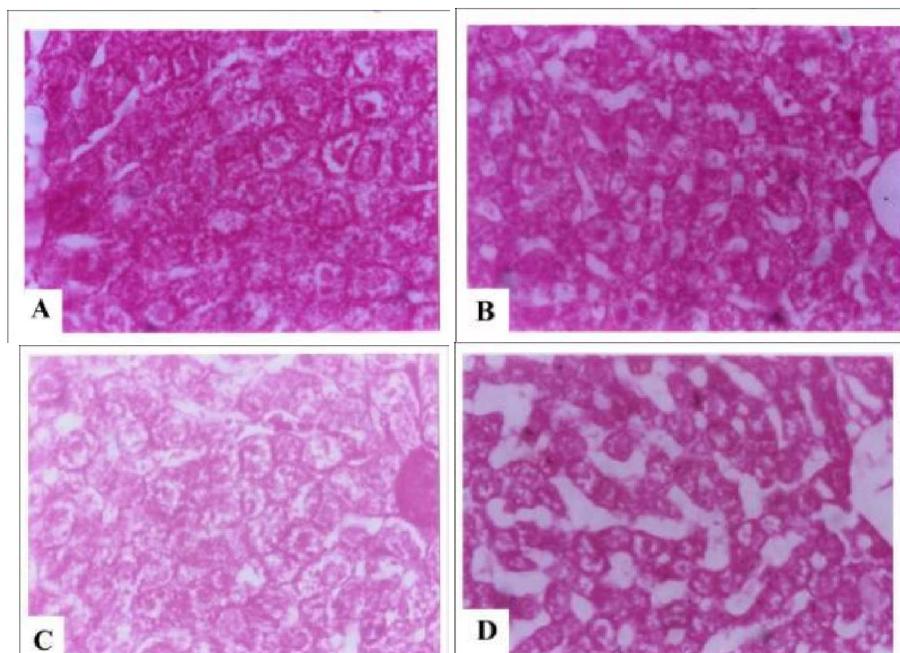
Figure 2: (A) Photomicrograph of a section of the liver of control rabbit showing strong PAS positive stain. (PAS, X 400).
(B) Photomicrograph of a section of the liver of rabbit treated with silymarin displaying PAS positive stain. (PAS, X 400)
(C) Photomicrograph of a section of the liver of rabbit treated with cisplatin displaying weak PAS stain. (PAS, X 400)
(D) Photomicrograph of a section of the liver of rabbit treated with cisplatin and silymarins showing a moderate to strong PAS positive reaction.   (PAS, X 400)
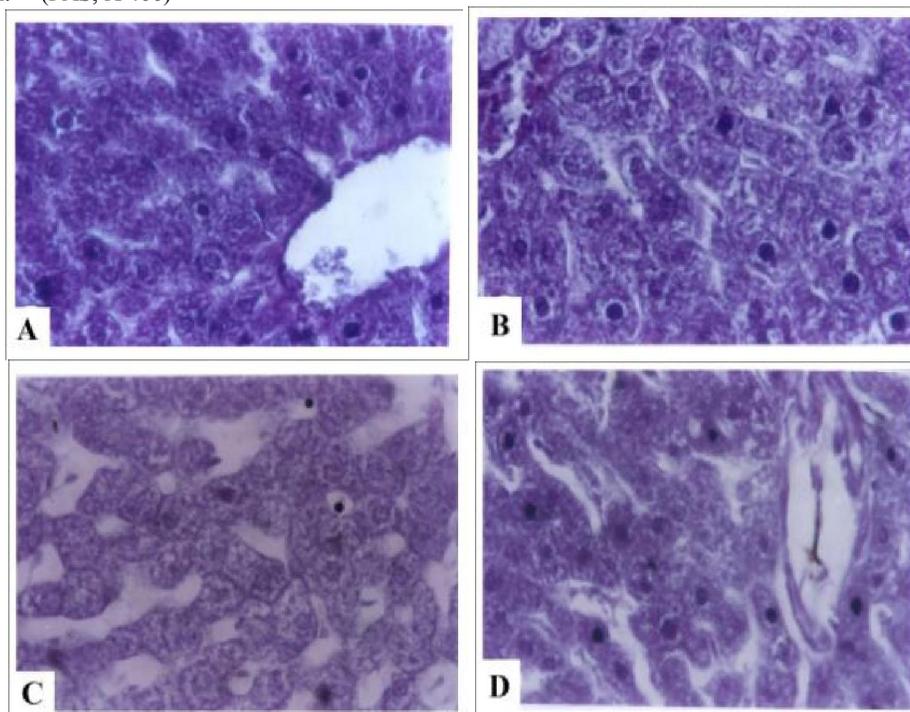

Figure 3: (A) Photomicrograph of a section of the liver of control rabbit showing strong stain for bromophenol blue. (Bromophenol blue, X 400); (B): Photomicrograph of a section of the liver of rabbit treated with silymarin displaying normal bromophenol blue stain.   (Bromophenol blue, X 400); (C) Photomicrograph of a section of the liver of rabbit treated with cisplatin showing weak bromophenol blue stain.   (Bromophenol blue, X 400); (D) Photomicrograph of a section of the liver of rabbit treated with cisplatin and silymarin illustrating moderate bromophenol blue stain. (Bromophenol blue, X400)
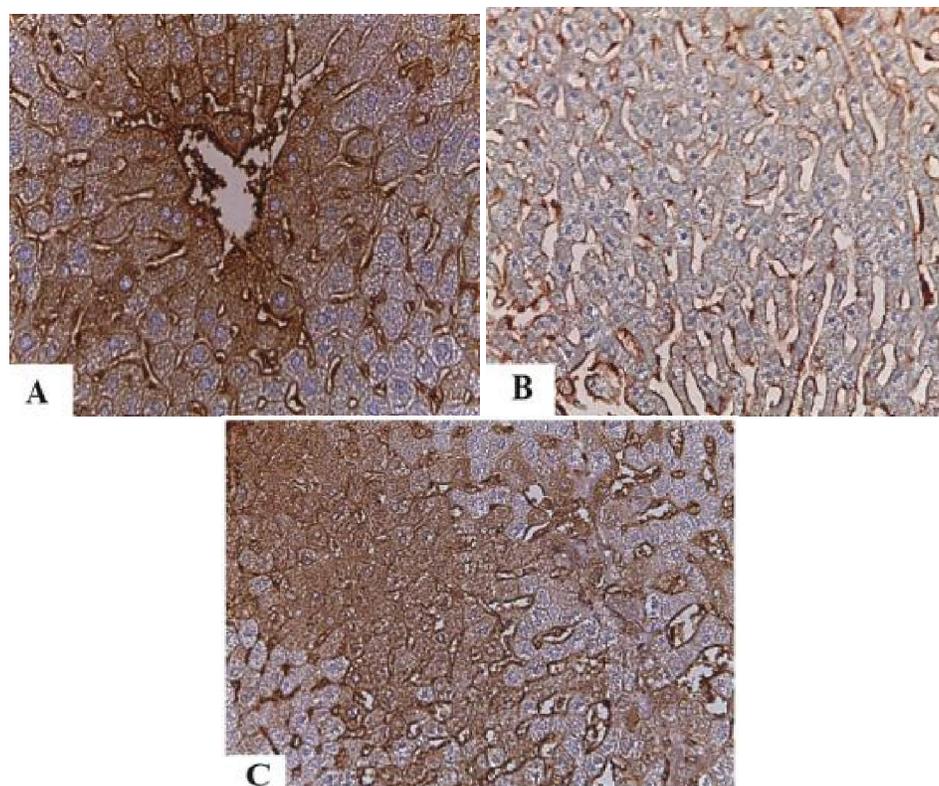
Figure 4: (A) Photomicrograph of a section of the liver control group showing strong positive immunoreaction for Bcl-2 protein of cytoplasm of most hepatocytes. (X, 400)
(B) Photomicrograph of a section of the liver of cisplatin group showing negative immunoreaction for Bcl-2 in cytoplasm of hepatocytes. (X, 400)
(C) Photomicrograph of a section of the liver of cisplatin plus silymarin showing moderate immunoreaction for Bcl-2 protein in cytoplasm of hepatocytes. (X, 400)

4. Discussion

Cisplatin, one of the most active cytotoxic agents against cancer, has several toxicities. Hepatotoxicity is one of them occurred during high doses treatment (Koc *et al.,* 2005; Prabbitibha *et al.,* 2006). It is well known that the liver is the center of detoxifying and eliminating the toxic substances that are carried to it via the blood. The liver is known to accumulate significant amounts of cisplatin, thus hepatotoxicity can be associated with cisplatin treatment (Liao *et al.,* 2008). Oxidative stress has been defined as a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses, which may lead to tissue injury. Cisplatin acts on cancer cells by releasing free radicals which damage liver cells. Free radicals are known to attack the highly unsaturated fatty acids of the cell membrane to induce lipid peroxidation and at the same time, decrease in the activities of antioxidant enzymes that protect against lipid peroxidation in the liver tissues. Liver, which is considered as the main central organ of metabolism and detoxification of most drugs and toxins, was chosen for the present study to examine both the

histopathological, histochemical and immunohistochemical changes when rabbits exposed to cumulative dose of cisplatin (17.5 mg/kg b. w.) in cisplatin, and the potential protective role of silymarin. The results demonstrated that rabbits treated with cisplatin showed development of swollen liver cells with vacuolated cytoplasm and pyknotic nuclei, wide and congested blood sinusoids. Meanwhile, the histochemical examinations of liver sections of cisplatin-treated rabbits showed decrease in carbohydrate and protein contents. In agreement with present study, (Dank, *et al*., 2008 and El-Sayyad *et al.* (2009) who reported that administration of cisplatin resulted in histopathological abnormalities, necrosis, hepatocyte degeneration and apoptotic cell.

The present study indicated that lipid peroxidation (MDA) in liver significantly increased in the liver of rabbits treated with cisplatin. This result agrees with previous studies which have demonstrated the oxidative stress and lipid peroxidation in cisplatin-induced liver toxicity (Cayir *et al.,* 2009). Our results also are in accordance with previous studies which have demonstrated the

involvement of oxidative stress, lipid peroxidation, and mitochondria dysfunction in cisplatin –induced liver toxicity (Kang, 2004 and Yilmaz *et al.,* 2005). In this study, cisplatin also decreased significantly the scavenger enzymes activities (GSH and SOD) in the liver of rabbits. This result may be due to that cisplatin induced increase generation of free radical and decrease the amounts of antioxidant enzymes against lipid peroxidation. These results are in accordance with (Al-Majed *et al.,*2006 and Aleisa *et al.,* 200 7) in kidney and liver tissues in which cisplatin and carboplatin injections caused low GSH and SOD. On the other hand, it was observed that levels of GSH and activities of SOD in cisplatin plus silymarin group were significantly increased in comparison with cisplatin group.

Apoptosis is a common feature of hepatotoxicity induced by chemicals or drugs. Cisplatin is considered as apoptotic agent (Kishimoto *et al.,* 2000). The Bcl-2 family proteins play an important role in regulating the apoptotic signaling pathway. Bcl-2 is an anti-apoptotic protein decreasing its expression with aging and when exposed to toxins (Hockenberry, *et al.,* 1990 and Numata *et al.,* 2002). A number of studies reported that cisplatin has been found to have an apoptotic effect on kidney and liver (Kang and Reynolds, 2009; Lieber, *et al.,* 2010 and Mohamed, *et al.,* 2013). This study demonstrated marked downregulation of the anti-apoptotic protein Bcl-2 after administration of cisplatin and a negative immunoreaction for Bcl-2 in cytoplasm of most hepatocytes.

Inspection of the data obtained from the present work regarding the histopathology and histochemistry displayed the occurrence of good improvement by silymarin against cisplatin-induced liver damage. This observation is in agreement with that of (Mansour, *et al.,* 2006) who reported that the administration of silymarin inhibited the effect of cisplatin in liver. Also, the present results are in accordance with that of many authors who who demonstrates that silymarin has protective effects against an antioxidative, lipid peroxidation and Histopathological changes (Eminzade *et al.,* (2008) ; Wu e*t al.,* (2008). In conclusion the present results added an additional evidence that cisplatin is a hepatotoxic agent and silymarin afforded protection against the liver injury induced by Cisplatin.

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