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Histopathological and biomedical parameters determination in the protective effect of crocin on hepatotoxicity induced by methotrexate in rats

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ABSTRACT

Introduction: Methotrexate (Met) as a chemotherapy drug has many side effects, such as infiltration of neutrophils and development of oxidative stress. Crocin (Cro), a carotenoid isolated from saffron, has numerous therapeutic characteristics including anticancer and antioxidant activities. This study was designed to evaluate the effects of Cro against hepatic damage in rats induced by Met.

Methods: In this study, 48 male Wistar rats were randomly assigned into 8 groups, control normal (saline), Met control-treated group (20 mg/kg), Cro groups (12.5, 25, 50 mg/kg) and Met + Cro treated groups (12.5, 25, 50 mg/kg). Treatments were administered by intraperitoneal injection daily for 28 days. Griess technique was hired for the determination of serum nitrite oxide (NO) level. Concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were determined in order to assess liver function disturbances. In addition, Thiobarbituric acid reactive species, antioxidant capacity, diameter of hepatocytes and central hepatic vein (CHV) were investigated.

Results: Met administration significantly increased the liver malondialdehyde (MDA) and NO level, the mean diameter of CHV, hepatocytes and hepatic enzymes. Met also decreased the tissue FRAP level compared to the normal control group ($P < 0.01$). The Cro and Cro + Met treatments in all doses significantly reduced the mean diameter of hepatocytes and CHV, hepatic enzymes, hepatic MDA and NO levels and increased the tissue FRAP level compared to the Met control group ($P < 0.01$).

Conclusion: It seems that Cro administration improves liver injury induced by Met in rats.

Introduction

By industrial revolution in human societies, the number of cancer incidences has increased considerably and subsequent use of anti-cancer drugs has found great applications (1). Anti-cancer drugs increase the longevity of many cancer patients, but apart from the therapeutic and beneficial effects, they may leave adverse effects on other tissues over time (2).

Methotrexate (Met) is an important anti-cancer compound with therapeutic applications (3). It is widely used as a chemotherapeutic agent in treatment of serious forms of malignant tumors including leukemia, lymphoma, breast and uterine cancers, as well as non-cancerous diseases such as rheumatoid arthritis and skin conditions (4). In clinic, it is proved that Met is a therapeutic option in the treatment of some cancers, yet various side effects have been reported. Met can accumulate in different tissues especially gall-bladder, liver and kidneys (5). It can be a potential hepatotoxic agent due to the formation of active oxygen radicals (6). Met is a folic acid synthesis inhibitor

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and causes impaired DNA synthesis and cell replications by inhibition of purines and pyrimidines synthesis (7). As one of the first anti-neoplastic drugs, Met was released to the market as a folate antagonist (8). It seems that the Met increases the development of free radicals and direct damage to renal tubules through oxidative stress accumulation (9). Lipid peroxidation is one of the important factors involved in oxidative stress formation in the cellular membrane, lipoproteins, and other lipid structures. Lipid peroxidation leads to production of peroxides and hydroxides, causing toxicity in the body and reduction in the level of antioxidant enzymes (10).

Saffron is a small and perennial plant belonging to the iris family. The applicable parts of this plant include style and three-branched stigma, known as saffron. The geographical distribution of saffron is mostly Iran and other countries around the Mediterranean Sea (11). In addition to being a highly consumed food flavoring, saffron has also various pharmacological effects (12). It has potentially anti-genotoxic and cancer-preventive effects which can be used alongside the chemotherapy drugs (13,14).

Crocin (Cro) is a glycoside which is composed of some types of carotenoids called crocetin and sugar, responsible for the color of saffron. Other carotenoids such as beta-carotene, lycopene, zeaxanthin, and vitamins especially riboflavin and thiamine are found in saffron. Cro is the active ingredient in saffron (15) owns free radical scavenging and antioxidant nature (16).

Considering the toxic effect of Met and, on the contrary, the beneficial effects of Cro especially anti-oxidant property, and since so far no study has been conducted on the effects of Cro related to the hepatic damage caused by Met, the present study was designed to investigate the inhibitory effects of Cro in hepatic injuries induced by Met in male Wistar rats.

Materials and Methods
Animals
Animal manipulation was conducted according to the guidelines of care and handling of animals prepared by Iran's Ministry of Health. Forty-eight male Wistar rats (220-250 g) were purchased from the Pasteur Institute (Tehran, Iran). According to the animal care facilities they were housed in routine keeping conditions including standard cages (n=6 per cage), 23 ± 2°C ambient temperature, and exposed to 12-12 h light/dark cycle. The animals had free access to water and food throughout the experimental period (17).

Groups and treatment
The rats were randomly divided into 8 groups (n=6 in each). The first group as normal control group received normal saline (through intraperitoneal injection) equivalent to the amount of experimental groups. Second group, as the control group of Met, received 20 mg/kg a single dose of Met (with normal saline as solvent), intraperitoneally once a day for 28 consecutive days. Third to fifth groups, the Cro administration groups, each animal in these groups respectively received 12.5, 25 and 50 mg/kg of Cro intraperitoneally, for 14 days at about 10 o'clock. Sixth to eight groups, Met + Cro administration groups, each animal received a single dose of 20 mg/kg Met in order to induce hepatic damage, which respectively followed by reception of 12.5, 25 and 50 mg/kg of Cro intraperitoneally for 28 days, at about 10 o'clock (11, 18).

Dissection and sampling
Twenty-four hours after the last injection, each group of rats was placed into a plastic box containing a packet of cotton impregnated with Ether, in order to kill the animals. The venipuncture procedure from the right ventricle of animals' heart was done by a 5 mL syringe. In order to form blood clot, the blood sample was incubated for 15 minutes at 37°C. Then, it was centrifuged (3000 rpm) for 15 minutes until the serum was separated. The resultant serum was stored in a -70°C refrigerator as stock for measuring the levels of liver biochemical parameters, nitric oxide, oxidative stress, and antioxidant capacity, Then, at the last stage of dissecting process, the liver was removed and fixed in 10% formalin solution for histological and morphometric investigations (15).

Oxidative stress assessment
In order to assess the oxidative stress level, thiobarbituric acid reactive species were measured using colorimetric analyze of malondialdehyde (MDA) as the last product of lipid peroxidation in the liver. In brief, 1400 μL of three following solutions were prepared separately including acetic acid, TBA, and sodium dodecyl sulfate (all from Sigma, USA) and were added to 100 μL of liver homogenate. The mixture was animated for 50 minutes. Four milliliters of 1-butanol (Sigma, USA) was added to the previous combination and vortexed for 15 minutes through centrifugation (5000 rpm). The absorbance of the higher layer was measured at 532 nm (Spectro, Germany) and sequential concentrations of tetraethoxypropane (Sigma, USA) were used as the external standard. The antioxidant capacity of the liver was measured using FRAP assay. The FRAP substance consisted of 30 mL of acetate buffer and 1.5 mL of chloride ferric (all from Sigma, USA). Briefly, 60 μL of liver homogenate was added to 1.5 ml of newly prepared solution. FRAP substance (Sigma, USA) was added to a test tube and incubated at 37°C for 10 minutes. The absorbance of the blue-colored complex was read at 593 nm. Sequential concentrations of $FeSO_4.7H_2O$ (Sigma, USA) were used as external standards (19).

Enzymes
The liver was split and turned into a homogenate solution.

To separate the biological enzymes, the obtained solution was centrifuged twice at 10 000 rpm for 15 minutes. The supernatant was separated in order to measure the enzymes activities. The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were examined by the method of Reitman and Frankel. The alkaline phosphatase (ALP) protocols were determined according to the procedure set out in the practical laboratory manual (11).

Nitrite oxide assay

The nitrite oxide (NO) level was measured by Griess assay using microplate technique. For this purpose, to measure the concentration of nitrite available in the serum, the supernatant (400 μL) of serum sample was deproteinized by zinc sulfate (6 mg zinc sulfate powder was mixed with 400 μL serum and vortexed for 1 minute). The supernatant (100 μL) was separated and 100 μL of vanadium chloride, 50 μL of N-(1-naphthyl) ethylenediamine dihydrochloride (NEED) and 50 μL of sulfonamide solutions were added to the supernatant. Sodium nitrite (0.1 M) was used for the standard curve, and increasing concentrations of sodium nitrite (5, 10, 25, 50, 75, and 100 μM) were prepared. The Griess solution was added to all microplates containing sodium nitrite and supernatant which finally was read through an ELISA reader (stat fax100, USA) at the wavelength of 540 nm (17).

Morphological and histopathological examinations

In order to evaluate the histological changes of the liver, the inferior 1-cm-long part of the right lobe of the liver was removed in the transverse section, washed in saline, and fixed in 10% formalin. In the next step, the tissue embedded in paraffin and the sections were cut (4 μm) using a microtome (Leica RM 2125, Germany). Thin sections were subjected to tissue processing including, clarification in xylene, dehydration in ascending concentrations of ethanol and then stained with hematoxylin and eosin. For each hepatocyte, the full cellular area was measured. The hepatocyte outline was measured after capturing an image with a 40× objective lens. The maximum and minimum axis was measured for each hepatocyte in order to measure the mean axis. At least 50 hepatocytes from each zone were measured in each liver sample. A separate measurement for central hepatic vein (CHV) was performed using the same assay. The planning was examined with an Olympus BX-51T-32E01 research microscope connected to a DP12 camera (3.34-million pixel resolution) and Olysia Bio-software (Olympus Optical Co. LTD, Tokyo, Japan) (10).

Statistical analysis

After data acquisition, the Kolmogorov–Smirnov test was first conducted to confirm the data compliance of the normal distribution. One-way analysis of variance (one-way ANOVA) was used for statistical analysis and Tukey post hoc test was used to determine the differences among the groups. SPSS 16 was used for data analysis, and the results were expressed as mean ± standard error, and $P < 0.05$ was considered as significant.

Results

Oxidative stress

The liver MDA level significantly increased in the Met control group compared to the normal control group ($P < 0.01$). The liver MDA level decreased significantly in all Met + Cro groups compared to the Met control group ($P < 0.01$). Similarly, the Met significantly reduced the level of liver FRAP in Met control group in comparison with normal control group ($P < 0.01$). Administration of Cro significantly increased the FRAP level in the liver in all Met + Cro groups compared to the Met control group ($P < 0.01$). In all groups, treatment with Cro caused no significant alteration in the liver FRAPS and liver MDA levels compared to the normal control group ($P > 0.05$) (Figure 1).
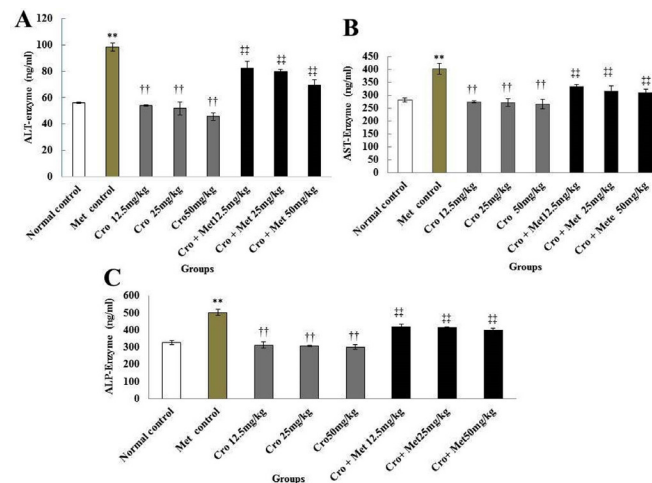
Liver enzymes

The Met led to a significant increase in the levels of ALT, AST and ALP enzymes in comparison with the normal control group ($P < 0.01$). The mean concentration of ALT, AST and ALP enzymes showed no significant differences in all Cro groups compared to the normal control group ($P > 0.05$). Also, all doses of Cro and Met + Cro presented a significant decrease in the mean concentration of ALT, AST and ALP enzymes in comparison with the Met control group ($P < 0.01$) (Figure 2).

Nitric oxide

The results of blood serum NO measurement showed a significant increase in Met control group compared to control normal group ($P < 0.01$). The mean NO concentration extracted from blood serum displayed no
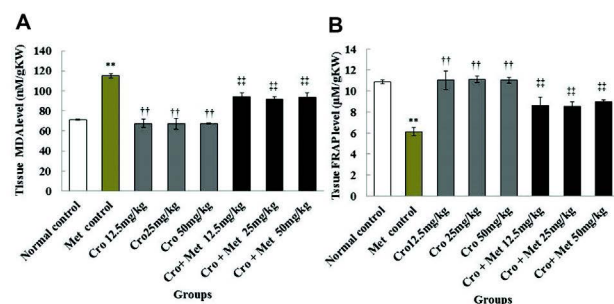
Figure 1. Effects of Crocin (Cro) and Crocin + Methotrexate (Met) on the tissue ferric reducing ability of plasma (FRAP) level (A). and liver MDA level (B). (**A**) tissue malondialdehyde (MDA) level; (**B**) tissue ferric reducing ability of plasma (FRAP) level. **Significant difference compared to control normal group ($P < 0.01$). ††Significant difference compared to Met control group ($P < 0.01$). ‡‡Significant difference compared to Met control group ($P < 0.01$). Crocin: Cro, Methotrexate: Met, Malondialdehyde: MDA, Ferric reducing the ability of plasma: FRAP.
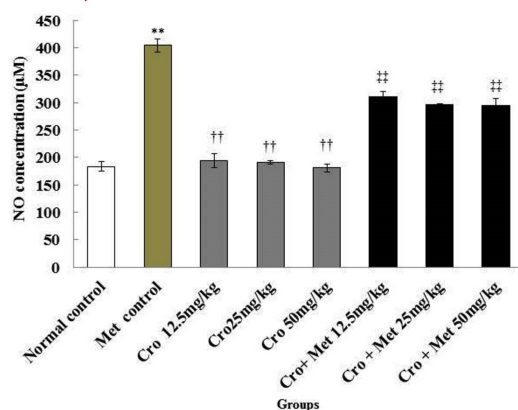
Figure 2. Effect of Methotrexate (Met), Crocin (Cro) and Cro + (Met) administration on liver enzymes of rats. (**A**) Alanine Aminotransferase (ALT) enzyme, (**B**) Aspartate Aminotransferase (AST) enzyme and (**C**) Alkaline Phosphatase (ALP) enzyme. **Significant difference compared to the normal control group ($P < 0.01$). ††Significant difference compared to Met control group ($P < 0.01$). ‡‡Significant difference compared to Met control groups ($P < 0.01$).

significant variations in all Cro groups compared to the normal control group ($P > 0.05$). Also, the mean NO in blood serum significantly reduced in Cro and Met + Cro in all doses compared to the Met control group ($P < 0.01$) (Figure 3).

Morphometric analysis

The mean diameter of hepatocytes and the CHV in experimental groups showed a significant increase among the normal and Met control groups ($P < 0.01$). No significant variations were detected in the mean diameter of hepatocytes and the CHV in all Cro groups compared to the normal control group ($P > 0.05$). Furthermore, the Cro and Met + Cro significantly reduced the mean diameter of hepatocytes and CHV in all treated groups in comparison with Met control group ($P < 0.01$) (Figure 4).

Figure 3. Effects of Crocin (Cro), Methotrexate (Met) and Cro + Met on mean levels of nitrite oxide (No). **Significant difference compared to the normal control group ($P < 0.01$). ††Significant difference compared to Met control group ($P < 0.01$). ‡‡Significant difference compared to Met control group ($P < 0.01$).

Histopathological changes

Histological analysis showed liver structure changes in normal control and Cro treatment groups. After treatment with Met in Met control group, the liver showed evident changes and injuries. These anomalies included increase in white blood cells (inflammation), increase in irregularities, sinusoidal space dilatation, and vacuolization of hepatocytes (necrosis). Treatment with Met + Cro in all doses reduced the hepatic toxicity caused by Met administration (Figure 5).

Discussion

The present study was designed to discover whether the Cro as an anti-oxidant operant could influence the pharmacological effects of Met in order to alleviate the Met-related hepatocyte damages. In addition to anti-tumor effects, Met also has anti-inflammatory, immunomodulatory, and therapeutic properties in different doses. Despite the effective clinical properties, Met develops various side effects due to the formation of active oxygen radicals (20). The results of the present study indicated a significant increase in the diameter of hepatocytes and the size of the central vein in the control group receiving Met compared to the normal control group. Furthermore, all groups receiving Met + Cro, showed significant reduction in central vein size and diameter of hepatocytes compared to the control Met-receiving group.

In this study, some changes were observed in the liver tissue of the control Met group characterized by hyper-perfusion in sinusoidal spaces, aggregation of inflammatory cells (macrophages) around the central veins, increased size of central vein and permeation of lymphoid cells in the portal space. Macrophages, i.e. Kupffer cells existed in sinusoids of the liver can be activated in response to tissue injuries and liberate toxic intermediates. Among these
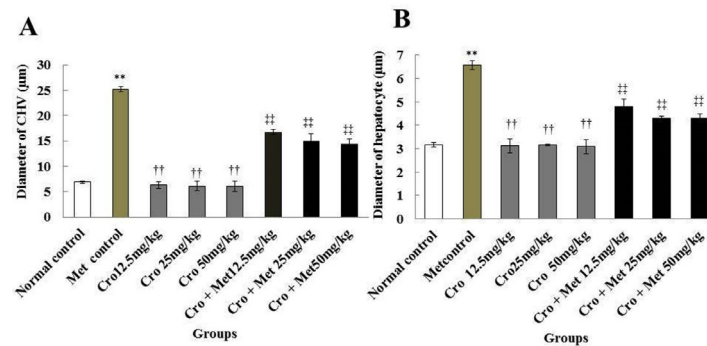
Figure 4. Effect of Methotrexate (Met), Crocin (Cro) and Cro + Met administration on the central hepatic vein (CHV) (**A**) and diameter of hepatocyte (**B**). **Significant difference compared to the normal control group ($P < 0.01$). ††Significant difference compared to the Met control groups ($P < 0.01$). ‡‡Significant difference compared to Met control group ($P < 0.01$).

highly toxic and active intermediates, there are also tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and NO. Aggregation of aforementioned cells and the secretion of toxic intermediates in some regions with no necrosis damage can induce the development of hepatotoxicity and necrosis of hepatocytes (21).
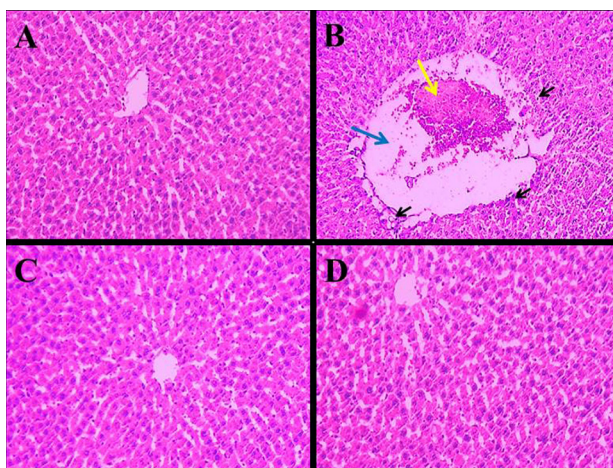
It seems that the lipid peroxidation as Met-related effects causes the production of ROS and free radicals which lead to altered enzymatic activity and eventually cellular damage and necrosis. This phenomenon occurs by the onset of ROS to unsaturated fatty acids, alkylation of proteins and other cellular macromolecules (6). Indeed, it seems that free radicals attack to hepatocytes and induce necrosis process in parenchymal cells. These cells can trigger hepatic inflammatory responses and cause infiltration of mononuclear inflammatory cells into the

Figure 5. Microscopic images of liver tissue in different groups (4 μm thick sections, H&E staining, magnification of 100×). Micrograph of the liver section in the control of normal groups (**A**), normal liver structure. Micrograph of the liver section of Met control group (**B**), increased white blood and macrophage cells (Inflammation) (black arrows), and Central hepatic vein (CVH) dilatation (blue arrow) and hyperemia (yellow arrow), due to the oxidative stress caused by Met. Micrograph of the liver section in Cro group (20 mg/kg) (**C**), normal liver structure. Micrograph of liver section in Cro + Met (20 mg/kg) group (**D**), normal liver structure. Crocin: Cro, Methotrexate: Met.

damaged tissue. In turn, the necrotic cells release pro-inflammatory intermediates and induce hepatic damage (10). The results of a study by Mahmoud et al are in line with the present study which showed that administration of Met caused breakdown and necrosis of hepatocytes, hyper-perfusion of sinusoidal spaces, and significant increase in the expression levels of TNF-α (22).

Met can indirectly cause mitochondrial damage, discharge of the mitochondrial enzymes, and diminished anti-oxidant activity (23). Indeed, the mitochondrion is a primary intracellular target for onset of oxidative stress and increased ROS/RNS production in mitochondrial damage (11). The results of the current study also showed that Cro was able to reduce lipid peroxidation (decreased MDA) and increase anti-oxidant capacity (increased FRAP) of liver tissue, reducing the oxidative stress.

Consistent with these findings, a large body of studies have shown anti-oxidant properties for Cro (11,15,17). Thus, it appears that Cro with its anti-oxidant properties could reduce MDA level and increase FRAP level by inhibition of ROS generation. In addition, it seems that an increase in the diameter of the hepatic central vein is related to the necrosis of hepatocytes and breakdown of the lipid in the liver in response to the Met administration (24). By prevention of lipids and proteins peroxidation, the Cro can inhibit reduction of glutathione content and modify the activity of antioxidant enzymes. In other words, elevation of antioxidant capacity alleviates the injuries which finally cause the cells to cope with the oxidative stress conditions (17). It also seems that by regulation of inflammatory pathways and inhibition of apoptosis process, Cro can act as cellular protective agent (25).

The results of a study by Salahshoor et al confirmed the results of the present study, suggesting that administration of Cro in mice treated with morphine can cause a significant reduction in the size of central vein and diameter of hepatocytes (11). Met can cause induction of oxidative stress condition, nitrosative stress and damage to cells through enhancing the expression of NG-Kβ and

P38 pathways (26).

The results obtained from a study by Lari et al indicated that administration of Cro against diazinon-induced toxicity in male Wistar rats led to a significant reduction in MDA levels, confirming the results of the present study (27). The results of this investigation indicated a significant increase in levels of AST, ALT, and ALP enzymes in control Met-receiving group compared to normal control group. Furthermore, across all groups receiving Cro + Met, a significant reduction was observed in ALP, ALT, and AST levels compared to the control group which received Met alone. Incidence of necrosis or cell-membrane damage of hepatocytes causes release of these enzymes into the bloodstream (10). It seems that Met causes liberation of receptors as speckled droplet through down-regulation of RAR-α, where these receptors can cause induction of hepatic damage (28). It seems that Cro causes stability of cellular membranes through prevention of lipid peroxidation and enzymatic leakage (17).

The results of a study by Mohajeri et al showed that the treatment with Cro significantly reduced the levels of cell damage markers of liver tissue present in the serum, which is in line with the results of the present study (29).

Cro can cause development of cellular protection and anti-apoptosis effects by scavenging the ROS through endoplasmic reticulum (11). The results of the present study showed that the level of NO present in blood serum of the control Met-receiving group could grow significantly when compared to the normal control group. Furthermore, in the group receiving Cro + Met, a significant reduction was observed in the serum level of NO in comparison with the nitride oxide level in the blood serum of the Met control group. These results confirm antioxidant and anti-inflammatory effects of Cro.

NO is a type of free radical produced in mammalian cells which interfere with the regulation of physiological processes. Elevation of NO production can be associated with the development of different diseases (15). The hydroxyl radicals produced by NO and superoxide anion can cause interference with the process of pathogenesis and hepatotoxicity (17). It seems that the reduction of NO production occurs through under-expression of iNOS (30). Antioxidants have the capacity to remove free radicals (31). Antioxidants can cause diminished NO production by damage induction to the nitride oxide system (protein enzymes, substrates, and cofactors) (10). The results of a study by Kim et al is in line with the results of the present research, suggesting that Cro causes inhibition of NO-inducing lipopolysaccharide through inducing expression of HO-1 and Calmodulin-calcium-dependent kinase-4 protein (32).

Based on the results obtained from the present study, it can be stated that administration of Cro as a potent antioxidant factor on animals with a healthy liver as well as in groups under Met treatment according to the studied doses can own positive effects on function and structure of hepatocytes. Furthermore, the results of the present study showed that the antioxidant properties of Cro can cause a diminished level of hepatic enzymes, enhanced antioxidant capacity, and diminished nitric oxide levels. It can exert its protective effect against MET-induced hepatic injuries through different mechanisms.

Conclusion

It appears that the Cro provides the hepatoprotective effects against Met-based oxidative stress which may be due to its strong potential antioxidant attributes. Cro administration moderates the activity of detoxification enzymes and antioxidant agents. Cro is involved in hepatic tissue recovery and prevention of Met adverse effects on liver enzymes as evidenced in the abovementioned examination of the male Wistar rats.

Authors' contribution

CJ conceived and designed the experiments; CJ and SR revised the manuscript; SR and AA analyzed the data; MRS wrote the paper. All authors read and confirmed the publication of the article.

Conflict of interests

The authors declare that there is no conflict of interest.

Ethical considerations

All investigations conformed to the ethical of research and were approved by the Ethics Committee of Kermanshah University of Medical Sciences (ethics certificate No.1396.562)

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