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Research Article
Cytoprotective Effect of Silymarin on Cisplatin Induced Hepatotoxicity and Bone Marrow Toxicity in Rats

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Abstract

Background and Objective: Cisplatin is considered one of the most effective and widely used anti-neoplastic drugs in chemotherapeutic regimes of cancer treatment. Toxic side effects associated with cisplatin including nephrotoxicity, neurotoxicity, hepatotoxicity and bone marrow toxicity, were limit its clinical uses. Nowadays, researchers were directed to use herbal medicine to overcome these side effects. Silymarin is one of herbal medicine which has anti-oxidant, anti-apoptotic and anti-inflammatory effects. This study was designed to investigate the efficacy of silymarin against hematological and pathological disorders, bone marrow toxicity and hepatotoxicity induced by cisplatin in rats. **Materials and Methods:** Thirty-two Albino rats were used and were divided into 4 equal groups as follow; control, silymarin-treated, cisplatin-treated and silymarin-protected group. The experiment continued for thirteen days through which blood and tissue samples were taken at 8th and 13th days of the experiment. Hematological evaluation includes: RBCs count, packed cell volume, hemoglobin concentration, platelets countas well as total and differential leukocytic counts. Bone marrow evaluation was done through applying the differential cell count, cellular density, myeloid/erythroid ratio, megakaryocyte percent and maturation index for both myeloid and erythroid series. Hepatic biomarkers were investigated including activities of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase. Cytological and histopathological examinations were also performed on all hepatic sections. The collected data were analyzed by one-way analysis of variance (ANOVA). **Results:** Cisplatin-treated group showed normocytic normochromic anemia with marked suppression in bone marrow cell proliferation manifested by diminishing of maturation indices in association with megakaryocyte hypoplasia. Elevated hepatic biomarkers in association with cytological findings in cisplatin-treated group documented the occurrence of severe hepatic lipidosis. Immunohistochemistry revealed hepatic toxicity through activation of caspase-3 and inhibition of anti-nuclear factor kappa beta activation in hepatocellular nuclei of cisplatin treated group. In silymarin protected group, most of hematological alterations, hepatic biomarkers as well as cytological and histopathological changes were significantly improved ($p<0.05$)toward control levels. **Conclusion:** It is concluded that prior-treatment with silymarin partially attenuated the cisplatin induced anemia, thrombocytopenia, bone marrow myelosuppression and hepatotoxicity through its anti-apoptotic and cytoprotective properties.

Key words: Cisplatin, silymarin, hepatotoxicity, bone marrow toxicity, cytology, immunohistochemistry

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diagnosis of cancer and the possibility of chemotherapy treatments are stressful for pet owners due to the potential side effects of chemotherapy. Chemotherapy may be used as the only treatment for certain metastatic disease or for tumors that cannot be removed surgically or may be used to shrink large tumors prior to surgery. Cisplatin (cis-diamine dichloro-platinum) (CDDP) is considered the widely and the most popular chemotherapeutic agent used in veterinary medicine for the treatment of different types of cancers especially in dogs including leukemia, lymphoma, multiple myeloma and sarcoma, as well as cancers of lung, mammary gland and ovary[1]. Cisplatin is one of alkylating agents that directly damage DNA resulting in cell apoptosis. Like most of chemotherapeutic drugs; cisplatin does not distinguish between cancer and normal cells and eliminates not only the fast-growing cancer cells but also other fast-growing cells in the body[2]. Treatment with cisplatin frequently causes hepatotoxicity nephrotoxicity, thrombocytopenia and bone marrow toxicity[3] in a dose dependent manners making difficulty to complete course of chemotherapy[4]. Unfortunately, the previous toxicity of cisplatin is an inherent adverse effect, where most of patients develop severe hepatotoxicity and myelosuppression during cisplatin treatment[5,6]. These side effects appeared when the drug reached its peak level during the first weeks of treatment[7] causing intolerable discomfort in cancer animals and worsen their quality of life[3].

Despite that oxidative stress and apoptosis seem to play a crucial role in the mechanism of hepatotoxicity and bone marrow toxicity. There is no precise treatment for cisplatin-induced bone marrow myelosuppression. Therefore, many investigations have been designed to assess the potential hepatoprotective effects of several anti-oxidants and anti-inflammatory agents against the adverse effects of cisplatin[8,9].

Some researches advised the use of enriched diets with herbal plants like silymarin[10]. Silymarin (*Silybum marianum*) known as milk thistle, is a member of Asteraceae family and is well recognized as a hepatoprotective herbal medicine. Silymarin is a lipophilic extract of the milk thistle seeds[10]. It is established that silymarin has been utilized medicinally to cure liver diseases including viral hepatitis, cirrhosis[9]. It is well known for its anti-oxidant, anti-inflammatory, anti-apoptoticproperties which contribute its ability to scavenge free radicals[11]. Besides the antioxidant effect, silymarin indicates effective antineoplastic, immunomodulating and membrane stabilizing[12,13] properties in different animal and human studies. Furthermore, according to the literature, protective effects of silymarin in different tissues including brain, heart, liver, kidney, lung, pancreas and skin[14,15] have been reported against some toxic materials and different disorders. This protective role may attribute to its active components, namely, silybin and silychristin isomers[10]. However, the study for the effect of silymarin against bone marrow myelosuppression induced by cisplatin has not been previously investigated.

This was encouraging to design the current study in order to assess the ameliorative role of silymarin in rats exposed to cisplatin injection. Moreover, hematological (with highlighting on bone marrow examination), cytological and histopathological alterations induced by cisplatin were investigated.

MATERIALS AND METHODS

Drugs: Cisplatin (CDDP) was obtained in the form of vial (1 mg mL^{-1}) from Egyptian International Medical Company, United Pharmaceuticals, Cairo, Egypt. Silymarin was obtained from Sedico Company, Egypt, in the form of capsule (140 mg).

Animals: A total of 32 Albino rats (weighing about 180±10 g) were obtained from Animal House, Faculty of Veterinary Medicine, Cairo University, Egypt. Rats were acclimated for a period of 7 days in Veterinary Clinical Pathology Laboratory condition prior to the experiment. Rats were fed with standard laboratory diet and allowed to drink water *ad libitum*. The study was carried out from January to April, 2016.

Experimental design: All rats were randomly divided into 4 main groups of 8 rats for each as follow: group I (control group); rats were orally received distilled water and intra-peritoneally (i.p.) injected with normal saline at the 5th and 10th days of the experiment. Group II (silymarin-treated group); rats were orally received silymarin at a dose of 100 mg kg^{-1} day^{-1} [9] all over the experimental period. Group III (cisplatin-treated group); rats were i.p. injected with cisplatin at a dose of 7.5 mg kg^{-1} [16] at the 5th and 10th days of the experiment. Group IV (silymarin-protected group): Rats were orally received silymarin at a dose of 100 mg kg^{-1} for 10 successive days and they were i.p. injected with cisplatin at a dose of 7.5 mg kg^{-1} at the 5th and 10th days of the experiment (cisplatin injection was carried out 2 h post silymarin administration).

Hematological examination and hepatic biomarkers: Blood samples were collected from each rat through venous plexuses at 8th and 13th days of the experiment. Blood samples were divided into two parts; first part was anti-coagulated by di-potassium salt of Ethylene Diamine Tetra-acetic Acid (EDTA) for evaluating hemogram according to Feldman *et al.*[17]. Second part was collected in a clean centrifuge tube and allowed to clot, then centrifuged at 3000 rpm for 10 min for serum separation. Clear non hemolysed supernatant serum was harvested for measuring the following hepatic biomarkers: Activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) and concentrations of total proteins, albumin together with calculation of globulins concentration and albumin/globulins ratio (A/G)[18]. All these biomarkers were assayed using reagent kits supplied by Stan Bio-Laboratories incorporation, USA.

Bone marrow and cytological examinations: Rats at the 13th day of the experiment were anesthetized by ether then abdominal dissection was carried out. Impression smears from liver were taken and stained with field stainfor cytological examination[19]. Bone marrow aspirations were collected from rats' femur and their smears prepared immediately and stained with field stain for bone marrow examination including: Differential cell count of bone marrow, estimation of bone marrow cellular density, myeloid/erythroid (M/E) ratio, megakaryocyte percentage and maturation index for myeloid and erythroid series[20].

Bone marrow cellularity was estimated using low power magnification by comparing the percentage of fat to bone marrow hematopoietic cells, while megakaryocyte percentage was estimated usinghigh power magnification. Differential cell count of bone marrow as well as myeloid/erythroid (M/E) ratio were done on 300 bone marrow hematopoietic cell[20]. Maturation index was calculated as a ratio between the number of proliferative phase cells to the number of maturative phase cells. Myeloid proliferative phase includes: myeloblasts, promyelocytes and myelocytes whereas myeloid maturative phase includes: metamyelocytes, band cells and segmented neutrophils. Cells of erythroid proliferative phase are: rubriblasts, prorubricytes and rubricytes, whereas cells of erythroid maturative phase are the metarubricytes[20].

Histopathological examination: Tissue specimens from liver were taken and were fixed in 10% formalin then routinely processed, dehydrated in different grades of ethanol, cleared in xylene and finally embedded in paraffin blocks. Then they were sectioned at 5-6 μm thickness and stained with hematoxylin and eosin stain (H and E) according to Bancroft and Gamble[21]. For immunohistochemical examination, the sections of hepatic tissues in phosphate buffered saline (pH = 7.2) were incubated overnight at 4°C with the respective primary monoclonal antibody [anti- Nuclear Factor Kappa Beta (NF-κB), dilution (1:100)]. Immunohistochemical staining was performed by the streptavidin-biotin complex method[22]. All sections were then counter stained with hematoxylin according to Al-Malki and Sayed[22]. All chemicals and solutions were of good quality and analytical grade.

Statistical analysis: Values were expressed as Mean±SD. Statistical comparisons among the means of different experimental groups were made with completely randomized one way ANOVA by COSTAT program version 6.4. p-value of <0.05 was assumed for statistical significance[23].

RESULTS

Hematological results: Mean values of erythrogram and leukogramat 8th and 13th days of the experiment are illustrated in Table 1. Results of silymarin-protected group exhibited significant improvement ($p<0.05$) in hematological parameters compared to cisplatin-treated groupthat suffered from normocytic normochromic anemia in association with thrombocytopenia. These findings were confirmed by increased values of Red Blood Corpuscles (RBCs) count, Packed Cell Volume (PCV %) and hemoglobin concentration as well as platelets count. However, both cisplatin-treated and silymarin-protected groups showed leukocytosis with neutrophilia and lymphopenia. Microscopical examination of blood smears revealed presence of hypochromacia, acanthocytes, target cells and toxic neutrophils in cisplatin-treated group, while silymarin-protected group exhibited anisocytosis, poikilocytosis, acanthocytes and polychromacia.

Hepatic biomarkers: Hepatic biomarkers statistical analysis of different experimental groups is illustrated in Table 2. Analysis of hepatic enzymes activities including ALT, AST and ALP weresignificantlyincreased ($p<0.05$) in cisplatin-treated group compared to control and silymarin-treated groups at 8th and 13th days of the experiment. However, the levels of these biomarkers were significantly decreased ($p<0.05$) in silymarin-protected group compared to cisplatin-treated group at both 8th and 13th days of the experiment. In addition, total proteins and albumin concentrations were

Table 1: Hematological parameters of different experimental groups at the 8th and 13th days of the experiment

	Days							
	8th				13th			
Parameters	Group I	Group II	Group III	Group IV	Group I	Group II	Group III	Group IV
PCV (%)	44.20±1.64^{a}	44.20±1.96^{a}	30.00±1.20^{b}	41.70±2.04^{a}	54.33±7.03^{a}	45.71±6.23^{ab}	36.33±1.36^{b}	44.2±6.58^{ab}
Hb (g dL^{-1})	16.04±0.59^{a}	15.72±0.98^{a}	10.24±1.27^{b}	15.04±1.08^{a}	12.5±0.98^{a}	11.18±1.28^{a}	8.46±1.57^{b}	10.24±1.18^{ab}
RBCs (×10^{6} µL^{-1})	10.19±1.50^{a}	9.54±1.40^{a}	7.13±1.02^{b}	9.53±1.32^{a}	8.08±0.61^{a}	7.13±1.03^{a}	6.58±0.63^{a}	7.01±1.05^{a}
MCV (fl)	53.21±5.11^{a}	49.23±4.52^{a}	42.71±3.50^{a}	43.97±5.50^{a}	67.20±7.05^{a}	67.10±6.35^{a}	55.91±5.31^{a}	62.62±6.14^{a}
MCHC (g%)	36.16±1.25^{a}	36.03±1.48^{a}	35.55±2.03^{a}	34.04±1.50^{a}	24.48±2.03^{a}	23.32±1.02^{a}	23.29±2.35^{a}	23.29±1.65^{a}
Platelet count (×10^{3} µL^{-1})	313.833±72.05^{a}	297.714±69.20^{a}	202.10±43.26^{b}	258.80±36.11^{b}	300.06±71.25^{a}	320.61±62.05^{a}	120.12±41.29^{b}	250.65±31.25^{a}
TLC (×10^{3} µL^{-1})	7.04±1.40^{a}	7.4±2.30^{a}	17.24±3.51^{b}	8.12±2.50^{a}	7.84±1.47^{a}	7.58±1.65^{a}	16.04±2.46^{b}	9.04±1.88^{a}
Neutrophil (×10^{3} µL^{-1})	6.64±1.12^{a}	6.84±1.50^{a}	15.11±3.50^{b}	6.67±0.87^{a}	6.58 ±1.05^{a}	6.98 ±1.29^{a}	14.38±2.25^{b}	7.96±1.35^{a}
Band cell (×10^{3} µL^{-1})	0.23±0.06^{a}	0.11±0.03^{a}	0.84±0.50^{b}	0.34±0.08^{a}	0.26±0.05^{a}	0.20±0.02^{a}	0.75±0.03^{b}	0.38±0.04^{a}
Lymphocyte (×10^{3} µL^{-1})	1.98±0.50^{a}	1.85±0.06^{a}	0.85±0.08^{b}	0.98 ±0.08^{b}	1.87 ±0.21^{a}	1.94±0.05^{a}	0.68 ±0.06^{b}	1.52±0.25^{b}
Monocyte (×10^{3} µL^{-1})	0.20±0.06^{a}	0.30±0.04^{a}	0.79±0.06^{a}	0.12±0.01^{a}	0.25±0.03^{a}	0.31 ±0.04^{a}	0.80±0.06^{a}	0.14±0.01^{a}
Eosinophil (×10^{3} µL^{-1})	0.82±0.07^{a}	1.42±0.16^{a}	0.64±0.24^{a}	0.52±0.06^{a}	0.81±0.06^{a}	1.40±0.16^{a}	0.62 ±0.24^{a}	0.53±0.06^{a}

Data are presented as Mean±SD, Group I: Control group, Group II: Silymarin-treated group, Group III: Cisplatin-treated group, Group IV: Silymarin-protected group, $^{a-c}$Means with different superscripts within a raw are significantly different at p<0.05

Table 2: Hepatic biomarkers levels of different experimental groups at 8th and 13th days of the experiment

	Days							
	8th				13th			
Parameters	Group I	Group II	Group III	Group IV	Group I	Group II	Group III	Group IV
ALT (U L^{-1})	37.18±2.38^{a}	36.63±2.72^{a}	73.14±3.47^{b}	57.45±3.99^{b}	40.38±2.54^{a}	40.20±2.58^{a}	79.75±3.42^{b}	52.43±3.83^{a}
AST (U L^{-1})	88.69±9.92^{a}	89.42±8.98^{a}	150.01±11.76^{b}	118.54±10.22^{a}	93.43±9.11^{a}	94.12±8.20^{a}	169.01±10.43^{b}	120.33±10.32^{a}
ALP (U L^{-1})	73.42±6.38^{a}	70.78±5.94^{a}	162.19±9.45^{b}	95.28±8.51^{a}	79.95±5.66^{a}	78.92±5.24^{a}	169.4±9.11^{b}	100.68±8.43^{c}
Total proteins (g dL^{-1})	5.09±0.13^{a}	5.12±0.06^{a}	4.03±0.08^{b}	4.88±0.05^{a}	5.40±0.12^{a}	5.42±0.11^{a}	3.86±0.10^{b}	4.48±0.09^{a}
Albumin (g dL^{-1})	2.19±0.08^{a}	2.04±0.04^{a}	1.69±0.07^{b}	1.96±0.03^{a}	2.05±0.09^{a}	2.06±0.08^{a}	1.55±0.07^{b}	1.98±0.06^{a}
Globulins (g dL^{-1})	2.40±0.06^{a}	2.38±0.05^{a}	2.34±0.08^{a}	2.32±0.04^{a}	2.45±0.05^{a}	2.56±0.10^{a}	2.48±0.11^{a}	2.54±0.07^{a}
A/G	1.12±0.11^{a}	1.15±0.09^{a}	0.72±0.14^{a}	0.80±0.07^{a}	1.04±0.09^{a}	1.12±0.10^{a}	0.63±0.06^{b}	0.76±0.04^{b}

Data are presented as Mean±SD, Group I: Control group, Group II: Silymarin-treated group, Group III: Cisplatin-treated group, Group IV: Silymarin-protected group, $^{a-c}$Means with different superscripts within a raw are significantly different at p<0.05, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase

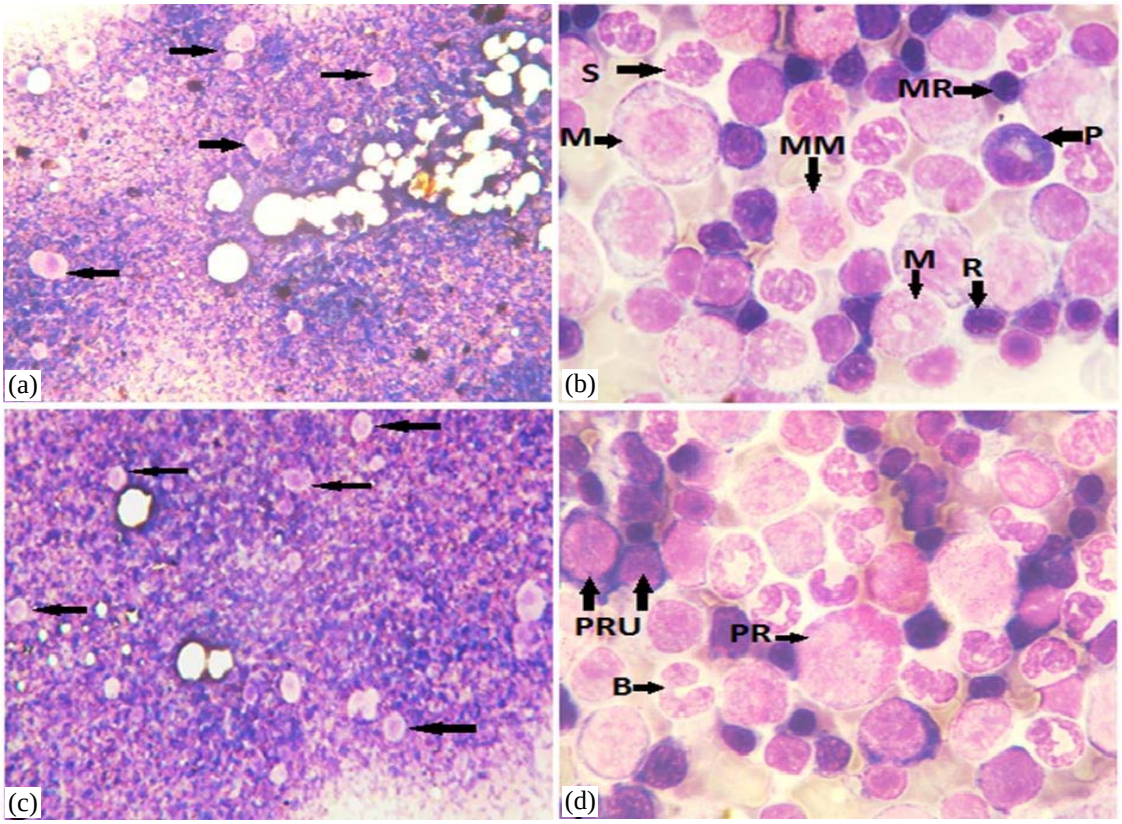

Fig. 1(a-d): Bone marrow smear of control and silymarin-treated groups (a) Bone marrow smear of control group showed normal cellular denisty with numerous megakaryocytes (arrows) (Field stain, ×100), (b) Bone marrow smear of control group showed myelocyte (M), metamyelocyte (MM), rubricyte (R), metarubricyte (MR) with segmented neutrophil (S) and plasma cell (P) (Field stain, ×1000), (c) Bone marrow smear of silymarin-treated group showed normal cellular denisty with numerous megakaryocytes (arrows) (Field stain, ×100) and (d) Bone marrow smear of silymarin-treated group showed promyelocyte (PR), prorubricyte (PRU) with band cell (B) (Field stain, ×1000)

significantly decreased ($p<0.05$) in cisplatin-treated group as compared to control group, meanwhile total proteins and albumin concentrationswere significantly higher ($p<0.05$) insilymarin-protected group than the cisplatin-treated group.

Bone marrow examination: Mean values of differential cell counts of bone marrow, cellular density, myeloid/erythroid (M/E) ratio, maturation index for myeloid and erythroid series are illustrated in Table 3 and 4.

Cisplatin-treated group showed severe hypocellularity, myeloid proliferative cells count including myeloblasts, promyelocytes and myelocytes and erythroid proliferative cells count including rubriblasts, prorubricytes and metarubricytes were significantly decreased ($p<0.05$). Additionally, myeloid maturative cells count including metamyelocyte and segmented granulocytes was significantly increased ($p<0.05$). Insignificant changes in M/E ratio and erythroid maturation index were noticed. Bothmyeloid maturation index and megakaryocyte were significantly lower ($p<0.05$) than control group as well as silymarin-treated group (Fig. 1a-d, Fig. 2a-b).

In comparison to cisplatin-treated group, silymarin-protected group was significantly decreased ($p<0.05$) in segmented granulocytesand metarubricytes count was significantly increased ($p<0.05$). Insignificant changes in M/E ratio and erythroid maturation index were also seen and both myeloid maturation index and megakaryocyte percentage were significantly increased ($p<0.05$) (Fig. 2c-d). Silymarin-treated group wassignificantly higher ($p<0.05$) than the control group in megakaryocyte percentage.

Cytological findings: Cytological examination of hepatic smears of control as well as silymarin-treated groups consisted largely of hepatocytes that were distributed in the smear as

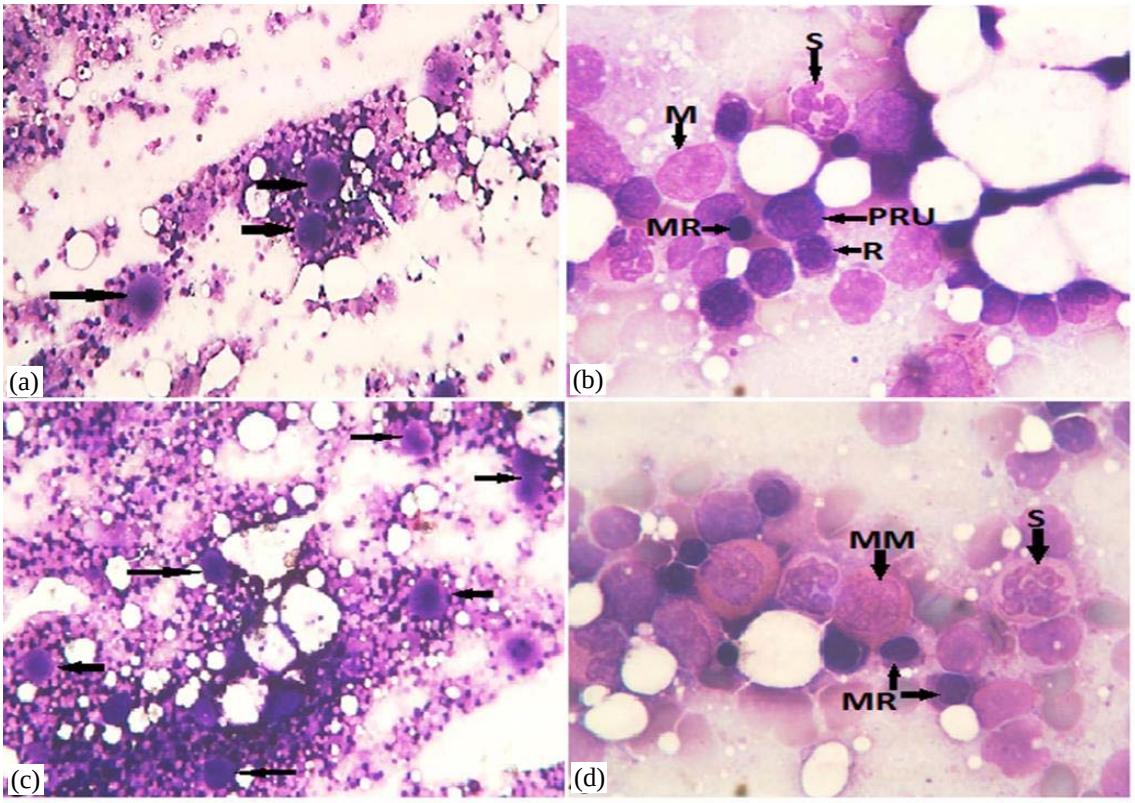

Fig. 2(a-d): Bone marrow smear of cisplatin-treated and silymarin-protected groups (a) Bone marrow smear of cisplatin-treated group showed severe hypocellular with three megakaryocytes (arrows) (Field stain, ×400), (b) Bone marrow smear of cisplatin-treated group showed myelocyte (M), rubricyte (R), prorubricytes (PRU), metarubricyte (MR) with segmented neutrophil (S) (Field stain, ×1000), (c) Bone marrow smear of silymarin-protected group showed improvement in cellular density than cisplatin-treated group with hyperplasia of megakaryocytes (arrows) (Field stain, ×400) and (d) Bone marrow smear of silymarin-protected group showed eosinophilicmetamyelocyte (MM), metarubricyte (MR) with segmented neutrophil (S) (Field stain, ×1000)

Table 3: Differential cell counts in rat femoral bone marrow of different experimental groups at the 13th day of the experiment

Femoral bone marrow	Group I	Group II	Group III	Group IV
Myeloblasts	1.82±0.39^{a}	0.93±0.04^{ab}	0.54±0.48^{b}	0.80±0.09^{ab}
Promyelocytes	10.87±1.52^{a}	10.50±2.60^{ab}	7.49±1.83^{b}	9.16±3.68^{ab}
Myelocytes	72.75±13.85^{a}	69.02±18.98^{ab}	33.63±12.47^{b}	55.14±22.76^{ab}
Metamyelocytes	31.42±1.40^{a}	33.94±15.14^{a}	49.89±2.42^{b}	54.08±11.86^{b}
Band cells	7.55±3.33^{a}	3.56±2.15^{a}	7.41±1.70^{a}	6.79±2.31^{a}
Segmented granulocytes	45.14±5.08^{a}	57.93±13.98^{ab}	75.59±8.72^{b}	44.37±6.95^{a}
Rubriblasts	2.18±0.12^{a}	1.59±0.45^{ab}	0.75±0.68^{b}	0.80±0.09^{b}
Prorubricytes	69.11±3.14^{a}	51.61±12.71^{b}	21.17±3.46^{c}	28.14±7.17^{c}
Rubricytes	33.05±17.22^{a}	33.11±0.84^{a}	27.22±2.23^{a}	31.83±3.59^{a}
Metarubricytes	65.19±16.99^{a}	69.67±18.58^{a}	31.06±24.83^{b}	43.71±8.70^{a}

Data are presented as Mean±SD, Group I: Control group, Group II: Silymarin-treated group, Group III: Cisplatin-treated group, Group IV: Silymarin-protected group, $^{a-c}$Means with different superscripts within a raw are significantly different at p<0.05

Table 4: Bone marrow evaluation of different experimental groups at the 13th day of the experiment

Parameters	Group I	Group II	Group III	Group IV
Cellular density	Normocellular	Normocellular	Severe Hypocellular	Hypocellular
Myeloid/erythroid ratio	1.01±0.30^{a}	1.13±0.12^{a}	1.09±0.51^{a}	1.63±0.32^{a}
Myeloidmaturation index	0.98±0.09^{a}	0.83±0.19^{a}	0.31±0.08^{b}	0.62±0.08^{c}
Erythroid maturation index	1.66±0.25^{a}	1.42±0.17^{a}	1.31±0.28^{a}	1.4±0.06^{a}
Megakaryocyte (%)	9.76±0.52^{a}	11.75±0.6^{b}	1.0±0.25^{c}	7.68±0.9^{d}

Data are presented as Mean±SD, Group I: Control group, Group II: Silymarin-treated group, Group III: Cisplatin-treated group, Group IV: Silymarin-protected group, $^{a-c}$Means with different superscripts within a raw are significantly different at p<0.05

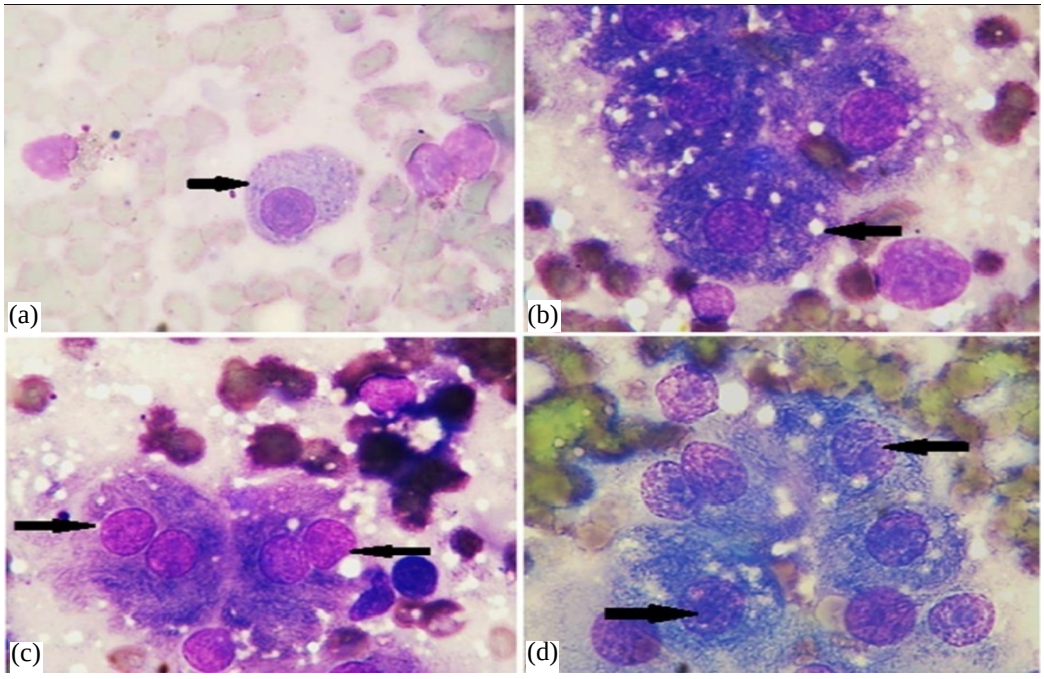

Fig. 3(a-d): Hepatic smear of control and cisplatin-treated groups (a) Normal hepatocyte of control group. Cells contain one round, centrally located nuclei (arrow) (Field stain, ×1000), (b) Hepatic smear of cisplatin-treated group exhibit hepatocytes with microvesicular vacuoles (arrow) (Field stain, ×1000), (c) Hepatic smear of cisplatin-treated group showed binucleated hepatocytes (arrows) (Field stain, ×1000) and (d) Hepatic smear of cisplatin-treated group showed vacuolization of hepatocytes with prominent nucleoli (arrows) (Field stain, ×1000)

single cells andclusters. Normal hepatocytes were seen with its uniform, large or slightly oval shape and basophilic cytoplasm. Nuclei were round, centrally placed, with coarse chromatin and a single large prominent nucleolus. Low numbers of binucleated hepatocytes were also observed. Mast cells, hepatic macrophages (Kupffer cells) with low numbers of lymphocytes were seen (Fig. 3a).

Hepatic smears examination of cisplatin-treated group exhibited severe hepatic lipidosis recognized as discrete, round vacuoles within the cytoplasm. These vacuoles were microvesicular; vacuoles smaller than the nucleus. Large numbers of binucleated hepatocytes with presence of mixed inflammatory cells including large number of neutrophils, Kupffer cells, eosinophils, lymphocytes and mast cells were clearly observed. Naked (free) nuclei with prominent nucleoli were metastasized all over the hepatic smears (Fig. 3b-d).

Cytological examination of hepatic smears of silymarin-protected group revealed absence of hepatic lipidosis and presence of regenerative hyperplasia of hepatocytes. Mild to moderate hepatocellular anisocytosis and anisokaryosis, slightly increased cellular and nuclear size and increased basophilia of the cytoplasm were markedly noticed. Binucleated hepatocytes were more numerous than in normal hepatic tissue and less than cisplatin-treated group. Naked (free) nuclei with prominent nucleoli were still noticed in hepatic smears but less numerous than in cisplatin-treated group. Mixed inflammatory cell infiltrates include neutrophils, eosinophils, macrophages and lymphocytes were seen (Fig. 4a-d).

Histopathological findings: Microscopic examination of control and silymarin-treated groups showed normal hepatic architecture (Fig. 5a). Microscopic examination of cisplatin-treated group revealed acute hepatic injury including hepatocytomegally, karyomegally with increased number of binucleated hepatocytes, increased number of apoptotic hepatocytes with diffuse vacuolization of hepatocytes (Fig. 5b).

Additionally, there was Kupffer cell hypertrophy with moderate infiltration of mononuclear cells (Fig. 5c). Hepatic parenchyma generally displayed congestion of central vein with sinusoidal congestion with multifocal hemorrhagic areas and intense mononuclear cell infiltration (Fig. 5d).

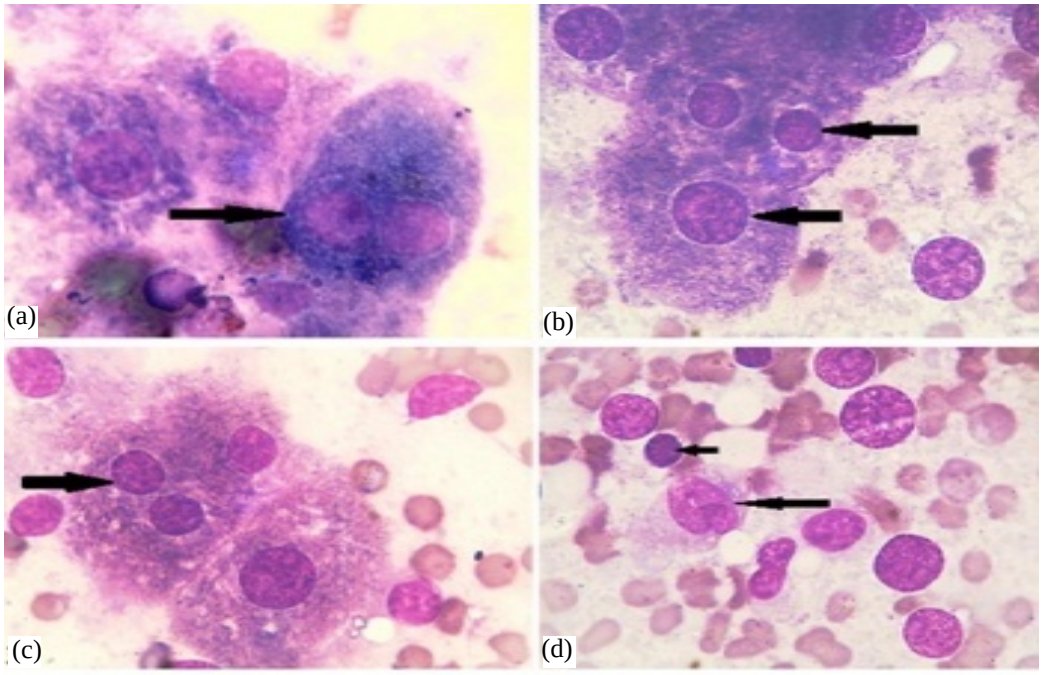

Fig. 4(a-d): Hepatic smear of silymarin-protected group (a) Hepatic smear of silymarin-protected group revealed absence of hepatic lipidosis with deeply basophilic binucleated hepatocyte (arrow) (Field stain, ×1000), (b) Hepatic smear of silymarin-protected group revealed regenerative hyperplasia of hepatocytes with hepatocellular anisocytosis and anisokaryosis (arrows) (Field stain, ×1000), (c) Hepatic smear of silymarin-protected group revealed binucleated hepatocyte (arrow) with hyperplastic hepatocyte (Field stain, ×1000) and (d) Hepatic smear of silymarin-protected group revealed naked (free) nuclei with prominent nucleoli with Kupffer cell (long arrow) and lymphocyte (short arrow) (Field stain, ×1000)

Pretreatment with silymarin alleviated the severity of cisplatin as the hepatocytes displayed uniform size of cell cytoplasm and nuclei with less number of binucleated hepatocytes with focal kupffer cell hypertrophy. Moreover, the apoptotic hepatocytes were extremely reduced with mild sinusoidal congestion of hepatic lobules. Mild vacuolization of hepatocellular cytoplasm was also detected with few mononuclear cell infiltrations (Fig. 5e-f).

Immunohistochemical findings: Immunohistochemical detection of caspase as an indicator for apoptosis revealed that, cisplatin-treated group had increased cytoplasmic and nuclear expression of caspase-3 including large number of hepatocytes in hepatic lobules (Fig. 6a). Caspase-3 expression was extremely reduced in hepatocytes either in intensity of brown positive hepatocytes or distribution among hepatocytes (Fig. 6b), while the strong and diffuse cytoplasmic expression of anti-Nuclear Factor Kappa Beta (NF-κB) in hepatocytes with negative nuclear expression were detected in cisplatin-treated group (Fig. 6c). On the other hand, cytoplasmic expression of NF-κB extremely reduced in silymarin-protected group with fain brown nuclear staining indicating the nuclear expression of NF-κB was achieved in silymarin-protected group (Fig. 6d).

DISCUSSION

Cisplatin is considered one of the most potent alkylating agents used as anticancer drug through its direct damaging of cancer cell DNA which will prevent them from division. Despite the positive effects of anticancer drugs, they are poisonous. Animals receiving these agents undergo severe side effects as hepatotoxicity, nephrotoxicity and bone marrow toxicity that limit the dose which can be administered. The ability to manage the before mentioned side effects isessential for the success of cancer chemotherapy[24]. Different natural compounds have been recently investigated as potential protective agents against cisplatin induced toxicity[25]. One of these compounds is silymarin which has been studied to ameliorate the toxic side effects of chemotherapeutic drugs through its hepatoprotectiveefficacy[2].

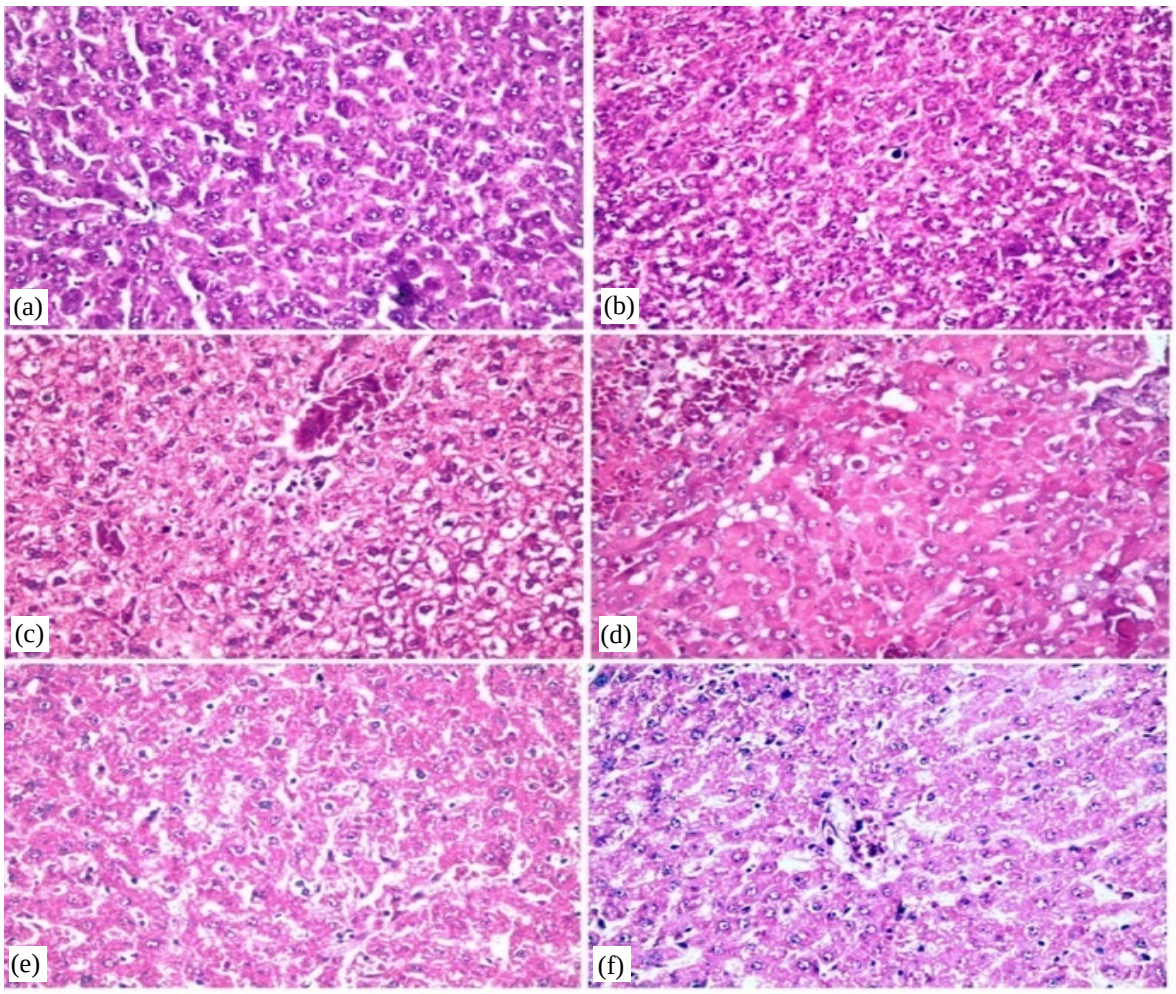

Fig. 5(a-f): Hepatic histological findings of different experimental groups (a) Silymarin-treated group showed normal hepatic histological structure (H and E, ×400), (b) Cisplatin-treated group showed anisokaryosis of hepatocellular nuclei, increased number of binucleated hepatocytes, hepatocytomegally with karyomegally and presence of apoptotic body associated hypertrophy of Kupffer cells (H and E, ×400), (c) Cisplatin-treated group showed diffuse vacuolization of hepatocytes, hepatocellular necrosis with mononuclear cell infiltration associated with central vein congestion (H and E, ×400), (d) Cisplatin-treated group showed apoptotic hepatocytes that surrounded by clear hallo associated with parenchymal hemorrhages (H andE, ×400), (e) Silymarin-protected group showed showing mild vacuolization of hepatocytes, focal hypertrophy of Kupffer cell lining the hepatic sinusoids, few mononuclear cell infiltrating the hepatic sinusoids and mild sinusoidal congestion (H and E, ×400) and (f) Silymarin-protected group showed hepatocellular necrosis with karyorrhexis of hepatocellular (H and E, ×400)

In the present study, cisplatin-treated group induced significant adverse effect on hematological parameters at both 8th and 13th days of the experiment and the pretreatment with silymarin had successfully ameliorated these hematological disturbances. Toxic effect of cisplatin on blood parameters was demonstrated by the significant decline in the values of RBCs count, PCV (%) and hemoglobin concentration. However, both silymarin-protected and cisplatin-treated groups showed leukocytosis with neutrophilia and lymphopenia at both 8th and 13th days of the experiment.

The previous results suggested that normocytic normochromic anemia induced in cisplatin-treated group may explained by the cytotoxic effect of cisplatin on bone marrow cells or due to increased RBCs osmotic fragility induced by cisplatin[26]. Anemia associated with cisplatin intoxication may

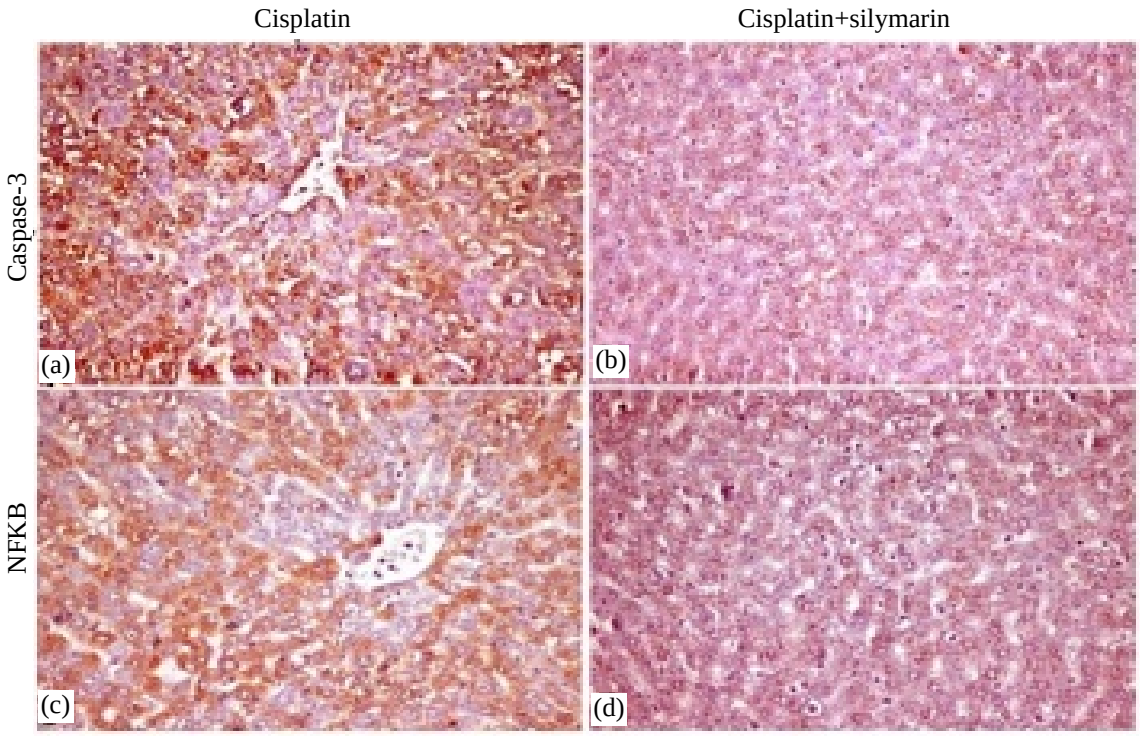

Fig. 6(a-d): Immunohistochemical analysis of caspase-3 and NF-κB in different experimental groups (a) Cisplatin-treated group showed strong caspase-3 positive brown reaction in hepatocellular cytoplasm (×400), (b) Silymarin-protected group showed faint expression of caspase-3 in hepatocellular cytoplasm (×400), (c) Cisplatin-treated group showed strong cytoplasmic expression and negative nuclear expression of NF-κB in hepatocytes (×400) and (d) Silymarin-protected group showed faint cytoplasmic and nuclear expression of NF-κB in hepatocytes (×400)

be produced either by suppressing the activity of hematopoietic tissues or by accelerating RBCs destruction because of the altered RBCs membrane permeability[26,27].

Lymphopenia and thrombocytopenia in cisplatin-treated group were resulted from the apoptotic effect of cisplatin on lymphocytes and platelets and thereby ultimately reduced the number of these cells in the blood. On the other hand, the observed leukocytosis in both cisplatin-treated and silymarin-protected groups could be the consequence of infection and inflammation[28].

Pretreated rats with silymarin revealed significant modulation in most of their hematological parameters which changed by cisplatin administration. Silymarin was found to have beneficial effects against cisplatin side effects on most of hematological parameters as it increased RBCs count, PCV (%), Hb concentration and platelets count toward normal levels by its anti-oxidant, anti-inflammatory and anti-apoptotic actions[2].

Bone marrow examination is considered an important and critical component for hematotoxicity assessment because bone marrow is the primary site in the body where the hematopoietic stem cells proliferate and differentiate[20,29]. Depending on the proliferating nature, bone marrow cells are very sensitive to cytotoxic chemicals and easily susceptible to DNA damage[30] especially the undifferentiated cell population as recorded in the present study with cisplatin-treated group. Microscopical examination of bone marrow smears in different experimental groups revealed hypoplasia of both myeloid and erythroid series in cisplatin-treated group together with relative increased metamyelocytes and segmented granulocytes. Inhibition of cell proliferation is one of the major causes of cisplatin induced myelotoxicity[6,31] which reflected in the present study by decreasing of maturation index in association with hypoplasia of megakaryocyte cell line. While, the insignificant change in M/E ratio is due to the parallel cytotoxic effect of cisplatin on both myeloid and erythroid series.

Silymarin-protected group IV showed an improvement in myeloid maturation index with megakaryocyte hyperplasia in comparison to cisplatin-treated group III. These changes may be attributed to the cytoprotective effect of silymarin[32,33],

in addition to the proliferative cells of both myeloid and erythroid series of group IV are less dramatically affected than group III.

Hepatic enzymesare the most sensitive biomarkers used for evaluating the function and integrity of liver cells. Thus, presence of such enzymes within the circulation is considered clear evidence for the damage of hepatocytes cell membrane.

In the present study, significant elevations ($p<0.05$) of serum hepatic enzymes in cisplatin-treated group give an evidence for severe hepatotoxicity. Cytological examination of hepatic smears exhibited severe hepatic lipidosis, large number of binucleated hepatocytes, presence of mixed inflammatory cells with large number of free nuclei as a sign of tissue necrosis[34,35]. Additionally, the reduction of serum total proteins and albumin levels in cisplatin-treated group could be attributed to the direct impairment effect of cisplatin on synthetic and execratory functions of hepatocytes[26].

Oral administration of rats with silymarin prior to cisplatin treatment (group IV) was significantly reduced ($p<0.05$) cisplatin toxic effect on hepatic enzymes activities compared to untreated rats (group III) together with increased total proteins and albumin concentrations. Reduction of hepatic enzymes activities in silymarin-protected group may be due to the role of silymarin in scavenging of free radicals, decreasing formation of reactive oxygen species and inhibiting of fatty acid peroxidation that produced by cisplatin[8,24].

Additionally, this oral administration of silymarin was significantly improved ($p<0.05$) most of cytological findings associated with cisplatin hepatotoxicity, this improvement appeared in the form of absence of hepatic lipidosis and presence of regenerative hyperplasia of hepatocytes. Binucleated hepatocytes and free nuclei are still noticed in hepatic smears but less numerous than those appeared in cisplatin-treated group[25,36,37].

Histopathological examination of cisplatin-treated group revealed acute hepatocellular degenerative changes, induction of apoptosis and initiation of inflammatory reaction as well as hemodynamic derangement of hepatic vasculatures. Degenerative and apoptotic cascades induced by cisplatin were attributed to increased oxidative stress in hepatic tissue which reported by Sinha *et al.*[38] who discussed the role of oxidative stress in induction of apoptosis via mitochondrial and non-mitochondrial pathways.

Expression of caspase-3 in cisplatin-treated group was increased in both hepatocellular cytoplasm and nuclei as cisplatin induced hepatocellular apoptosis[39]. In silymarin-protected group this expression was reduced as the silymarin reduced the oxidative stress which subsequently decreased the apoptotic cascade in hepatocytes[40].

Expression of NF-κB in cisplatin-treated groupshowed strong cytoplasmic and negative nuclear expressions indicating strong oxidative stress activities and DNA binding interference in hepatocytes that resulted in inhibition of NF-κB activation and translocation into the nuclei[41]. Pretreatment with silymarin demonstrating faint NF-κB expression in hepatocytes cytoplasm and their nuclei indicating the initiation of NF-κB activity and translocation in the nuclei that attributed to low level of oxidative stress induced by anti-oxidant activity of silymarin[42].

The results suggested that, cisplatin induced hepatic injury and apoptosis through activation of caspase-3 and inhibition of NF-κB activation in hepatocellular nuclei. Increased cytoplasmic expression of NF-κB without nuclei in cisplatin treated group is related to increasing apoptotic expression of caspase-3 as referred by Jeschke *et al.*[43] who found the increased hepatic cytoplasmic NF-κB expression without nucleus reflecting the anti-apoptotic effect of NF-κB. On the other hand, pretreatment with silymarin limited the massive hepatocellular loss by apoptosis, through its activation of hepatocytes NF-κB and enhancement of hepatocellular regeneration[44]. The NF-κB activation resulted in activation of cytoprotective genes, inhibition of apoptosis and supporting cell viability[45].

In the current study, the oral intake of silymarin prior to chemotherapy significantly improved ($p<0.05$) most alterations of hematological parameters, hepatic biomarkers as well as cytological and histopathological finding. Hematological alterations revealed the myelosuppressive effect of cisplatin including hypoplasia of both myeloid and erythroid series as well as megakaryocyte hypoplasia. Results of this study showed that, silymarin have the ability to be anti-myelosuppressive agent through its improvement in myeloid maturation index with megakaryocyte hyperplasia. Moreover, silymarin administration revealed potent protective effect against severe hepatic lipidosis and elevation of hepatic biomarkers induced by cisplatin. Before recorded results, the study recommended using silymarin as anti-myelosuppressive and hepatoprotective agent prior to chemotherapy regimes in animals.

CONCLUSION

Silymarin had successfully ameliorated the hematological disturbances induced by cisplatin and provided adequate

protection to rat bone marrow hematopoietic cells against cisplatin induced myelosuppression as evident from cellularity of femoral bone marrow. Pretreatment with silymarin limited the massive hepatocellular injury that was observed by caspase-3 and NF-κB expression and cytological examination.

SIGNIFICANCE STATEMENTS

This study discovers the possible cytoprotective effect of silymarin that can be beneficial for hepatic and bone marrow toxicities induced by cisplatin in rats. This study will help the researcher to uncover the critical role of silymarin against bone marrow toxicity and could be used as a dietary protective agent during cancer chemotherapy that many researchers were not able to explore. Thus, a new theory on this herbal medicine as anti-myelosuppressive agent could take place.

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