



Ameliorative Effects of Hydroalcoholic Extract of *Lavandula officinalis* L. on Methotrexate-Induced Oxidative Stress in Rats

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Article Info

Article History:

Received: 18 October 2016
Accepted: 31 December 2016
ePublished: 30 March 2017

Keywords:

-Methotrexate
-Oxidative Stress
-*Lavandula officinalis*
-Rat

ABSTRACT

Background: Methotrexate as a chemotherapy drug can cause chronic liver damage and oxidative stress. The aim of this study was to evaluate the protective effect of hydroalcoholic extract of *Lavandula officinalis* on methotrexate-induced oxidative stress in rats.

Methods: In this experimental study, thirty five Wistar male rats weighing 200-250 g were randomly divided into 5 groups (n = 7 in each group). Negative control group (normal saline 5ml/kg); positive control group received normal saline orally for 10 days, and a single dose of methotrexate (MTX, 20mg/kg, i.p.) was administered on the 9th day. Groups 3-5 received respectively 100, 200 and 400 mg/kg of *Lavandula officinalis* extract (LOE) orally for 10 days, and a single dose of MTX was injected on the 9th day. 24 h after the last injection, animals were sacrificed. Blood samples were collected to determine serum AST, ALT and ALP levels. Malondialdehyde (MDA), glutathione (GSH) levels and catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity were assayed in liver tissue. A portion of liver was maintained in 10% formalin for Hematoxylin and Eosin (H&E) staining and histological examination.

Results: The result obtained from current study was showed a significant increase in the levels of AST, ALT, ALP, MDA and decrease of GSH, CAT and SOD by MTX administration. Pre-treatment with LOE showed reduction in the levels of AST, ALT, ALP, MDA and increase of GSH, CAT and SOD in all doses but the most significant alteration was observed in doses of 200 and 400 mg/kg (P<0.05). Histological results showed that methotrexate could lead to liver damage. Also the hepatoprotective effect of the LOE was confirmed by the histological examination of the liver.

Conclusion: Our results indicate that hydroalcoholic extract of *Lavandula officinalis* have produced amelioration in biochemical and oxidative stress parameters against MTX -induced oxidative stress.

Introduction

Nowadays, herbal remedies such as the use of supplements and plants extracts rich in polyphenols, especially flavonoids and phenolic acids is usual throughout the world. Because it has been shown that phenolic compounds have protective effects against reactive oxygen species (ROS) and also improved the body's antioxidant

system.¹

Lavender (*Lavanda*) with scientific name of *Lavandula officinalis*, commonly known in Iran as "Ostokhoddous", is a medicinal plant largely used and included for centuries in the pharmacopoeia of several countries, including Iran.² The chemical composition and pharmacological evaluation of *Lavandula angustifolia* have been the subject of

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several studies over the years.³ Lavender is comprised of over 100 constituents, among which the primary components are linalool and linalyl acetate, α -pinene, limonene, 1,8-cineole, cis- and trans-ocimene, 3-octanone, camphor, caryophyllene, terpinen-4-ol.⁴

Among the other components of the plant can be cited tannins, coumarin, flavonoids and phytosterols.

Many of its applications in Iranian traditional medicine in the treatment of many diseases have been proven. Researchers have reported multiple health properties of this plant, such as, anti-anxiety, antidepressant, anti-inflammatory, antispasmodic, analgesic, antibacterial, antiparasitic, antiviral, sedative and antioxidant.⁵⁻⁷

Kalantar *et al.* showed that hydroalcoholic extract of *Lavandula officinalis* (100, 200 and 400 mg/kg for 10 consecutive days) reduces biochemical indices and oxidative stress parameters against gentamicin-induced nephrotoxicity.⁸

HajHashemi and colleagues examined and demonstrated the anti-inflammatory effect of this extract and polyphenols, as well as essential oil.³

Methotrexate (MTX) is a folate antagonist first used to cure some types of cancer. It was used at higher doses for curing cancer and two decades ago it is used at much lower doses to cure rheumatic illnesses according to studies MTX has a potential to induce renal toxicity, neurotoxicity, hematologic toxicity, pulmonary toxicity, cutaneous toxicity and gastro-intestinal toxicity.⁹ Hepatotoxicity is the most serious side effect of MTX. Hepatotoxicity of MTX seems to relate to the generation of reactive oxygen species¹⁰. The straight toxic effect of MTX; inhibition of various enzymes relating to DNA synthesis increase of the generation of ROS.^{10,11} Moreover, MTX inhibits cytosolic NADP-dependent dehydrogenase and NADP malic enzyme and makes a reduction in intracellular NADPH amounts. NADPH is necessary for glutathione reductase enzyme that maintains the levels of reduced glutathione (GSH), which is a substantial cytosolic antioxidant substance. Thus, the reduction in the rates of GSH due to MTX leads to a weakening of the affectivity of the antioxidant defense system protecting the cell in opposition to ROS.¹⁰

Since Lavender plant has a powerful antioxidant and anti-inflammatory effects, the protective effect of the extract of this plant in MTX-induced liver oxidative stress checked out in this study, as if to show the preventive effects of this plant, simultaneous use of daily specified amount of Lavender and MT can be recommended, to prevent the hepatotoxicity complications caused by MTX.

Materials and Methods

Chemicals

5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), trichloro acetic acid (TCA), 1,1,3,3-tetraethoxypropane (TEP), 2-thiobarbituric acid (TBA), methotrexate (MTX), Bovine Serum Albumin (BSA) and Bradford reagent were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO), USA. All other chemicals were of analytical grade and prepared from Merck Company (Darmstadt, Germany).

Animals

Adult male Wistar albino rats weighing 200–250 g were obtained from animal house of Ahvaz Jundishapur University of Medical Science, Iran. Rats were kept in polypropylene cages and given standard rat chow and drinking water ad libitum. The animals were maintained at a controlled condition of temperature ($20 \pm 2^\circ\text{C}$) with a 12 h light: 12 h dark cycle. Animal were acclimated to the environment for a minimum of one week prior to inclusion in experiment. The investigation was performed according to the Animal Ethics Committee Guidelines for the use of experimental animals.

Extract preparation

Lavandula officinalis was collected from Shiraz, Iran (Figure 1). Samples of the plant were identified by botanist from the Division of Pharmacognosy, Ahvaz Jundishapur University of Medical Sciences. The plant material was shade-dried, powdered and soaked in a 70% aqueous-ethanol solution in a large container for 3 days with occasional well shaking.¹² The solvent was filtered through a whatman paper and then was removed under vacuum in a rotary evaporator until dryness.¹³ The percentage yield was 16% for dried hydroalcoholic extract (w/w).



Figure 1. *Lavandula officinalis*.

Experimental design

In this experimental study, male Wistar rats were randomly divided into five groups, each group consisting of seven rats. Control group (normal saline 5ml/kg); MTX group received normal saline orally for 10 days, and a single dose of MTX (20mg/kg, i.p.) on the 9th day.¹⁴ Groups 3-5 received respectively 100, 200 and 400 mg/kg of LOE orally for 10 days,⁸ and a single dose of MTX (20mg/kg, i.p.) on the 9th day.

Sample Collection

After 24 h of last administration, the animals were anaesthetized with a combination of ketamine/xylazine (60/6 mg/kg, i.p.) and blood samples were collected from the jugular vein. Serum was separated by centrifugation for 10 min at 3000 rpm and stored at -20 °C until analysis. Then animals were sacrificed by decapitation and liver was isolated, washed with saline quickly. For histological studies, a part of this tissue was fixed in 10% phosphate buffered formalin.

For biochemical estimations the second part of tissue was homogenized (1/10 w/v) in ice-cold Tris-HCl buffer (0.1M, pH 7.4). Protein content in homogenates was measured by the method of Bradford,¹⁵ using crystalline BSA as standard.

Serum Biochemical Parameters

The most commonly used markers of liver injury are aspartate aminotransferase (AST, formerly serum glutamic-oxaloacetic transaminase [SGOT]), alanine aminotransferase (ALT, formerly serum glutamate-pyruvate transaminase [SGPT]) and alkaline phosphatase (ALP).

Colorimetric determination ALT or AST was estimated by measuring the amount of pyruvate or oxaloacetate produced by the formation of 2, 4-dinitrophenylhydrazine according to the method of Reitman and Frankel¹⁶ ALP was assayed using kits provided by Randox Laboratories Co; according to the methods described by Belfield and Goldberg.¹⁷

Tissue Biochemical Parameters

GSH level assay

The levels of GSH in the tissue homogenate were measured following the method described by Ellman¹⁸ based on the formation of a yellow colored complex with Ellman's Reagent (DTNB). Briefly, 2 ml Tris-EDTA buffer (pH = 8.6) was added to 40 µL homogenate in 2 ml cuvettes and then, 40 µL DTNB reagent (10 mM in methanol) was added to the mixture. The reaction mixture was incubated at room temperature for 20 min and the yellow color developed was read at 412 nm using a spectrophotometer (UV-1650 PC, Shimadzu, Japan). The standard curve was constructed over the concentration range of 1–10 µM of GSH. Results were expressed as nmol/mg protein.

MDA level assay

The lipid peroxidation was expressed by measuring the amounts of malondialdehyde via the TBA color reaction by the method described by Buege and Aust.¹⁹ Briefly, 0.5 ml of tissue homogenate was mixed with 2.5 ml of TCA (10%, w/v), the samples were centrifuged at 3000 rpm for 10 min and 2 ml of each sample supernatant was transferred to a test tube containing 1 ml of TBA solution (0.67%, w/v). The mixture was kept in boiling water for 10 min, forming a pink color solution. The mixture was then cooled immediately and the absorbance was measured at 532 nm by spectrophotometer (UV-1650 PC, Shimadzu, Japan). The standard curve was constructed over the concentration range of 1–10 µM of TEP. Results were expressed as nmol/mg protein.

CAT activity assay

CAT activity in the tissue was assayed by following the procedure of Aebi.²⁰ In a cuvette containing 200 µl phosphate buffer and 50 µl of tissue supernatant (obtained after centrifugation of tissue homogenate at 12000g for 20 min at 4°C), was added 250 µl of 0.066 M H₂O₂ and decrease in OD was measured at 240 nm for 60 s. One unit of activity equals to the moles of H₂O₂ degraded (per min), divided by the number of milligrams of protein in the tissue supernatant. The molar extinction coefficient of 43.6 M⁻¹ cm⁻¹ was used to determine catalase activity.

SOD activity assay

Tissue supernatant obtained after centrifugation at 12000g for 20 min at 4°C was measured spectrophotometrically by calculating the rate of inhibition of auto-oxidation of hematoxylin for the assay of SOD according to the method described by Martin²¹ and expressed as units/mg protein.

GPx activity assay

GPx is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function of GPx is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. GPx activity was measured with the GPx kit (Randox Labs, Crumlin, UK).

Histopathological examination

For the histological examination, small pieces of liver were fixed in 10% phosphate buffered formalin, embedded in paraffin, sectioned at 5 µm; and stained with hematoxylin and eosin for light microscopic observations.

Statistical analysis

Results were expressed as Mean±SD. Data were

analyzed by one-way ANOVA test followed by Tukey's post hoc test for multiple comparisons. Data analysis was performed using the Prism 5.0 (San Diego, CA, USA) statistical package program. P-value less than 0.05 was considered significant.

Results

Effects of LOE and MTX on serum analysis

Result showed that 24 hour after MTX administration rat developed hepatotoxicity that reflected by a significant increase ($p < 0.05$) in the levels of AST, ALT, ALP (Table 1). Pre-treated groups with LOE showed decrease in the level of in all doses but it was significantly decrease in doses of 200 and 400mg/kg ($p < 0.05$).

Effects of LOE and MTX on liver Tissue Parameters

Figure 2 shows the effect of MTX and LOE

pretreatment on the level of MDA and GSH in liver tissue. The results clearly revealed that MTX intoxication markedly increase the amount of MDA in rats ($p < 0.05$).

As shown in Figure 2, the decrease in MDA level was significantly observed in pretreated rats by LOE (400 mg/kg). GSH level significantly decreased in the liver of rats exposed to MTX and pretreatment with LOE (200 and 400 mg/kg) significantly inhibited the MTX-induced reduction of GSH content.

As shown in Figure 3, MTX significantly decreased CAT, GPx and SOD activity in compared to control group ($p < 0.05$). Pretreatment with LOE (200 and 400 mg/kg) significantly inhibited the MTX-induced reduction of CAT, GPx and SOD activity.

Table 1. Effect of LOE on serum parameters in MTX induced toxicity in rats.

Groups	AST(IU/L)	ALT(IU/L)	ALP(IU/L)
Control	64.23±4.27	51.12±3.07	178.2±6.57
MTX	124.18±5.96 ^a	137.51±5.12 ^a	259.4±8.29 ^a
LOE(100mg/kg) + MTX	108.09±4.81 ^a	112.23±4.13 ^a	227.6±7.31 ^{a,b}
LOE(200mg/kg) + MTX	87.16±4.20 ^{a,b}	97.45±3.95 ^{a,b}	209.1±6.85 ^{a,b}
LOE(400mg/kg) + MTX	75.12±3.93 ^b	82.67±3.22 ^{a,b}	198.8±6.12 ^b

Values are means ± SD (n = 7). Data were analyzed by one-way ANOVA test followed by Tukey's post hoc test for multiple comparisons.

LOE: *Lavandula officinalis* extract

MTX: Methotrexate

^asignificant difference in comparison with the control group ($P < 0.05$).

^bsignificant difference in comparison with the MTX group ($P < 0.05$).

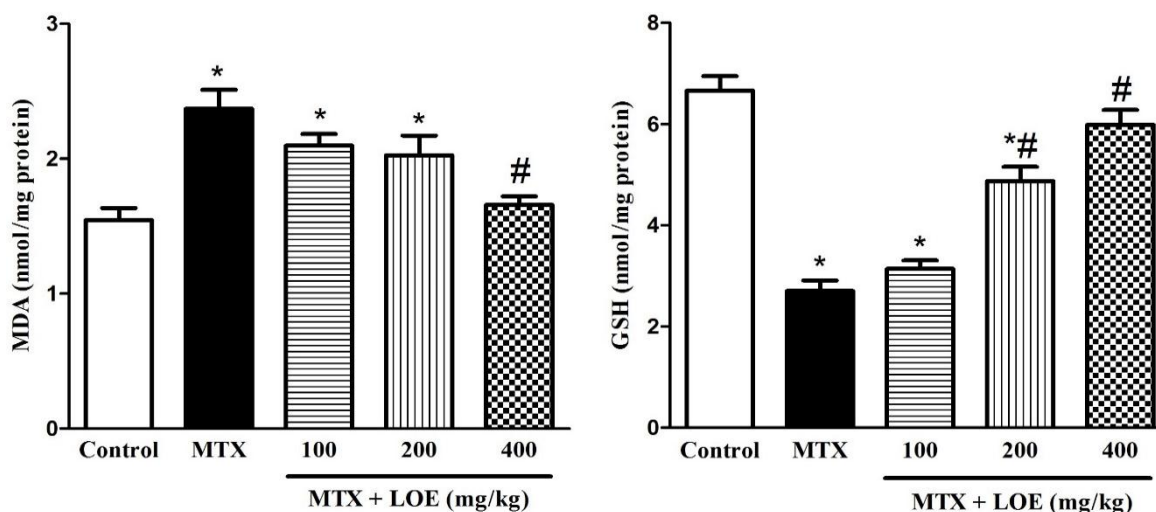


Figure 2. Effect of pretreatment with LOE on MDA and GSH levels in liver tissues in MTP- induced oxidative stress. MDA level increased and GSH content decreased significantly in MTX -treated group. Pretreatment with LOE decreased MDA and increased of GSH levels.

LOE: *Lavandula officinalis* extract

MTX: Methotrexate

Values are means ± SD (n = 7). Data were analyzed by one-way ANOVA test followed by Tukey's post hoc test for multiple comparisons.

*significant difference in comparison with the control group ($*P < 0.05$).

#significant difference in comparison with the MTX group ($#P < 0.05$).

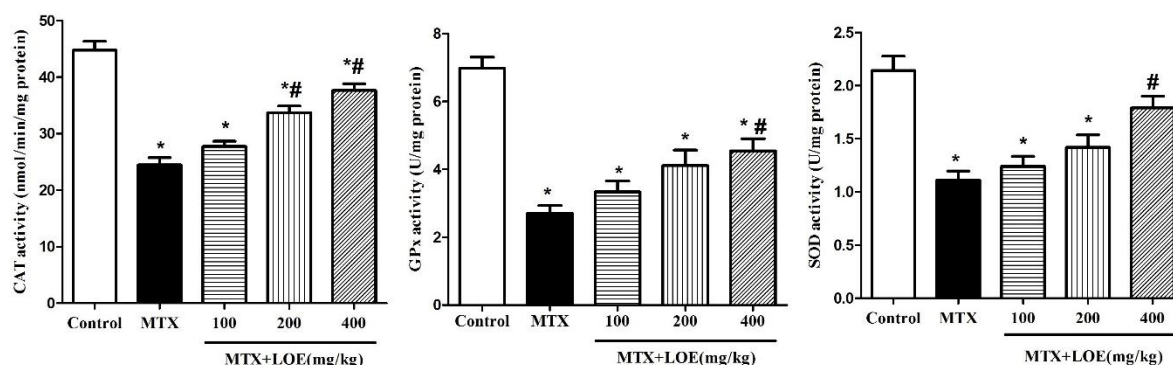


Figure 3. Effect of pretreatment with LOE on CAT, GPx and SOD activity in MTX- induced oxidative stress. CAT, GPx and SOD activity decreased significantly in MTX -treated group. Pretreatment with LOE (400 mg/kg) increased significantly CAT, GPx and SOD activity.

LOE: *Lavandula officinalis* extract

MTX: Methotrexate

Values are means \pm SD (n = 7). Data were analyzed by one-way ANOVA test followed by Tukey's post hoc test for multiple comparisons.

*significant difference in comparison with the control group (*P < 0.05).

#significant difference in comparison with the MTX group (#P < 0.05).

The light microscopic findings

The histopathological changes in rat liver were examined in H&E stained sections.

In MTX-treated group (Figure 4B), severe changes were observed in liver morphology, including hepatocytes necrosis (circle area), accumulation of inflammatory cells in periportal area, cell swelling in hepatocytes around the central vein compared with the control group (Figure 4A)

Treatment with LOE has improved histological changes including inflammation and necrosis in comparison with MTX-treated group (Figure 4C-E).

Discussion

Drugs employed for cancer chemotherapy are renowned to generate acute toxic side effects in numerous organ systems. It has been reported that bone marrow, gastrointestinal tract, mucosal membranes, hair follicles and liver damage may occur especially high doses or following chronic administration of MTX¹¹. Methotrexate, is a folate antagonist drug, is specifically utilized in the remedy for malignant tumors as well as non-neoplastic illnesses.²² However, the medical use of this drug is usually restricted by its unwanted effects including serious nausea and extensive gastrointestinal ulceration. The conclusions of the studies demonstrate that MTX causes oxidative tissue damage by increasing lipid peroxidation in the liver tissue and also reducing the amount of antioxidant enzymes. Also, increased AST and ALT values, biochemical indicators of liver damage, and histopathological detections supported this conclusion.¹¹ The conversion of MTX to its main extracellular metabolite, 7-hydroxymethotrexate, happens in the liver, where it is oxidized by a soluble enzymatic system. Inside

cells, MTX is saved in a polyglutamated structure. Long-term drug administration can lead to depletion of MTX polyglutamates and reduced folate levels. The attendance of higher levels of polyglutamates leads to an extended intracellular attendance of the drug, and this has been offered as a mechanism for MTX hepatotoxicity. It appears that MTX can reduce the availability of NADPH in cells by inhibiting the pentose cycle enzymes. Hence, the malfunction in the antioxidant defence system, may consequence with increased sensitivity of cells to ROS affiliated damage.²³ MTX lead to enhances in serum AST, ALT and also ALP quantities have been related to hepatic structural injury because these enzymes, important markers of hepatocellular damage, are usually localized in the cytoplasm as they are eventually released into the circulation after cellular damage has occurred.²⁴ In our study, increased AST, ALT and also ALP quantities in MTX-treated rats show liver toxicity. Results obtained from pervious study has been well supported our result that revealed elevation in liver enzymes level, who have demonstrated that MTX causes hepatotoxicity.²⁵⁻²⁷ All these changes in the liver function tests in MTX -treated animals were considered the major evidences of the hepatotoxicity of MTX.

The present study showed oral administration of LOE significantly lowered the MTX induced serum levels ALT, AST and ALP as hepatic enzyme markers (Table1).

These results were similar to several studies who reported that LOE reversal of enhanced serum enzymes on induced liver damage by Malathion and Alloxan.^{28,29}

Reactive oxygen species (ROS; \bullet OH, O₂⁻, RO, ROO, NO) play main role in enhance of MTX-induced cellular damages in liver tissue.

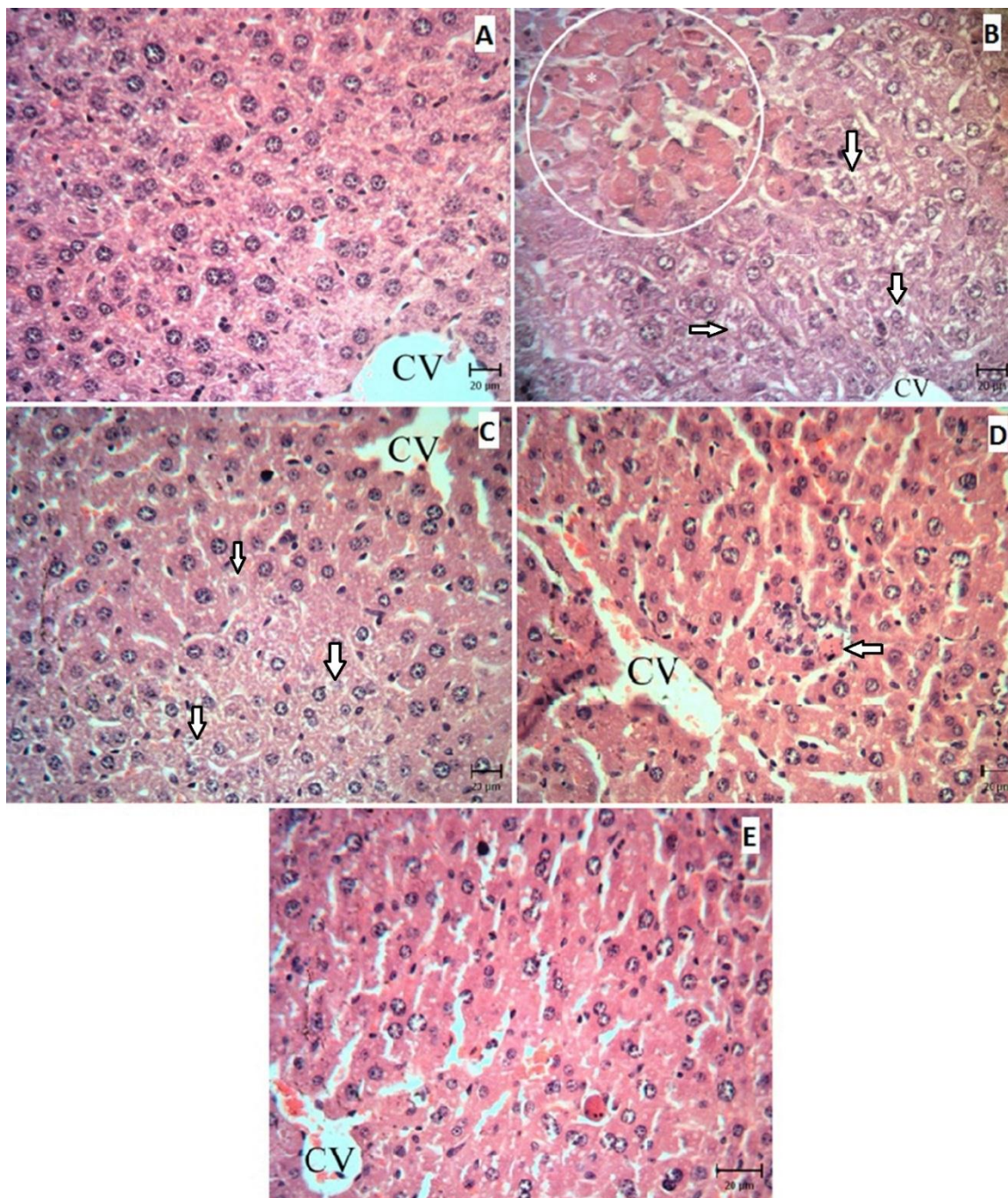


Figure 4. Photomicrographs of liver in the seven experimental group (H&E). Magnification x 400
 (A) Control group, normal morphology of histological section of rat liver
 (B) MTX-treated group, Note to necrotic cells (circle) with eosinophilic cytoplasm (arrows) in compare with normal cells and pyknotic nuclei also ballooning degeneration with free space in hepatocytes is evident.
 (C-E) MTX+LOE (100, 200,400mg/kg)-treated group.

Induced of oxidative stress due of ROS may lead to initiation and progression of some disease such as cardiovascular, diabetes and neurodegenerative disorders.³⁰

The human body is equipped with possesses defense systems against free-radical damage like the non-enzymatic antioxidants such as reduced glutathione and endogenous antioxidant enzymes such as GPx, SOD and CAT. Hence, generation of high levels of ROS or any disturbance in the

oxidant–antioxidant status can result in oxidative damage to macro molecules (DNA, proteins and lipids), tissues or organs.³¹

In view of the presented results the level of GSH and the activities of antioxidants enzymes; SOD, CAT and GPx were significantly ($p < 0.05$) decrease in the liver tissue of MTX-treated rats, in comparison to the control group, which indicated that MTX has caused severe oxidative stress. Our results were parallel to some studies which

indicated, the after MTX intoxication, significant depletion of the GSH level and also significant decreases in activities of SOD, CAT and GPx was evidenced, corroborating the state of oxidative stress.^{23,32,33}

MDA is one of the most commonly markers of lipid peroxidation. Lipid peroxidation is a well-known mechanism of cellular injury in the human body. MDA is an extremely reactive three carbon dialdehyde and main oxidative degradation product of membrane unsaturated fatty acid, owning toxic attributes. High quantities of MDA have been related to various disorders in humans.³⁴ Considering that the MTX toxicity causes reactive oxygen metabolites in liver tissue, measurement of level of MDA, will be valuable in the diagnosis for liver toxicity.³⁵

The present study revealed intoxication of rats by MTX significantly increase MDA levels in liver when compared with control rats. Consequently, it can be hypothesized that oxidative stress may be certainly one of the contributing causes for MTX-induced liver disorders. Some previous studies have been reported that enhanced ROS throughout MTX exposure. Therefore, ROS attacks almost all cell components such as membrane lipids and increased MDA level due to producing lipid peroxidation.²³

The results obtained in this study recommend that the protective effects of LOE against MTX-induced oxidative stress are considerable; and that LOE facilitated in the reduction of the oxidative damages. In the present study, LOE administration produced an increase in the level of GSH in both liver and also attenuated the decrease in GSH induced by MTX. Our data with LOE are actually in line with earlier published reports.³⁶

LOE administration to MTX treated rats significantly increased the SOD and CAT activities. This could be because of the free radical scavenging and antioxidant property of LOE. These results are similar to the observation of another study where LOE was shown to decrease SOD and CAT activities in malathion induced damage in experimental rats. Furthermore our results demonstrated that LOE supplementation to the MTX treated rats increased the concentration of GPx. An increase in intracellular GPx level induced by LOE has been indicated in earlier reported.²⁹

At the same time, oral administration of LOE significantly decreases the hepatic MDA of the rats of groups 4 and 5 when compared with the MTX group ($p < 0.05$). These results of LOE might be the schedule for the defense against pathological alterations in liver in rats induced by MTX. Similar results, indicating that LOE diminished formation of MDA level, have been reported by some other researcher.^{23,36}

Results from literature demonstrated that LOE antioxidant property confirmed here is because of its ability to scavenge ROS, such as hydroxyl radicals, hydrogen peroxide and superoxide anions in the liver.²⁸ Results obtained from our study recommend that the antioxidant attributes may be accountable for LOE liver protective effect which was in keeping with the earlier studies which hypothesized that LOE conveys protection to cells by reducing the generation of free radicals.

We generated oxidative stress in the rat liver by using MTX. It has been reported that MTX is a useful compound for the study of oxidative stress, because its toxicity is mediated by free radicals.

This study illustrate for the first time that MTX-induced oxidative stress was protected by LOE treatment, indicating the antioxidative effect of LOE. In agreement with previous reports, we found diminished GPx, SOD and CAT activities in MTX-treated rat liver.

However, major limitation in this study was a phytochemical analysis to determine the composition of the plant extract.

Conclusion

The results of this study suggest that oral LOE can protect liver tissue from oxidative damages induced by MTX through modification of antioxidant enzymes activities, non-enzymatic antioxidant levels. LOE is able to reverse oxidative stress-induced damages by MTX in the liver tissue, due it direct as well as indirect antioxidant potential and capacity of improve body antioxidant status. In addition, the results showed here confirm the beneficial for the protection against pathological changes induced by MTX, in rat's liver. From the foregoing, it is apparent that LOE is a promising candidate in the management of liver damage in patients receiving chemotherapeutic drugs.

Acknowledgement

This work was supported by the grant number 94s49 provided by Deputy of Research of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Conflict of interests

The authors claim that there is no conflict of interest.

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