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Original Article

Potential Protective Effect of Pretreatment with Caraway Essential Oil *in vivo* Model of Iron Nanoparticle-induced Liver Injury

Abolfazl Dadkhah[1]*, Faezeh Fatemi[2], Mohammad Reza Mohammadi Malayeri[3], Fatemeh Torabi[4], Mostafa Sarbazi[5] and Salome Dini[6]

[1]*Department of Medicine, Faculty of Medicine, Qom Branch, Islamic Azad University, Qom, Iran*
[2]*Materials and Nuclear Fuel Research School, Nuclear Science and Technology Research Institute, Tehran, Iran*
[3]*Department of Pathobiology, Faculty of Veterinary Medicine, Garmsar Branch, Islamic Azad University, Garmsar-Iran.*
[4]*Department of Physiology, Faculty of Science, Qom Branch, Islamic Azad University, Qom, I.R. Iran*
[5]*Graduated Student, Faculty of Veterinary Medicine, Garmsar Branch, Islamic Azad University, Garmsar-Iran*
[6]*Young Researchers and Elite Club, Karaj Branch, Islamic Azad University, Karaj, Iran*

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Abstract

Recently, there has been a great deal of interest in the use of the medicinal plants with high antioxidant compounds for curing liver injuries induced by hepatoxcitic agents. This study was to assess the protective effect of *Carum carvi* L. essential oil (E.O) on the hepatoxitic rats induced by overdose of iron oxide nanoparticles (NP_s). The rats were distributed to 4 groups. In negative control group (NC), the rats received normal saline and DMSO daily for 3 days. In control group (C), iron oxide nanoparticles (Fe_2O_3) (200 mg/kg b.w) was injected daily for 3 days. In the treatment groups, iron oxide nanoparticles plus E.O at 100 & 200 mg/kg b.w were injected daily for 3 days. In following, cytochrome P450 (CYP450), glutathione S-transferase (GST), glutathione (GSH), aspartate transaminase (AST), alkaline phosphatase (ALP) and alanine transaminase (ALT) were estimated at 72 h after NP-treatment. The administration of the E.O could return considerably the decrease of the GST and GSH levels as well as the increase of the level of AST induced by iron oxide NP_s. These results are in agreement with histopathological results. The data indicated that E.O provided an efficient prevention against iron oxide NP_s-induced hepatotoxicity in rats.

Keywords: *Carum carvi*, Essential oil, Iron oxide nanoparticles, Liver enzymes, Detoxification enzymes, Hepatotoxicity

Introduction

Liver is the major organ for keeping the body's internal environment constant. No organs are not able to function similar to the liver. . The flow of nutrients and controls the metabolism of protein, carbohydrate, and fats were mainly affected [1]. The liver located in the circulatory system accumulates and collects metabolites as well as eliminates and neutralizes toxins [2, 3].

Iron as a vital factor in the cells of body is effective in the metabolic producer such as O_2, electron transport, oxidative phosphorylation and energy production, xenobiotic metabolism, DNA synthesis, cell growth, apoptosis, gene regulation, and inflammation. On the other hand, iron which is a negotiator of the liver injuries and local inflammation may activate per oxidative process and produce oxygen-free radicals, lipid peroxidation (LP) damage to protein and DNA and stimulation of fibrosis [4].

Overdoses of iron as a poisonous element could cause various disorders due to oxidative stresses and weakness of the immune system. In many illnesses with a difference of end-stage hepatic disorders, overindulges of iron were commonly found. Iron

*Corresponding author: Department of Medicine, Faculty of Medicine, Qom Branch, Islamic Azad University, Qom, Iran.
Email Address: dadkhah_bio@yahoo.com

overload is also relatively common in liver disorders. . The pathogenesis of liver fibrosis in patients with iron overload is not well mentioned [5].

Iron nanoparticles (NP_s) can be widely applied for cancer treatment, drug delivery, medical imaging, disease diagnosis, and gene therapy [3]. As the accumulation of Iron NP_s in the cells and cancerous tissues, has been widely applied for rapid diagnosis of cancers at early stages [6]. Also, the application of iron NP_s as a drug transporter for the treatment of cancer dates back to 1970 and has continued ever [7]. Otherwise, these particles due to their small size have special physical, chemical, mechanical, electric and magnetic properties; for example, they freely enter the cell and can leave adverse effects. However, our knowledge is very limited [8]. For example, high doses of iron NP_s stimulate an increase of reactive oxygen species (ROS) generation agent. Then, free radicals are generated following the damage of different organs especially the liver [9].

There is a large-scale of interests in the natural antioxidants which induce the natural defense by seizing the oxidative injuries. Otherwise, the usage of natural antioxidants with probable fewer side effects for modulation of the liver oxidative damages cannot be ruled out.

Carum carvi L., belonging to the family Apiaceae, is commonly known as Kala Jira, caraway, meridian fennel, and Persian cumin. Caraway is a globally distributed spice with a long history as a traditional medicinal plant. It is originally cultivated in Asia, Africa, and Europe [10]. In traditional medicine, its seeds were used for treating several diseases *e.g.* colic pain, flatulence, and bronchitis [11]. In addition, its main biological properties are carminative, intestinal spasmolytic, antiseptic, antimitotic, antiasthma tic, anti-inflammatory and antiulcer genic gastro protective [10, 12-19]. Several studies also proved that the antioxidant properties of the extract and the essential oil isolated from caraway seeds [20-22]. *In vivo* studies indicated that caraway seeds essential oil has hepatoprotective activity in the liver against carbon tetrachloride induced toxicity by modulating the oxidative stress/antioxidant parameters such as glutathione (GSH) and Lipid peroxidation (LP) [21,22].

Based on our previous GC and GC/MS analysis, the essential oil derived from Persian caraway seeds consisted of 17 known compounds, three major compounds were cumin aldehyde, γ-terpinene, γ-terpinene-7-al and p-cymene [23, 24]. The protective effect of caraway essential oil in the sepsis model was confirmed [23]. The essential oil probably has a protective role in the kidney against oxidative injury in advanced stages of sepsis [24]. Our recent research informed that the remarkable chemo preventive properties of the caraway seeds essential oil [25]. Regarding the importance of achieving the effective and safe drugs for liver disorders, there is no study on the role of the caraway essential oil against NP_s toxicity. The present study aimed –for the first time- to investigate the probable potential protective effect of the *C. carvi* essential oil against iron oxide NP_s induced hepatotoxicity in the rats, through measuring major hepatic metabolizing enzymes as well as the liver function markers.

Material and Methods

Adult male Wistar rats (3-4 months of age, weighing 150±20 g) were obtained from the Pasteur Institute of Iran, Tehran, and maintained at 25±2°C with a 12-h light/12-h dark cycle. The animal studies had been approved by the Medical Ethics Committee of Tarbiat Modares University based on the World Medical Association Declaration of Helsinki. Rats had unrestricted access to a commercial pellet food and tap water ad libitum.

Preparation of Caraway Essential Oils

Dried caraway seeds (C. L.) cultured in Iran were purchased from Kerman city. Essential oils were extracted from *C. carvi* (Apiaceae) aerial parts using a Clevenger-type apparatus. The oil compounds were analyzed by GC/MS analysis as described earlier [23,24].

Animal Treatments

Male rats with Wistar race were applied whole the present study. All animals were distributed 4 groups (n=5). First group called negative control (NC), the rats received normal saline (nanoparticle solution) and DMSO (essential oil solution) daily for 3 days. In control group (C), iron oxide NP_s (Fe_2O_3) (200 mg/kg b.w) was i.p injected daily for 3 days. In the treatment groups, iron oxide NP_s (200 mg/kg b.w) plus caraway essential oil (E.O) at 100 and 200 mg/kg b.w were i.p injected daily for 3 days. At day 3 (72 h after treatments), the rats were anesthetized and the blood was collected by heart puncture. The rats were then killed and liver were removed and processed for histological and biochemical assays as described below.

Preparation of Tissue Homogenate and Plasma

The blood and liver samples were gathered from all animals for assessing the biochemical parameters.

Biochemical Assays

Cytochrome (CYP450) Protein Assay
CYP450 protein level was performed by ELISA on liver preparations according to the procedure described in the kit from Bioassay Technology Laboratory, China.

Glutathione S-transferase (GST) Activity

Liver cytosolic GST activities were measured spectrophotometrically using CDNB as substrate as described by Habig *et al*. (1974). The specific activity was calculated based on the nmol/min/mg protein in samples which was measured by Bradford assay [26, 27].

GSH Estimation

GSH was estimated in tissue homogenates according to the procedure of Seldak & Lindsay (1968) [28].

Liver Function Tests

To confirm the liver function and injury, serum alanine transminase (ALT), asparate transminase (AST), and alkaline phasphatase (ALP) were determined spectrophotometrically according to the procedure described in the kit purchased from the Pars Azmoon, Co, Iran.

Histopathological Studies

Preparation of tissues and staining
The liver specimens of all animals were fixed in 10% buffered neutral formalin solution. Then, they processed routinely for paraffin embedding and sectioned for hematoxillin and eosin staining and Lillie's method as iron staining.

Lillie's Method Staining [29]

The sectioned liver tissues were deparaffinised and hydrated to distilled water. For ferric iron accumulation staining, the sections were placed in potassium Ferro cyanide solution for one hour. Then, they were washed well in 1% aqueous glacial acetic acid and rinsed in distilled water. Finally, the sections were dehydrated in 95% alcohol, absolute alcohol and cleared in xylene and mounted. Iron accumulations were visible as dark Prussian blue.

Histopathological Scoring

In histopathological assessment nuclear chromatin clumping, iron accumulation and fatty degeneration were the prominent changes. The histopathological changes were graded numerically to be comparable statistically. The histological scoring was performed as described by Eidi [30], as followings: score 0= no visible cell changes, score 1= focal hepatocyte change on less than 25% of the tissue, score 2=focal hepatocyte change on 25-50% of the tissue, score 3= extensive, but focal hepatocyte change, score 4 = global hepatocyte change.

Statistical Analysis

Data are shown as means ± Standard Error of Mean (SEM) of three samples gained from 5 rats. The results were analyzed by one-way ANOVA followed by Tukey's HSD.

Results

Effect of *C. carvi* E.O on the GSH and GST and CYP450 Activities

The data illustrated that administration of iron NP_s to rats led to a significant ($P<0.05$) decrease in GSH level (342.4 ± 19.2) as compared to the negative groups (Table 1). Similarly, the level of the detoxifying enzyme GST activity (509.8 ± 28.92) (Table 1) diminished in the rats treated with toxic dose of iron oxide NP_s at 72 h ($P<0.05$). In contrast, co-administration of rats with caraway E.O could significantly ($P<0.05$) raise GSH level (423 ± 23.16) and (428 ± 25.28) at 72 h, respectively (Table 1). Likewise, the administration of caraway E.O at 100 and 200 mg/kg b.w surprisingly elevated the reduction of serum GST activity with (717.5 ± 39.99) and (751 ± 46.08) respectively due to iron oxide NP_s (Table 1). It should be noted that the CYP450 activity did not change ($P>0.05$) significantly in all groups.

Effect of *C. carvi* E.O on the Liver Enzymes

Serum activities of AST, ALT and ALP enzymes were represented in Table 2. Iron oxide NP_s considerably elevated the level of AST (125.60 ± 8.38) in NC group at 72 h as compared to those of the control group (Table 2). However, the pretreatment of rats with caraway E.O at 100 (118.5 ±6.61) and 200 (113±8.13) mg/kg b.w remarkably ($P<0.05$) protected the liver by decreasing the AST level (72 h) as compared to iron oxide NP_s intoxicated group, (Table 2). The levels of ALT and ALP unchanged significantly in all groups even after iron oxide NP_s treatments (Table 2).

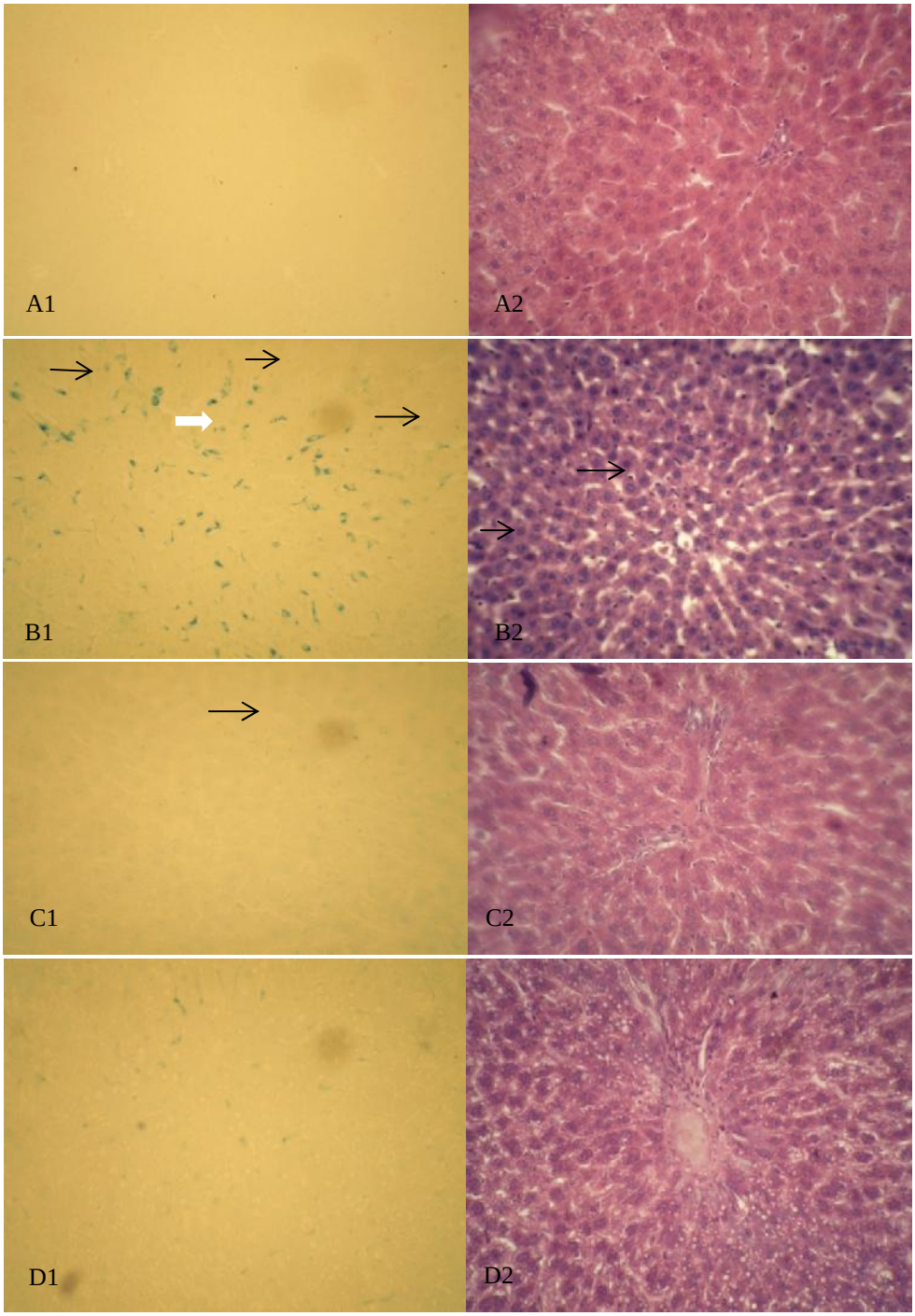

Fig. 1 Effect of caraway essential oil on histopathological changes 72 h after iron nonoparticle administration. A: negative control group, A1; liver tissue in negative control group were completely normal, H&E 400*. A2; there is any iron accumulation in the liver tissue, Lillie's method staining 100*. B: control group, B1; periportal changes consist of clumped chromatin, increased phagocytic cells as dark small nuclei (small arrows) and apoptosis (large arrows), H&E 400*. B2; blue stain accumulation of iron particles in hepatocytes and kupffer cells of periportal region (arrows), Lillie's method staining 400*. C: E.O treatment group (100 mg/kg b.w), C1; mild fatty degeneration could be seen as bright vacuoles in hepatocytes of periportal region (arrow), H&E 400*. C2; iron aggregation has decreased in liver in compare with iron oxide NP_s treated group. Lillie's method staining 400*. D: E.O treatment group (200 mg/kg b.w), D1; extended fatty degeneration in periportal region in comparison with C1 group, H&E 400*. D2; mild aggregation of iron particles in periportal region, Lillie's method staining 400*.

Table 1 Effects of iron NPs on GST and CYP450 at 72 h

Groups	CYP450 (pg/mg protein)	GST (n mol/min/mg protein)	GSH (n mol/mg protein)
	72h	72h	72h
NC	1722±91.29	784.4±47.33	460.4±13.03
C	1668±77.09	509.8±28.92*	342.4±19.2*
E.O100	1735±101.03	717.5±39.99**	423±23.16**
E.O200	1765±71.23	751±46.08**	428±25.28**

NC: negative control group; C: control group; E.O: caraway essential oil (100 & 200 mg/kg b.w) treated groups. *P<0.05 is considered significantly between negative control group (NC) and control group (C). **P<0.05 is considered significantly between control group (C) and caraway essential oil treated groups.

Table 2 Effects of iron NPs on three liver enzymes activities (AST, ALT & ALP) at 72 h

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)
	72h	72h	72h
NC	112±7.26	37.80±2.63	360±20.24
C	125.60±8.38*	39.40±3.55	384±22.04
E.O100	118.5±6.61**	36±2.16	356.2±21.34
E.O200	113±8.13**	35±2.16	371.2±22.94

NC: negative control group; C: control group; E.O: caraway essential oil (100 & 200 mg/kg b.w) treated groups. *P<0.05 is considered significantly between negative control group (NC) and control group (C). **P<0.05 is considered significantly between control group (C) and caraway essential oil treated groups.

Table 3 Analysis of liver histopathological changes in different study groups

Groups	NC	C	E.O 100	E.O 200
Iron accumulation	0±0	4±0*	2.6±0.5	1±0**
Fatty degeneration	0±0	0±0*	1.3±0.5**	3±0**
Chromatin clumping	0±0	3±0*	0±0**	0±0**

NC: negative control group; C: control group; E.O: caraway essential oil (100 & 200 mg/kg b.w) treated groups. *P<0.05 is considered significantly between negative control group (NC) and control group (C). **P<0.05 is considered significantly between control group (C) and caraway essential oil treated groups.

Histological Findings

Histopathologic analysis showed any obvious lesion in the liver of negative control group indicating any iron accumulation in iron staining (Fig. 1A and Table 3). In contrast, iron oxide NP_s accumulated in the periportal hepatocytes and phagocytic cells in the liver of positive control group due to iron oxide NP_s injection. Increasing number of phagocytic cells, clumping of nuclear chromatin and apoptosis of hepatocytes observed in the periportal region of iron oxide NP_s treated group (Fig. 1 and Table 3). Administration of caraway E.O decrease in the iron aggregation which was more efficient (P<0.05) in 200 mg/kg b.w E.O treated group in comparison with positive control group. The use of caraway E.O induced fatty degeneration in the periportal hepatocytes, while the degeneration did not extended considerably by 200 mg/kg b.w E.O (Fig. 1 and Table 3).

Discussion

Our current studies have informed that the adverse effects of xenobiotic can be controlled by the treatment of the rats with natural resources enriched antioxidant compounds. They balance the liver and detoxifying enzymes [31-38]. Therefore, the research was designed to the effect of caraway E.O against iron oxide NP_s induced hepatotoxicity in *in vivo* system.

According to the data, the administration of iron oxide NP_s reduced GSH content to 200 mg/kg b.w when it exposed to overdoses of iron oxide NP_s. Generally, GSH is a vital intra/extra-cellular protective antioxidant against oxidative/nitrosative stress. Reduction of hepatic glutathione level,

increase shock and oxidative reactions in liver cells could be other effects of iron NP_s [6]. Likewise, there is a significant decrease in the level of GST as a metabolizing enzyme in the rats treated with iron oxide NP_s (Table 1). The GST_s as a family of cytosolic enzymes plays the main role in the detoxification of a range of xenobiotic agents by conjugation their metabolite produced by CYP450 to glutathione that is necessary in the maintenance of normal physiological processes [39]. So, the fall of GST level indicated probable active NP_s disposal by the liver. Moreover, the estimation of AST and ALT enzymes are excellent markers of hepatocellular injury. If the liver is injured -in cases of liver cell death inducing by shock or drug toxicity-, the enzymes will release into blood [40-42]. Previously, the level of AST ($P<0.05$) significantly went up due to 200 mg/kg b.w iron oxide NP_s in comparison with the normal group (Table 2).

On the contrary, the GSH content and GST activity in the liver treated by caraway E.O at both doses (100 and 200 mg/kg b.w) increased significantly (Table 1). The GST induction following essential oil treatments may reflect decreased oxidative stress arising from the oxygen radical scavenging activity of essential oils. Moreover, GST, a secondary antioxidant enzyme used in drug detoxification, helps in the radical-scavenging activity of GSH. Our previous results also recorded that essential oils derived from *Achillea wilhelmsii* K.Koch have protective effect on the liver damaged by over doses of paracetamol through modulating of acetaminophen-metabolizing enzyme activities, such as GST and CYP450 [32]. Furthermore, the pretreatment of the rats with two doses of caraway essential oil could restore the liver enzyme AST (Table 2). Other researchers also showed that iron oxide NPs have toxic effects on the liver due to increase the ALT, AST and ALP activities [43-44]. Another study demonstrated that the significant rise of AST, ALT and ALP levels over using maximum concentration of iron oxide NPs (150 µg/kg) [3]. Jensen *et al*. [45] confirmed that there was a direct link between serum levels of ALT and AST and iron toxicity. one study informed that the toxic influences of inhalation exposure to ferric oxide (Fe2O3) NPs in the rats. The amount of iron in the liver and the lung considerably evaluated and the rates of serum ALT, AST and ALP in iron oxide NPs-exposed group significantly lowered as compared to the unexposed controls confirmed by histopathological examinations [46]. In addition, caraway E.O at 100 and 200 mg/kg b.w could balance the level of AST activity.

Moreover, pathological assessments confirmed our biochemical data indicating that iron NP accumulated in the periportal hepatocytes and phagocytic cells in the liver of control group due to NP_s injection. Increased number of phagocytic cells, clumping of nuclear chromatin and apoptosis of hepatocytes were observed in the periportal region of iron oxide NP_s treated group (Fig. 1B and Table 3). Iron oxide NP_s accumulate in the liver and affect on the liver, stem cells and fibroblast cells that decrease of mitochondrial activity and lead to morphological changes [6].

Finding effective medicine with the least side effects is prioritized by research scientists [47]. In this case, herbal medicine with no side effects would be suitable alternatives. The natural compounds isolated from plant extracts possess multiple activities. It has been also observed that the isolated form is less active than the natural formulated compound [48]. Accordingly, the present study indicated that caraway essential oil inhibited iron oxide NP_s induced liver damages through modulating antioxidant/oxidative stress parameters in liver and plasma. Actually, it is assumed that the antioxidant activity of caraway essential oil may be responsible for protecting the liver from toxicity. In other words, caraway essential oils with the major antioxidant components as cumin aldehyde, ϒ-Terpinene, p-cymene and ϒ-terpinene-7-al might enhance the innate mechanisms of the antioxidant system or its antioxidant capacity against NP-induced oxidative stress are provided [23, 49]. Recently, we have informed the *in vitro* antibacterial and antioxidant properties of *C. carvi* essential oil [23, 49]. Indeed, we recently pointed that *C. carvi* E.O modulate the oxidative parameters of the liver and lung injuries *i.e.* myeloperoxidase (MPO) activity, thiobarbituric acid reactive substances (TBARS) and GSH levels in cecal ligation and puncture (CLP) rat system [37]. Other study also are agreement with these results that *C. carvi* oils containing the linalool, monoterpene alcohols, carvone, limonene, carvacrol, anethole, and estragol, flavonoids and other polyphenolic compounds had a considerable potential of antioxidant capacity [50]. In addition, cumin aldehyde exited in the caraway oil can eliminate the superoxide anions [51]. Another study reported the hepatoprotective effect of caraway oil against a hepatotoxin by maintaining the activities of enzymes (GST) and glutathione peroxidase (GSH-Px) and

GSH level as well as preventing LP which is the major consequence of the action of this hepatotoxic action [22]. The essential oil of *Thymbra capitata L.* has γ-terpinene and p-cymene, showing significantly high antioxidant activity [52].

Conclusion

The hepatoprotective activity of the caraway E.O against iron oxide NP_s which induced the liver injuries, approved when it modulated the levels of GST and GSH. . These findings were also confirmed by the liver enzymes (AST) and histopathological findings.

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Authors' contribution: All data and methods of the current study were confirmed by all authors.

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