

Hydroalcoholic extract of *Zataria multiflora* mitigates cisplatin-induced oxidative stress, apoptosis and hepatotoxicity in mice

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ABSTRACT: Cisplatin (CP), as an anticancer drug, causes hepatotoxicity. *Zataria multiflora* Boiss (ZM), as a herbal medicine, has antioxidant property and decrease oxidative stress-induced toxicity and side effects. This study investigated the effects of hydroalcoholic extract of ZM against cisplatin-induced hepatotoxicity. In this experimental study, thirty-two male mice were randomly divided into four groups (8/group). The control group; ZM group was received ZM (200 mg/kg) during 7 days by gavage; CP group was received CP (10 mg/kg) intraperitoneally in 5th day of study; ZM + CP group. Serum biochemical [alanine aminotransferases (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP)], tissue biochemical [malondialdehyde (MDA), protein carbonyl (PC) and glutathione (GSH) content], histopathological and immunohistochemical assays were examined to determine hepatotoxicity. CP caused significant increase in ALT, AST and ALP, oxidative stress (increased of MDA, PC and decreased of GSH in liver tissues), histological changes (hepatocellular degeneration, necrosis, inflammatory cell infiltration, congestion, sinusoidal dilatation). ZM significantly mitigated the toxic effects of CP on the liver tissue and oxidative stress ($p < 0.01$ and $p < 0.05$, respectively). Furthermore, CP increased caspase-3 immunoreactivity (apoptosis) (21.85 ± 6.81) and ZM administration significantly reduced immunoreactivity of caspase-3 compared with CP-treated mice (10.66 ± 3.23). The findings of this study showed that the ZM as a potential antioxidant compound and with scavenging free radicals, hepatic injury and suppression of caspase-3 expression attenuated the hepatotoxicity induced cisplatin.

KEYWORDS: Cisplatin; *Zataria multiflora*; oxidative stress; hepatotoxicity; caspase-3.

1. INTRODUCTION

Cisplatin cis-[Pt(NH₃)₂Cl₂] (cis-diamminedichloroplatinum(II)), as an anti-cancer drug, used for cancer treatment [1, 2] Despite anti-tumoral effect, CP has important side effects such as nephrotoxicity, hepatotoxicity, neurotoxicity and ototoxicity [3]. Liver as an important organ in detoxification and excretion metabolites of drugs, frequently injured [4]. So, liver is the main target of drug-induced damage. The severity of the damage depends on exposure time and its concentration [5]. Liver toxicity is very common in patients treated with high dose cisplatin [6]. Oxidative stress is involved in the pathogenesis of liver toxicity caused by cisplatin. Cisplatin induced toxicity in different organs through the generation of reactive oxygen species (ROS) and inhibition of endogenous antioxidant enzymatic and non-enzymatic molecules [7, 8]. In previous studies reported CP-induced hepatotoxicity decrease by exogenous antioxidants [9, 10]. The use of herbal medicine with natural antioxidants is recommended to reduce the toxicity caused by the chemotherapy drug. Many studies recently were showed herbal medicine with antioxidant property alleviate cisplatin-induced toxicity and side effects [11, 12].

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Zataria multiflora Boiss (ZM) (Shirazi avishan extract), a member of Lamianaceae family, is one of the largest families of herbal plants growing in the all world [13]. ZM is a medicinal herb that is used in various diseases. Pharmacological properties such as anti-angiogenesis [14], anti-inflammatory [15], radioprotective [16], chemoprotective [17], antiulcerogenic [18, 19], antibacterial and antioxidant [20] properties have been reported in the numerous studies. Thymol and carvacrol as main biologically active components of ZM are phenolic compounds [21, 22]. Previous studies were shown that thymol by having antioxidant and anti-inflammatory properties suppress lipid peroxidation and following that protect normal organs against toxicity induced oxidative stress [23] and chemotherapy [24]. Also, previous study was showed ZM by inhibiting lipid peroxidation and restoring the antioxidant enzymes improve hepatotoxicity induced cisplatin in rat [25]. However, no study showed the anti-apoptotic effect of ZM on the hepatotoxicity caused with CP.

Hence, this study aimed to assess the protective effect of ZM against cisplatin-induced hepatotoxicity. Therefore, in present study, we evaluated the effects of ZM against cisplatin-induced hepatotoxicity in mice via biochemical, histopathological and immunohistochemical assessment.

2. RESULTS

2.1. HPLC characterization of the ZM extract

The ZM extract was standardized based on thymol content. The thymol content was 9.24 ± 0.11 mg/g in ZM extract.

2.2. Effects of ZM on oxidative stress in CP treated mice

The MDA level, as the final product of lipid peroxidation (LPO), was significantly increased ($P < 0.001$), and GSH content as endogenous antioxidant, was decreased in CP treated group ($P < 0.001$) compared with control group. In addition, PC increased in CP treated mice ($P < 0.001$) when compared with control group. In contrast, ZM pretreatment in CP treated mice was significantly decreased the MDA and PC levels (respectively $P < 0.01$, $P < 0.03$) when compared with CP group, whereas the GSH content were significantly increased ($P < 0.05$) Figure 1.

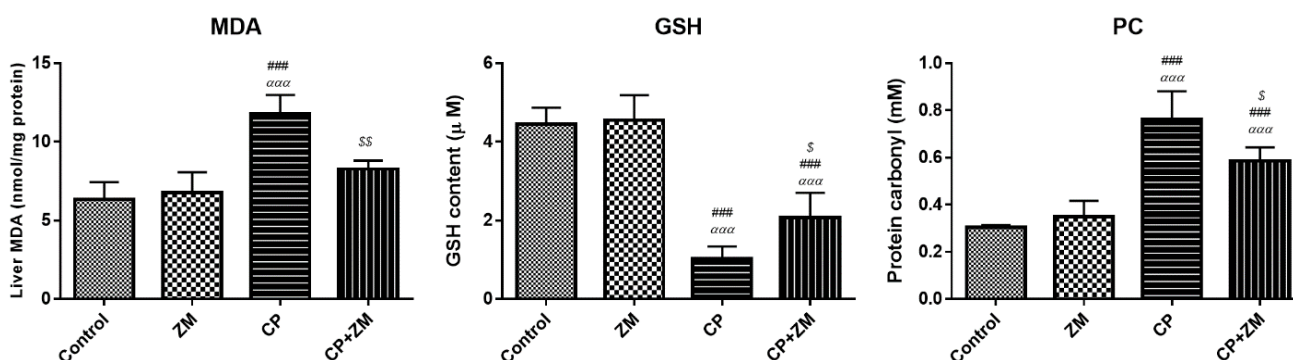


Figure 1. Histogram shows the levels of MDA, GSH and PC. All values are expressed as mean \pm SD. ### $P < 0.001$ vs the control group, $\alpha\alpha\alpha$ $p < 0.001$ and $\alpha\alpha$ $p < 0.01$ vs ZM group; \$\$\$ $p < 0.001$ and \$\$ $p < 0.01$ and \$ $p < 0.05$ vs CP group. ZM; *Zataria multiflora*, CP; cisplatin, MDA; malondialdehyde, GSH; glutathione, PC; protein carbonyl.

2.3. Effects of ZM on Serum enzymes in CP treated mice

Levels of the serum marker enzymes of hepatic damage, AST, ALT and ALP were increased in the CP treated mice, which were statistically significant compared to the control group ($P < 0.001$). ZM administration in CP treated mice significantly decreased the level of these markers compared to the CP group Table 1.

2.4. Effect of ZM on histopathology of liver in CP treated mice

The photomicrographs of liver in the all groups were presented in Figure 2. Normal histoarchitecture of lobules in liver (hepatocytes, sinusoids and Kupffer cells) with normal hepatic lobules were observed in control group (Fig. 2. A). Structure of the liver in ZM treated mice was close to control group (Fig. 2. B). The sections of liver in CP treated group showed disorganization in lobules such as periportal leukocyte infiltration, dilation sinusoids, focal necrosis with pyknotic and enlarged nuclei, hepatocyte vacuolization, congestion and hemorrhage (Fig. 2. C, D and E). However, ZM administration in CP treated mice alleviated

the pathological changes compared CP treated group (Fig. 2. F). Liver injury's mean scores of all groups were showed in Figure 3. CP increased liver injury score. Score of liver injury in ZM+CP group were lower compared to CP group ($P < 0.05$).

Table 1: Effect of ZM on serum marker enzymes of the liver affected by CP-induced hepatotoxicity.

Groups	AST	ALT	ALP
Control	123.3 ± 18.77	50 ± 8.87	161.3 ± 9.53
ZM	120.3 ± 19.62	48.5 ± 6.25	157.3 ± 19.77
CP	245.3 ± 53.26 ^{a***b***}	93 ± 7.87 ^{a***b***}	317.8 ± 19.47 ^{a***b***}
ZM+CP	168.8 ± 20.76 ^{c*}	67.25 ± 6.65 ^{a*b*c**}	229.5 ± 23.53 ^{a***b***c***}

All values are expressed as mean ± SD. *a* significant vs control, *b* significant vs ZM; *c* vs the CP groups; *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$. ZM; *Zataria multiflora*, CP; cisplatin, ALT; alanine aminotransferase, AST; aspartate aminotransferase and ALP; alkaline phosphatase.

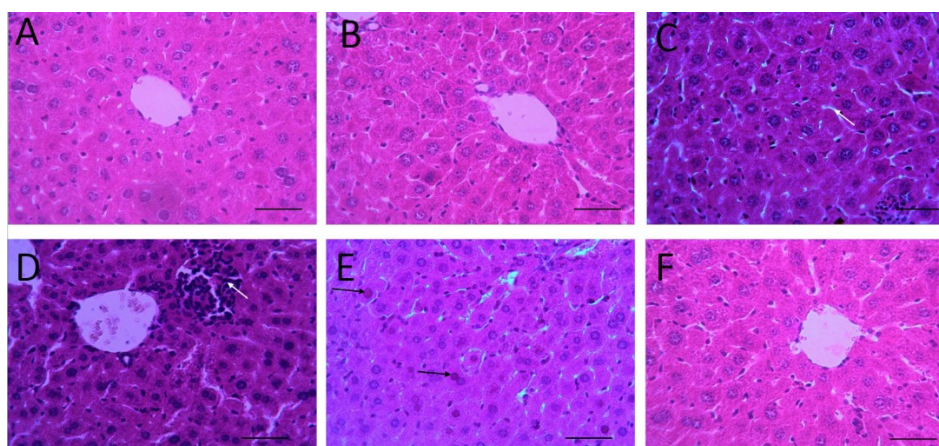


Figure 2. Photomicrographs show the effect of ZM and CP on the histological architecture of liver in mice. Control (A), ZM (B), CP (C, D and E) and ZM + CP (F) groups. Dilatation of sinusoids (C. white arrow), granulomatous formations (D. White arrow) and slightly cytoplasmic eosinophilic (E. black arrow) in CP group. (H & E staining. ×40, Scale bar = 100 μm. ZM; *Zataria multiflora*, CP; cisplatin.

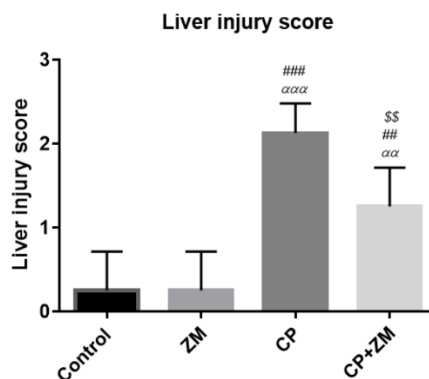


Figure 3. Histogram shows Liver injury score in liver tissue. Data are presented as Mean ± SD. ### $p < 0.001$ and ## $p < 0.01$ vs control group; and ZM groups; ## $p < 0.01$ vs the control and ZM groups; ααα $p < 0.001$ and αα $p < 0.01$ vs ZM group; \$\$ $p < 0.01$ vs the CP group. ZM; *Zataria multiflora*, CP; cisplatin.

2.5. Effect of ZM on immunoreactivity of caspase-3 in CP treated mice

Immunohistochemical photomicrographs of the liver were showed in Figure 4. Caspase-3 positive cells stained as brown color. Section of liver in the control group showed no caspase-3 immunoreactivity. Expression of caspase-3 was similar in the ZM and control groups. Increase immunoreactivity level of caspase-3 revealed in CP-treated mice. Immunoreactivity was showed in the hepatocytes especially around the central vein (Fig 4.A). Mild immunoreactivity staining of caspase-3 displayed in ZM + CP group (Fig 4.B) compared to CP alone group.

The histograms of the semi-quantitative analysis of caspase-3 staining in all groups are shown in Fig 5. The most intense immunoreactivity of caspase-3 was confirmed by semi-quantitative analysis in CP treated mice (21.85 ± 6.81) compared with the other groups ($p < 0.05$). ZM treatment decreased severity of immunoreactivity of Caspase-3 (10.66 ± 3.23). Immunoreactivity level of caspase-3 in the control group was similar to ZM group.

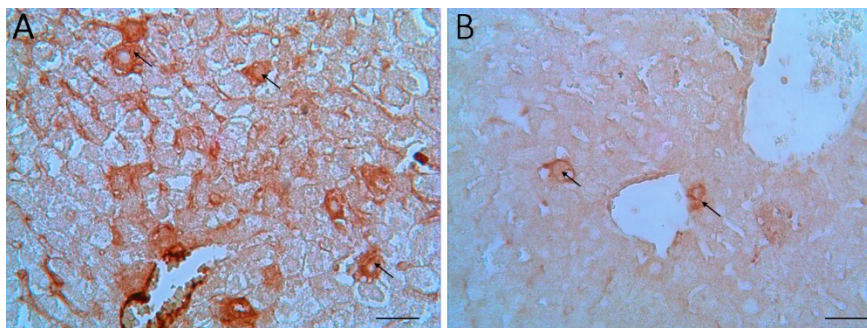


Figure 4. Photomicrographs show the effect of ZM and CP on immunoreactivity of caspase-3 in mice. Caspase-3 positive cells are shown with brown color. (A) Immunohistochemical staining demonstrated the caspase-3 immunoreactivity in CP group that were remarkable in hepatocytes of pericentral vein (black arrow). (B) ZM treatment diminished caspase-3 immunoreactivity in CP treated mice. $\times 400$, Scale bar = 100 μm , CP; cisplatin, ZM; *Zataria multiflora*.

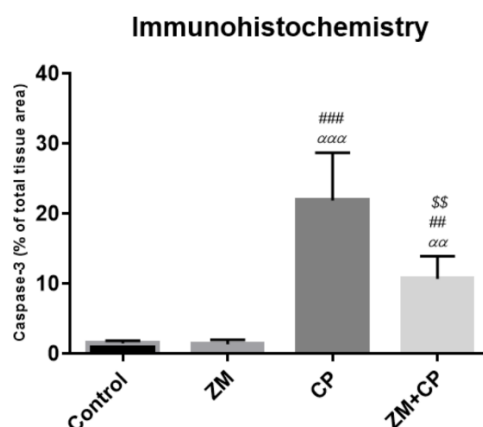


Figure 5. Histogram shows densitometry analysis of immunohistochemical staining for caspase-3. Data were presented as a percentage of total tissue area. ### $p < 0.001$ and ## $p < 0.01$ vs control group; ααα $p < 0.001$ and αα $p < 0.01$ vs ZM group; \$\$ $p < 0.01$ vs the CP group. ZM, CP; cisplatin, ZM; *Zataria multiflora*.

3. DISCUSSION

In the present study, cisplatin induced oxidative stress. Serum hepatic enzymes in CP treated mice markedly showed impaired liver function. Also, CP changes structure of liver tissue and increased immunoreactivity of caspase-3 in the hepatocytes. ZM could improve serum and tissue biochemical, histological and immunohistological parameters.

The present study demonstrated that CP considerably increased MDA, PC and decreased GSH in male mice. All these oxidative stress parameters (MDA and PC) were significantly mitigated with ZM treatment. Also, ZM were able to prevent reduction of GSH in CP-treated mice. Oxidative stress is one of the most important mechanisms in CP induced toxicity. CP with ROS production induced oxidative stress. ROS with damage of cellular components, such as lipids, proteins, and DNA is mediator of cell death [1, 8]. On the other hand, free radicals react with the membrane lipids and leads to lipid peroxidation [26, 27]. MDA, as the final product of lipid peroxidation, is a suitable marker for determining ROS level in an organ [28]. In this study, increase of MDA confirmed oxidative stress. PC is an indicator of protein carbonyl aggregation and protein oxidation. In this study, CP led to significant increase in PC content of hepatocyte cells [29]. In normal physiological conditions, generation and elimination of ROS is adjusted by an endogenous antioxidants such

as GSH, CAT and SOD. Reduction of PC contents in ZM + CP group confirmed inhibition protein oxidation in CP-treated mice that might have been via its antioxidant property. GSH, as a strong antioxidant with having SH-groups regulate inner mitochondrial membrane permeability and enzyme functional. In CP treatment, CP conjugate with sulfhydryl (SH) groups of GSH and decrease intracellular glutathione level that leads to mitochondrial injury and oxidative stress [9]. This finding was consistent with other that demonstrated that the ZM improve oxidative stress parameters in paracetamol-induced hepatotoxicity [30].

Our results showed that the administration of ZM significantly decreased lipid peroxidation as an indicator of oxidative stress in CP-treated mice. In previous studies showed that ZM effectively scavenged ROS and inhibited the production of oxidative damage induced by radiotherapy and CP [16, 31]. *Z. multiflora*, by having phenolic compounds and antioxidant property, scavenge free radicals and inhibit oxidative stress in body [32]. This study is consistent with other study that showed ZM with free radicals scavenging can inhibit lipid peroxidation [17]. Thymol and carvacrol as main biologically active components of ZM [21] alleviate cisplatin-induced nephrotoxicity [31, 33] and doxorubicin-induced cardiotoxicity [34]. ZM with having antioxidant [23], anti-inflammatory [35], and anticancer [36, 37] properties can be protect the body against chemotherapy. In most studies antioxidants are used as pre-treatment, post-treatment or combined treatment [38, 39]. In this study, ZM treated 200 mg/kg for 5 days before and 3 days after receiving of CP. In previous studies revealed that ZM have protective effect against Cyclophosphamide-induced genotoxicity and acetic acid-induced colitis [15, 17]. There is also evidence that ZM markedly reduced DNA damage induced by cyclophosphamide in mice bone marrow cells at optimum dose 200 mg/kg [17].

Histopathological changes in the liver were determined by light microscope. Parenchymal degeneration, central vein dilatation, congestion, sinusoidal dilatation, inflammatory cell infiltration, vacuolization in hepatocytes and biliary duct proliferation were revealed in the CP group. On the other hand, in this study, the serum parameters of oxidative stress in tissue damage increased in CP treated mice. Increased ALT, AST, ALP in CP treated mice proved hepatotoxicity. In the liver injury condition, leakage of cytosol transaminase enzymes occurs and those are released into blood [8]. The high level of the hepatic enzymes in the serum display the cellular leakage and integrity of cell membrane in hepatocytes [40]. Due to the high levels of oxidative stress markers, can be concluded that damage is due to oxidative stress. In ZM + CP, histoarchitecture of liver preserved. In this study, ZM mitigated side effects of CP treatment on serum parameters and significantly improved the liver functions. These data were evident that ZM protect liver against CP-treated. ZM by having strong antioxidant activity and scavenging of free radicals [25] can prevent of the damage caused by free radicals. It has been documented that ZM can improve structure of liver in cyclophosphamide-induced hepatotoxicity [41].

In the current study, results of immunohistochemical assay and caspase-3 positive cells proved significant apoptosis and DNA damage which was induced with CP in the hepatocyte cells. Our study demonstrated that pre and post treatment with ZM in CP- treated mice significantly decreased apoptosis of liver. In previous studies, was showed cisplatin induces DNA damage and genotoxicity, in vivo [42] and in vitro [43] conditions. Cisplatin with binding to nuclear DNA induces mitochondrial DNA damage [8]. Then, Release of activators from mitochondria into the cytosol, increased caspase-3 and caspase-9 [44]. El-sayaed *et al* showed thymol and carvacrol, as bioactive components of ZM decrease oxidative stress and inflammation [34]. On the other hand, antiapoptotic activities of these compounds by reducing TNF- α proved against nephrotoxicity induced CP [45]. While we have shown in this study for the first time that immunohistochemical findings revealed significantly increase in the expression of caspase-3 as apoptosis marker and ZM administration significantly decreased these up regulations.

4. CONCLUSION

Our study concludes that CP treatment could induce histopathological alternation, oxidative stress and apoptosis in the liver. Liver injury in cisplatin-treated mice was markedly mitigate by ZM extract. The protective effect of ZM was associated with decrease of oxidative stress, hepatic injury and caspase-3 expression. These findings proved that ZM has a hepatoprotective effect in cisplatin-treated mice via antioxidant and anti-apoptotic properties.

5. MATERIALS AND METHODS

5.1. Plant material and extraction

Dried aerial parts of ZM were collected in the flowering season from the city of Firozabad in the Fars province of Iran. Homogenous powder was macerated in 70% ethanol for 72 hours (1:10 w/v), after which the hydro alcoholic extract of dried ZM was processed by removing the solvent using a Rotary (Heidolph, Germany). The ZM extract was standardized based on thymol as the major active ingredient according to our previous reports [46, 47].

5.2. HPLC analysis

Analyses were developed by HPLC system, equipped with Eclipse DB-C18 column (5 μ m, 4.6 250 mm i.d.). A mobile phase was prepared consisting of acetonitrile: H₂O: acetic acid (65:34:1), at a flow rate of 1 ml/min. Detection was done using a diode array detector (DAD) at 274 nm and the chromatographic data analyses were done using Chrom Gate soft-ware. The filtered ZM extract was injected in HPLC system and chromatogram was recorded. In this mobile phase, the peaks of thymol and carvacrol were successfully separated in the ZM extract and pure peak of thymol was reached for quantification. Thymol standards were quantified by preparing in methanol solution, and serial dilutions were carried out by double-distilled water. Various standard concentrations of thymol were used into HPLC system to draw standard calibration curves. The amount of thymol in ZM extract expressed as micrograms per gram of dry extract weight (mg/g).

5.3. Animals

Thirty two adult male BALB/c mice (25 \pm 30 g) used in this study were obtained from the Institutional Animal Care and ethics Committee of the Medical science of Mazandaran University, Sari, Iran. For acclimatization period of one week to the Experiment environment, the animals were maintained under standard laboratory conditions of constant 12:12 dark/light cycle and 20°C–25°C for one week. They had free access to food and water during the study period.

5.4. Study design

In this study, the animals were randomly divided into 4 groups (8/ group): Group I (control); mice were received normal saline by gavage for 7 days. Group II (ZM); mice were received 200mg/kg hydroalcoholic extract of ZM daily by oral administration for 7 consecutive days. Group III (ZM+CP); on the 5 day, mice received a single intraperitoneal injection of CP (10 mg/kg). Group IV; mice received ZM 200 mg/kg for 7 days and CP 10 mg/kg on the 5 day. In ZM+CP group, ZM was administered one hour ago CP treated. The 8 day of the study, biochemical, histopathological and immunohistochemical assays were evaluated. The entry and exit criteria of this investigation were male mice, without receiving any medication before starting the study and the use of healthy mice without anatomical abnormality.

5.5. Specimen collections

One day after drug administration, the animals were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg). Blood samples were collected from the heart. Serum were separated for evaluating serum liver enzymes. Then animals were sacrificed and liver was immediately removed. For biochemical evaluation, part of the liver tissue washed with PBS, weighed and was freshly used. The other pieces fixed in 10% buffer formalin for histological and immunohistochemical assay.

5.6. Biochemical analysis

The concentrations of liver lipid peroxidation were measured by measure of MDA using the thiobarbituric acid with a spectrophotometric method. MDA content was expressed as nmol/mg protein. Tetramethoxypropane (TEP) was used in this experiment as standard.

The protein carbonyl were assessed spectrophotometrically by reading the absorption at a wavelength of 365nm with an absorption coefficient of 22,000 M⁻¹ cm⁻¹ was expressed as mM.

Content of the glutathione in the samples was determined by spectrophotometer with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as an indicator at 412 nm and expressed as μ M.

5.7. Serum markers of liver damage

The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined using Pars Azmoon kits, Iran.

5.8. Histopathological assay

For histopathological assay and to determine the effect of CP and ZM on liver, samples were fixed in 10% (w/v) buffer formalin for 24 hours. After processing and embedding in paraffin using standard protocol, sections with 5µm thickness stained with hematoxyline and eosin (H & E) for evaluation of liver damage. Sample sections were evaluated using 40× magnification for assessment of the degree of liver injury by a histologist that was blinded to the treatment groups. For the quantitative analysis, histological photomicrographs were evaluated by scoring system. According to extent of sinusoidal dilatation, inflammatory cell infiltration, congestion, degeneration and cytoplasmic vacuolization scored as 0 (normal), 1 (mild), 2 (moderate), or 3 (severe).

5.9. Immunohistochemical assay

Caspase 3, as one of the key markers of apoptosis was evaluated with immunohistochemistry. Immunohistochemical technique was performed according to the instructions kit manufacturer (Abcam Company, USA). At first, serial sections of tissues were mounted on polylysine-coated slides, then were deparaffinized and rehydrated in alcohol series. After that, they were incubated by 0.3% H₂O₂ in MeOH is better to block endogenous peroxidase activity for 15 minutes. Then, the tissue sections were incubated with protein blocker for 10 minutes. After incubation at 4°C overnight with primary antibodies (anti-caspase 3 rabbit polyclonal antibody, 1:100 in PBS, v/v, Abcam, Lat: GR224831-2) at 4°C overnight, serial sections of tissues were incubated with secondary antibody conjugated with horseradish peroxidase (Mouse and Rabbit Specific HRP/DAB, Abcam, Lat: GR2623314-4) for 20 minutes, sections were incubated with diaminobenzidine tetrahydrochloride for 5 minutes [48]. Then the slides were rehydrated in alcohol series and dehydrated in xylene. Finally, all the slides were assessed under light microscope with magnification of ×40. For the quantitative analysis, immunohistochemical photomicrographs were assessed using MacBiophotonics ImageJ 1.41a software by densitometry method. The positive staining severity was assessed as the ratio of the stained area to the entire field assessment.

5.10. Statistical analysis

Statistical data analysis was done using SPSS 19 version (Chicago, USA). All of the data are presented as mean ± SD. Different groups were compared with each other by using One-Way ANOVA followed by the Post hoc Tukey test. $p < 0.05$ was considered statistically significant.

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