



***Cichorium intybus* Improves Hepatic Complications Induced by Oxymetholone: An Animal Study**

Running title: Chicory Improvement of Hepatic Side Effect of Oxymetholone

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Abstract

Oxymetholone (OM) is a 17- α -alkylated anabolic-androgenic steroid used at low doses for the treatment of diseases such as anemia and osteoporosis. The present study aimed to evaluate the hepatoprotective effect of *Cichorium intybus* L. (Chicory) extract on OM-induced changes in liver enzymes, serum antioxidant status and liver tissue in rats. 42 Wistar rats were divided into 6 groups (n=7): control group (0.5 ml distilled water), OM group (5 mg/kg), *C. intybus* groups (100 or 200 mg/kg), and OM+*C. intybus* groups (5 mg/kg OM+100 or 200 mg/kg extracts). After two weeks, the animals were weighed and serum was separated to assay liver enzymes [aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatases (ALP)], nitric oxide (NO) and total antioxidant capacity (TAC). The liver was separated, weighed, fixed and stained with the hematoxylin-eosin method. OM significantly increased NO and liver enzyme (AST, ALP, ALP) concentrations, and significantly decrease serum TAC, animal and liver weights (P<0.05) and changed liver histology. Different concentrations of *C. intybus* extract (100 or 200 mg/kg) improved these OM-induced changes significantly (P<0.05). Chicory reversed serum biochemical and histological changes due to OM consumption.

Keywords: Liver, *Cichorium intybus*, Oxymetholone, Nitric oxide, Antioxidant

Introduction

Oxymetholone (OM) is an anabolic-androgenic steroid with an effect similar to male sex hormones such as testosterone and dihydrotestosterone. It is used in low doses to treat diseases such as anemia and osteoporosis. Some athletes use its higher doses as body enhancers, which have reported side effects such as premature baldness, aggression, liver tumor, sadness, irritability, and mania [1]. The use of anabolic steroids alters serum levels of liver aspartate (AST) and alanine (ALT) transaminases, alkaline phosphatase (ALP) and affects the liver structure [2]. Hepatic enzyme deficiency can indicate liver damage or alteration in bile flow, which may be related to other biochemical abnormalities or symptoms of liver disease [3]. Because the liver is a vital

organ and performs various biochemical, synthetic, and secretory functions, several tests for liver failure are used. The evaluations of AST, ALT, and ALP are the most important ones. The AST and ALT are the main enzymes in the liver and are biochemically interdependent [4].

Free radicals are produced constantly, and their circulation in the body causes extensive damage to many macromolecules. In healthy individuals, there is a balance between the production of free radicals and the antioxidant defense system [5]. The uncontrolled production of free radicals can disrupt this equilibrium, could damage the cell membrane structure and disrupt the integrity of various tissues. Antioxidant compounds prevent oxidative damage by keeping these radicals at an optimum level [6].

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Determination of plasma total antioxidant capacity (TAC) is one of the promising methods for diagnosing and treating various diseases such as cardiovascular system and diabetes. Plasma antioxidant molecules come from two sources: 1) endogenous such as uric acid, albumin, and thiols, and 2) exogenous such as vitamins E and C. TAC measurements provide a tool for establishing links between antioxidant capacity and the risk of disease as well as for monitoring of antioxidant therapy [7].

Nitric oxide (NO) is a free radical produced by endothelial, epithelial and hepatocyte cells [8]. NO and its derivatives play an important role in the pathophysiology of the liver. Despite its diverse and complex roles, specific patterns of the effect of NO on the pathogenesis and progression of liver disease have been observed. In general, NO in endothelial cells of the liver sinusoids has a protective role against disease progression, whereas NO-derived Nitric oxide synthase (NOS) plays a role in pathological processes [9]. NO and peroxynitrite affect liver tissue by different mechanisms and thus induce cell death and damage such as dilation and congestion of sinusoids, along with infiltration of mononuclear inflammatory cells [10,11]. Increased levels of NO due to sodium nitrate consumption in rats also increased the level of liver enzymes (ALT, ALP, AST), which indicates hepatocellular damage [7,9]. During hepatotoxicity induced by acetaminophen administration, high oxidative stress is observed. This leads to a release of inflammatory mediators such as NO via inducible NO synthase and cellular dysfunction [12].

Medicinal plants that possess anti-inflammatory and antioxidant activities could be effective in the treatment of liver diseases [13,14]. Many of them are popular in traditional medicine [15] and are used for their hepatoprotective properties. Chicory (*C. intybus* L.) belongs to the Compositae family and possesses a variety of pharmacological activities. It has antipyretic and hypoglycemic effects and is popular as a liver enhancer [14]. The leaves and flowers are a good source of anthocyanins, vitamins C and A. The herb is known as a strong stimulator of the immune system, limiting the processes of infection and inflammation [15]. The antimicrobial and antioxidant activities of chicory have been proven. Chicory can be effective in improving

nitrosamine-induced liver damage by increasing the level of antioxidants [16].

Our previous studies showed that OM degraded testicular tissue, decreased TAC, and increased NO levels and other plants' extracts (*Nasturtium officinale* and *Vaccinium arctostaphylos*) improved these changes [17,18]. The antioxidant and hepatoprotective activities of *C. intybus* have been reported in previous studies [14-16] but there is no information about its effect on OM-induced hepatic changes. This study aimed to investigate the protective effect of *C. intybus* on OM-induced changes in liver enzymes, liver tissue structure and its antioxidant status using animal model of rat.

Material and Methods

Laboratory Animals

The present study is an experimental animal investigation conducted after the approval of the University Ethics Committee of Kermanshah University of Medical Sciences (IR.KUMS.REC.1398.556) and the ethics of working with animals. In this experiment, 42 adult male Wistar rats weighing 200 ±15 g (2–3 months age) were purchased from the Laboratory Animal Center of University. They were maintained in a room with 12h light/dark cycle condition and received food pellet and water *ad libitum*.

Plant Material

The aerial parts of the *C. intybus* were collected from Kermanshah, Iran and identified by a professional botanist (School of Pharmacy) with the herbarium No. 1081 (Kermanshah Agricultural and Natural Resources Research and Education Center), and after that dried in the shade and powdered by electric milling. Percolation method was used for extraction; 20 g of the plant powder were added to 400 ml 70% ethanol and kept in the laboratory for 48 hours with stirring and away from light. The extract was filtered through filter paper (Watman 22), and the obtained solution was completely dried by evaporation of alcohol. The dried powder was stored at 4 °C until use [19].

Table 1 The chemical compositions isolated from the aerial parts of *C. intybus* L.

Group compositions	Compounds
Phenolic compounds	Caftaric acid, Chlorogenic acid, Cichoric acid, Isoquercitrin, Rutin, Quercitrin, Luteolin, Apigenin
Minerals	Fe, Cu, Zn, Mn
Vitamins	B1, B2, B3, B5, B6, B9, A, K, C
Others	Tannins, Saponins
	Flavonoids, Polyphenols, Gallic acid, Quercetin glucuronide,

Animal Grouping

42 adults' male Wistar rats were randomly divided into 6 groups of 7 each:

- 1) Control group: received 0.5 ml distilled water (extract solvent) daily;
- 2) Oxymetholone (OM) group: received OM (5 mg/kg) daily;
- 3 and 4) Chicory groups: Take one daily dose of *C. intybus* extract (100 or 200 mg/kg);
- 5 and 6) Chicory + OM groups: received OM (5 mg/kg) + *C. intybus* extract (100 or 200 mg/kg) daily [20].

The treatment period was two weeks, and the intraperitoneal injection method was applied. The rats were weighed and anesthetized 24 hours after the last injection. Blood samples were taken from the heart and serum was separated, and stored at -20 °C. The liver was separated, weighed, and fixed in 10% formalin. Isolated serum was used to measure the level of liver enzymes and the antioxidant status. Serum levels ALT, AST and ALP were measured by a diagnostic kit (Bio System, Spain).

Ferric Reducing Ability of Plasma (FRAP) Assay

Total antioxidant reducing power was measured by the FRAP method. This method is based on the ability of the serum to reduce Fe⁺³ ions to Fe⁺². FRAP reagents include acetate buffer (300 mM), FeCl₃.6H₂O solution (20 mmol/L), and 2, 4, 6 tripyridyl, 1, 3, 5 triazine. 200 µl of serum was added to 1500 µl of FRAP reagent, and their absorbance was read at 593 nm using a spectrophotometer (Pharmacia, Novaspec II, Biochrom, England) after that calculated using standard regression equation on serum FRAP curve expressed in µM [21].

Nitric Oxide Assay

Griess colorimetric method was used to measure NO level. Zinc sulfate was added to the serum and centrifuged for deproteinization. 100 µlit of the deproteinized samples were added to 100 µlit of vanadium chloride solution (1%), followed by Griess reagent containing 50 µlit of sulfanilamide (2%) and 50 µl of N-(1-naphthyl) ethylenediamine dihydrochloride

Changes in Liver Enzymes

OM significantly increased the AST enzyme (194.8±16.8) compared to the control group (156.3±5.8). *C. intybus* extract dose-dependently decreased AST in OM-treated groups (P<0.001). *C. intybus* alone insignificantly decreased AST (P> 0.05) compared to the control group (Fig. 2A). OM significantly increased ALT enzyme (P=0.018), *C. intybus* extract significantly decreased it in the OM-treated groups. *C. intybus* extract alone did not show a significant change in ALT compared to the control group (P>0.05) (Fig. 2B). Serum ALP levels were significantly increased in the OM group, and co-treatment with 100 and 200 doses of the extract

was added and incubated at 37 °C for 30 min. The samples were read at 540 and 630 nm by ELISA reader (STAT Fax100, USA), and the results obtained were compared with standard adsorbents (0 to 200 µM of sodium nitrate) [21].

Histopathological Examination

Livers were fixed in 10% formalin after tissue processing and preparations of 5 µm sections. The sections were stained with the hematoxylin-eosin method. Histopathology survey was performed using a light microscope (10x magnification) equipped with a Motic camera and software (Moticam 2000, Spain). Five random microscopic slides were taken from each specimen and images of each slide were obtained. Liver tissue structure, including the cellular arrangement of hepatocytes and sinusoids; portal spaces; the presence of inflammation and leukocyte infiltration was investigated [22].

Statistical Analysis

The normality of data was determined by the Kolmogorov-Smirnov test (P>0.05), and differences among data were statistically analyzed using one-way ANOVA followed by Tukey's test as post hoc (P<0.05 were considered significant). The charts were designed with Graph Pad Prism software package version 8 (Graph Pad Prism Software Inc., San Diego, California).

Results

Changes in Serum NO and TAC

The obtained results show a significant decrease in serum TAC during the administration of OM (P=0.008). Both doses of *C. intybus* prevented this decrease in OM-treated groups. However, the difference between *C. intybus* groups and control groups was not significant (Fig. 1A). OM significantly increased serum level of NO (P<0.001), while *C. intybus* treatments (100 and 200 mg) decreased it. The differences between *C. intybus* groups and control groups were not significant (Fig. 1 B). *C. intybus* significantly decreased it (P =0.004). *C. intybus* single extract caused a slight and insignificant decrease in ALP (Fig. 2C). The amount of total protein in the OM group (4.4 ± 0.46) was significantly decreased (P<0.001). In the OM + *C. intybus* extracts of 100 and 200 mg total protein content was 5.8 ± 0.16 and 5.8 ± 0.11 respectively, and its reduction improved (Fig. 2D).

Changes in Body Weight and Liver

At the end of the experiment, the body weight of the animals in all groups except the OM group was significantly higher than the initial weight and the difference of final weight in all groups compared to OM was significant (P<0.001). OM administration (14 days)

prevented the normal weight gain of the animals (Fig. 3A). Liver weight was also significantly decreased in the OM group ($P < 0.001$), and *C. intybus* (100 and 200 mg) treated OM groups dose-dependently increased the liver weight (Fig. 3B). The weight gain of *C. intybus* groups was not significant compared to the control group.

Liver Tissue Changes

The histological examination of the liver revealed normal structures of the liver lobules in the central vein region, sinusoids, portal spaces, and hepatocytes in control and *C. intybus* (100 and 200 mg) groups. However, the OM leads to hepatic tissue destruction and disruption; the central vein of the lobule was dilated, and the sinusoidal spaces were larger. Hepatocytes lacked regular arrangement and compact nuclei with low cytoplasm (Fig. 4D). Pathological changes were not observed in the liver tissue of OM + chicory extracts.

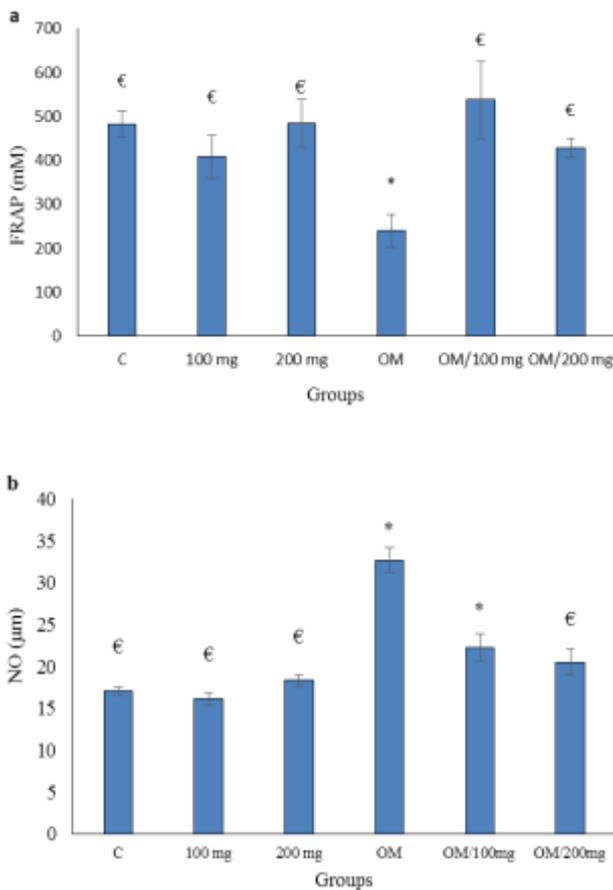


Fig. 1 Changes in TAC (a) and NO (b) in the experimental groups. *: Significant difference with the control group ($P < 0.05$). €: Significant difference with the oxymetholone (OM) group ($P < 0.05$).

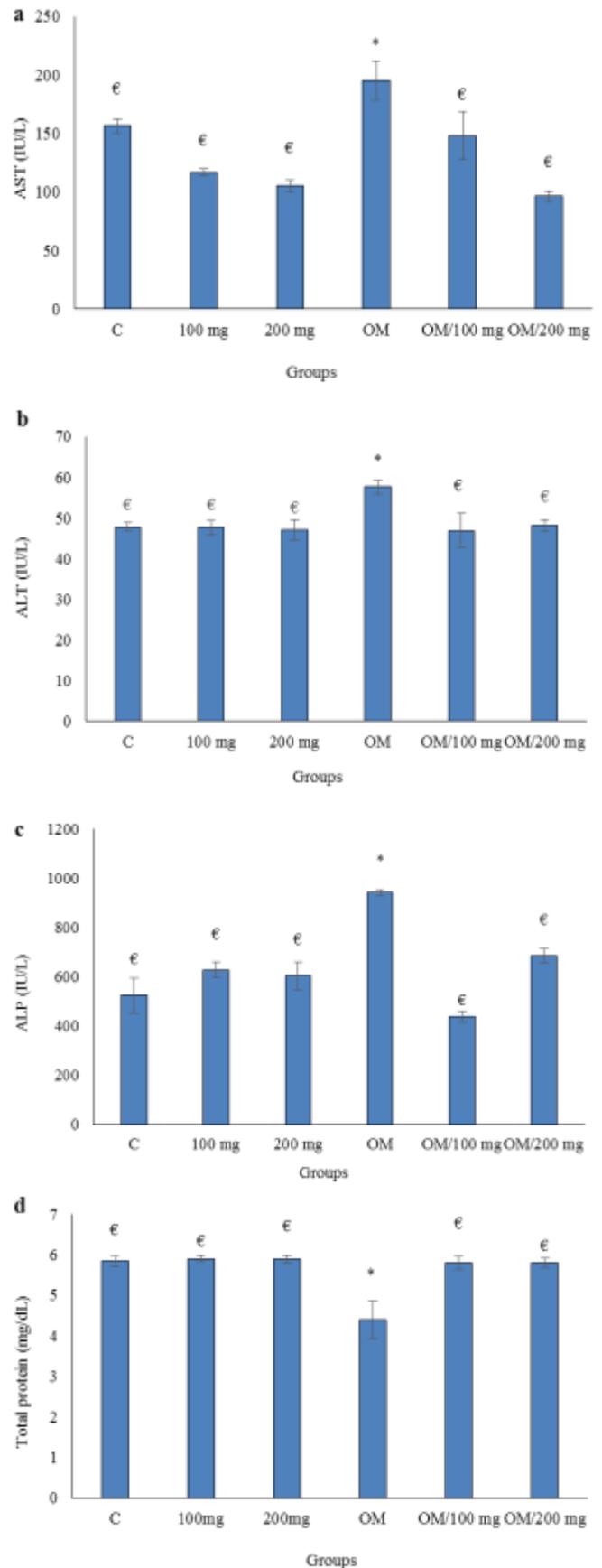


Fig. 2 Changes in serum levels of AST (a), ALT (b), ALP (c) and TP (d) in the different test groups. *: Significant difference with the control group ($P < 0.05$). €: Significant difference with the oxymetholone (OM) group ($P < 0.05$).

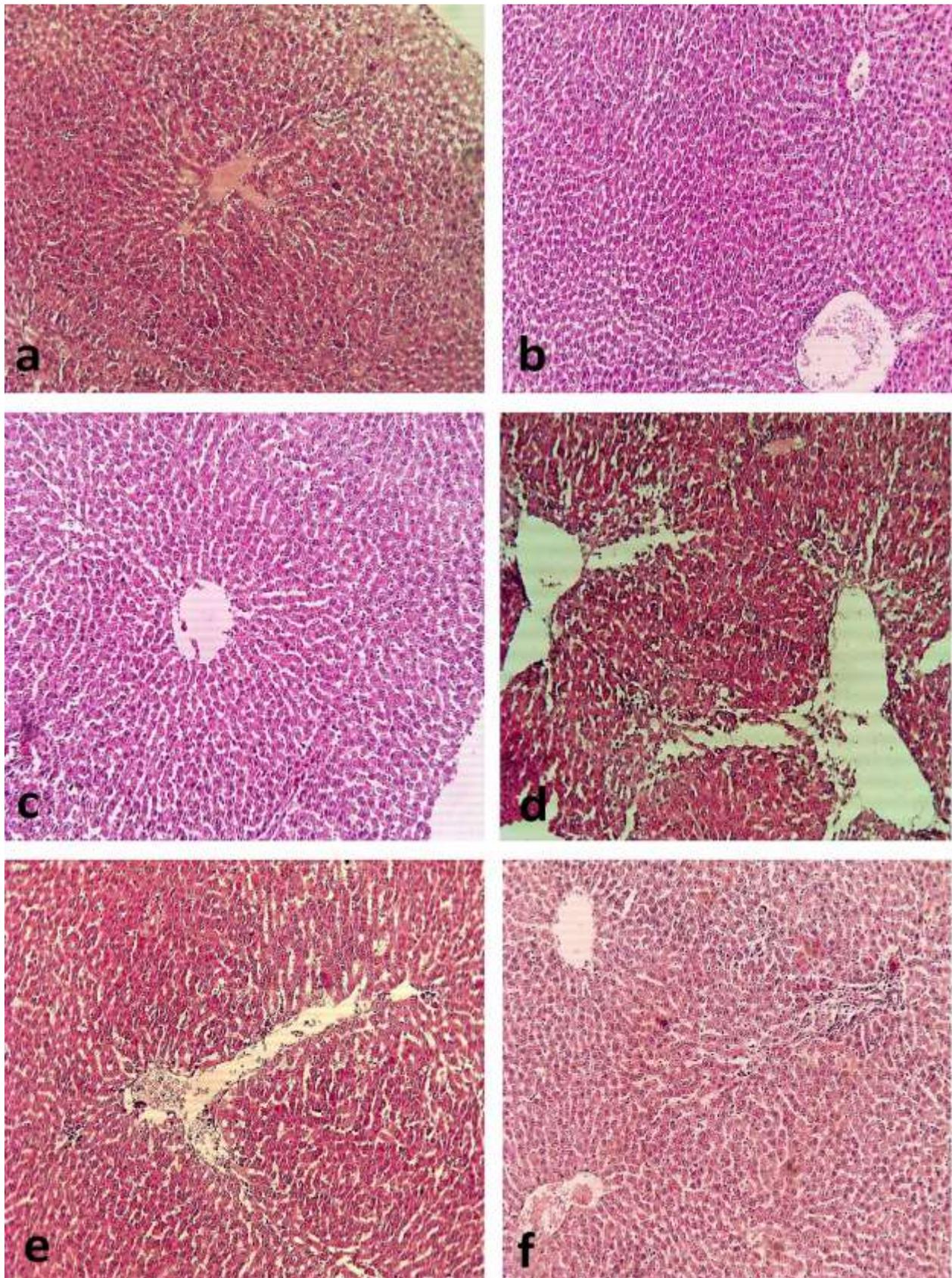


Fig. 4 Changes of liver tissue in different groups, a: Control; b: 100 mg/kg extract; c: 200 mg/kg extract; d: OM; e: OM+100 mg/kg extract; f: OM+200 mg/kg extract (x10).

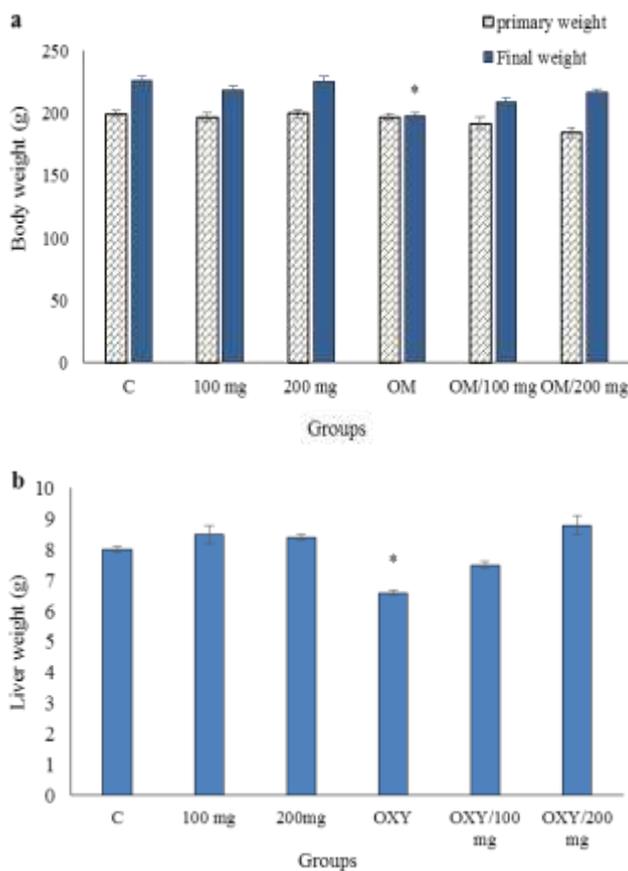


Fig. 3 Changes in the body weight (a) and liver weight (b) in animals of different experimental groups. *: Significant difference with the control group ($P < 0.05$).

Discussion

OM significantly increased NO and liver enzymes (ALT, AST and ALP), and decreased serum TAC, animal and liver weights. Hydroalcoholic extract of chicory (*C. intybus*) improved OM-induced serum biochemical changes, decreased NO and liver enzymes and increased TAC, animal and liver weights. The extract also improved liver tissue changes induced by OM. This study is the first scientific report on the protective effect of *C. intybus* on OM-induced liver damage. The use of anabolic steroids has undesirable side effects in various organs and can lead to serious liver problems such as cell destruction, increase transaminases, chronic vascular injury, liver tumors and fatty diseases [23]. In the present study, as well as in other similar studies, [1,17] OM increased serum levels of liver enzymes (ALT, AST, ALP). Nejati *et al.* (2016) investigated an OM-induced liver injury (elevated ALT, AST, ALP enzymes) in rats that were improved with royal jelly [1]. However, a similar effect was observed in the present study, indicating the high content of antioxidant compounds and hepatoprotective agents in both royal jelly and chicory [24].

The study of the effect of hydroalcoholic extract of *C. intybus* on carbon tetrachloride-induced liver fibrosis also showed that the extract decreased the activity of oxidant agents such as malondialdehyde and increased the activity of antioxidant agents such as superoxide dismutase, a decrease of AST and ALT serum levels and by this way protected liver tissue. [25], our results showed that chicory extract increased TAC in a dose-dependent manner and by that way could protect the liver from OM harmful effects. Moloudi *et al.* (2014) showed that during obstructive cholestasis induced liver injury, chicory extract reduced AST, ALT and ALP enzymes [26]. In the present study, chicory extract improved the biochemical changes induced by OM in rats. Histological studies also showed that both doses of chicory extract prevented liver damage.

Chicory has the *in vivo* oxidative stress-reducing effect through an increase of the antioxidant enzyme activity and reduction of malondialdehyde levels. However, its antioxidant properties have also been confirmed *in vitro* [15]. It contains high amounts of caffeine and chlorogenic acids, sinarin, luteolin and other flavonoids. The hepatoprotective effects of chicory are due, mainly to its chlorogenic acid and sinarin [16]. Other studies show *C. intybus* insulin-resistance stimulation by activation of pancreatic β -cells to synthesize insulin [27]. In another study, daily consumption of chicory and cinnamon reduced liver enzymes in patients with nonalcoholic fatty liver [28]. The methanolic and aqueous extracts of chicory seed have potent antioxidant and hepatoprotective activities, which could be due to the presence of flavonoids or polyphenolic compounds [29]. In the present study, chicory extracts increased TAC and decreased oxidative stress induced by OM and increased antioxidant enzyme activity.

Furthermore, chicory can be effective in improving liver damage caused by nitrosamine and carbon tetrachloride by increasing the level of antioxidants. Based on the results of the present study and previous research, it seems that the use of antioxidants or drugs that protect the liver cells from oxidative stress greatly contributes to the reduction of the adverse effects of OM and liver damage caused by it. Similar effects on the oxidative changes induced by alcohol and carbon tetrachloride in rat liver were observed by using the extracts of *Tragopogon* [30] and *Anethum graveolens* [31].

Hassan and Yousef (2010) showed that nitrosamine precursors induced a significant increase in liver Thiobarbituric acid reactive substances (TBARS) and higher activity of the enzymes AST, ALT, and ALP in rats. Additionally, there was a significant decrease in glutathione, glutathione reductase, superoxide dismutase, catalase, total protein and albumin levels. However, the diet containing chicory has been able to modulate these abnormalities caused by nitrosamine compounds, which

were reduced TBARS and significantly improving the biochemical and antioxidant parameters [16]. The results of our study showed that total protein decreased significantly in response to OM administration, which is due to its deleterious effects on liver tissue. Administration of chicory extract with OM prevented this decrease.

In the liver, NO is produced by eNOS (endothelial nitric oxide synthase) or iNOS (inducible nitric oxide synthase). Whereas eNOS is expressed only in sinusoidal endothelial cells, iNOS is mainly regulated by the induction of inflammatory cells. In inflammatory liver conditions, the NO produced by iNOS reacts with free radicals such as superoxide and that leads to the production of more dangerous radicals like peroxynitrite.[32] INOS-derived NO is an important factor in the pathogenic processes of many acute and chronic liver diseases, including drug-induced liver damage. iNOS is detectable in several cell types, including Kupffer cells and hepatocytes [27]. In the present study, OM increased serum NO and liver tissue damage. However, both doses of *C. intybus* extract prevented the increase of NO. Researchers have shown that the production of NO derivatives, such as peroxynitrite and n-nitrosamine could damage liver cells [33].

Chicory root extract contains phenolic and flavonoid compounds and its methanol extract has an antioxidant activity equivalent to ascorbic acid. It was also found that there was a significant relationship between FRAP and extract concentration. Therefore, the high FRAP content of chicory extract probably is due to its high phenolic and flavonoid content. The methanolic extract of chicory root is also comparable to ascorbic acid in terms of deactivation of NO, hydroxyl and superoxide-free radicals [34]. In the present study, NO was significantly increased in the OM group and treatment of chicory extract was decreased. Thereby, it is necessary to investigate the molecular mechanisms of chicory's protective effect against OM in this regard.

Conclusions

Chicory extract prevents tissue damage and biochemical changes caused by oxymetholone.

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Conflict of Interests

The authors declare that there are no conflicts of interests.

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