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# Protective Effects of *Origanum onites* Essential Oil in the Methotrexate-Induced Rat Model: Role on Apoptosis and Hepatoxicity

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**Abstract:** Methotrexate (MTX) is an effective cytotoxic agent which is used to treat malignancies and inflammatory diseases. *Origanum onites* (*O. onites*) is found throughout the Eastern Mediterranean region and has been used in traditional medicine. The essential oils (EOs) of *O. onites* rich in highly bioactive phytochemicals such as carvacrol (CVR) and has antiviral, antioxidant, anticancer and proapoptotic properties. The aim of this study was to investigate the protective effects of CVR and *O. onites*-EO treatment in MTX-induced hepatorenal toxic rats' liver and kidney tissues. The bcl-2/bax ratio, glutathione (GSH) level, malondialdehyde (MDA) level, and myeloperoxidase (MPO) activity of liver and kidney tissues were evaluated in the MTX-induced rat model. Results showed that the administration of CVR or *O. onites*-EO significantly increased bcl-2/bax expression and GSH levels as well as reduced MDA level and MPO activity in the kidney and liver tissues of MTX-induced rat model. In conclusion, our results suggest that *O. onites*-EO and CVR have protective effect in MTX-induced hepatorenal toxic rats' liver and kidney tissues by decreasing oxidative stress and apoptosis.

**Keywords:** Methotrexate; *Origanum onites;* essential oil; hepatorenal toxicity. © 2020 ACG Publications. All rights reserved.

## 1. Introduction

Methotrexate (MTX), a folic acid-analogue and effective cytotoxic agent, is used to treat malignancies and inflammatory diseases [1,2]. MTX-induced toxicity occurs as a result of the

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interaction of several factors such as dose, duration of treatment and apoptotic factors [2-6]. In the literature, high doses of MTX treatment have been reported to be associated with liver hepatotoxicity including cirrhosis and hepatic fibrosis [3]. MTX hepatotoxicity is related with elevated level polyglutamate prolonged drug residues accumulation in cell, and indirect suppression of DNA synthesis by thymidylate [3-8]. Recently, MTX-induced animal models are being developed to investigate hepatotoxicity and used for studying its pathogenesis and developing new therapeutic drugs [9,10]. Many plants contain a variety of antioxidant phytochemical and bioactive molecules that can be used in the treatment of oxidative stress related diseases. The medicinal properties of plant products are cause of increasing demand and usage worldwide. Essential Oils (EOs) are examples of natural products. They have crucial chemical characteristics and biological activities [4,11]. Essential oils consist of complex constituents of plant origin which may contain from 20 to 100 or so different volatile chemicals at varying concentrations. Due to their varied functional groups, they exhibit different bioactivities. Recent studies showed that *O. onites*-EO has antiviral effect, antioxidant, anticancer and proapoptotic properties [12,13]. *O.onites* is found throughout the eastern Mediterranean region and has been used in traditional medicine [13]. *O. onites*-EO is rich in highly bioactive phytochemicals. Carvacrol (CVR) and thymol were demonstrated to be major phytochemicals in *O. onites*-EO. Also, γ-terpinene, α-terpinene, p-cymene, linalool, β-bisabolene and α-thujene are the other main constituents of *O. onites*-EO [13-15]. Hepatotoxicity is a liver damage, and alcohol, medications and chemicals are possible reasons. Great efforts are being made by scientist to identify drugs that may reduce hepatotoxicity by interfering with the formation of hepatotoxicity with free radical scavengers. It is important that the approaches used in research are directed towards curative and / or preventive agents. In recent years, therapeutic strategies for hepatotoxicity have generally focused on possible antioxidant and anti-inflammatory substances derived from medicinal plants by scavenging reactive oxygen species and strengthening antioxidant activity. Cetin et al. [14] reported that *O. onites*-EO significantly decreased the histopathological scores and malondialdehyde (MDA) levels in cisplatin-induced hepatotoxicity rats. Additionally, *O. onites*-EO administration elevated superoxide dismutase and glutathione peroxidase (GSH-Px) activities. Aristatile et al. [2009 and 2012] showed that CVR had an important hepatoprotective and antioxidant activities in rats with D- galactosamine- induced hepatotoxicity [16,17]. Assuming that free radical scavengers (antioxidants) are protective against drug-induced nephrotoxicity and hepatotoxicity, this study aimed to investigate the possible protective effects of the intraperitoneal (i.p) administration of CVR and *O. onites*-EO on apoptotic proteins in MTX-induced hepatorenal toxicity in rats via.

## 2. Materials and Methods

### *2.1. O. onites-EO, Drugs and Chemicals*

The commercial essential oil of *O. onites* was acquired from TÜRER Inc (İzmir, Turkey) and identification of used plant (*O. onites*) to extract essential oil was done by Prof. Dr. K.H.C. Baser via comparison of the specimen has deposited at Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskisehir (ESSE 14567). Carvacrol (CVR-%98) and MTX (50 mg/mL; flacon) were purchased from Sigma (cat no: 282197; St Louis, MO, USA) and David Bull Laboratories, (Mulgrave-Victorica, Australia;). All chemicals were supplied by Sigma Aldrich Co. (St. Louis, Missouri, USA). All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

### *2.2. Analysis of O. onites-EO*

The *O. onites*-EO was analyzed by GC-MS using an Agilent 5977B GC-MSD system. Helium (0.8 mL/min) was used as carrier gas in the Innowax FSC column (L × I.D.= 60 m x 0.25 mm; $d_f$: 0.25 µm). The temperature of the oven was first maintained at 60°C for 10 min and then programmed to 240 °C at a rate of 1 °C/min after programmed to 220°C at a rate of 4°C/min. The mass spectra were recorded at 70 eV by adjusting the split ratio to 40: 1 and the injector temperature to 250 °C. The injected sample (1µL) was dissolved in 10% with *n*-hexane. The relative percentage of the separated compounds was

calculated using flame ionization detector (FID) chromatograms with a detector temperature of 300 °C. The same elution sequence was obtained by GC-MS (an Agilent 7890B GC) by simultaneous automatic injection of three copies of the same column.

*2.3. Identification of Compounds*

A comparison of the relative retention times with those of the authentic samples or comparison of the linear retention indices and relative retention indices with the n-alkane series was used to identify the essential oil components. Computer matching (Wiley GC/MS Library, NIST Chemistry Webbook, in-house ''Baser Library of Essential Oil Constituents'', and MS literature were used evaluating genuine compounds and components of known oils as well as MS literature data [18-21]. Relative percentage amounts of the separated compounds were calculated automatically from peak areas of the total ion chromatogram. *n*-alkanes were used as reference points in the calculation of relative retention indices [22,23].

*2.4. Animals and Conditions*

All experimental procedures involving animals were performed in accordance with the NIH (Publications No. 8023, revised 1978). After obtaining approval from the Marmara University Local Committee on Animal Research Ethics, the experiments were started (MUHDEK approval no: 04.11.2019, Ref. No: 81). Rats were supplied by the Marmara University Experimental Animals Research and Implementation Centre (DEHAMER). Forty-eight female/male Wistar albino rats weighing 200-250 g were housed in accordance with ethical rules, at 22 ± 2 °C, 12 h light / dark cycle, humidity of 50 ± 5 %, and accessed to water and food *ad libitum*. Rats were randomly divided into six experimental groups including six rats in each group.

*2.5. Experimental Design and Hepatotoxicity Protocol*

The *O. onites*-EO was diluted in neutral olive oil prior to administration into the rats. After a single dose of physiological saline (PS) was administered to the vehicle groups, PS (1 mg /kg/ ip, Vehicle-treated rats were used as a control), *O. onites*-EO (1 mg /kg/ ip, OO group) [14,24,25], or CVR (1 mg /kg/ ip, CVR group) [25] was administered for 5 days. Following administration of a freshly prepared PS as a single dose of MTX (20 mg /kg/ ip) to the hepatotoxicity groups on the first day, PS (1 mg /kg/ ip, MTX group), *O. onites*-EO (1 mg /kg/ ip, MTX+OO group), or CVR (1 mg /kg/ ip, MTX+CVR) was administered for 5 days. The last day of experiment ($6^{th}$ day), the rats were killed by decapitation. The kidney and liver tissues were excised and stored at -80° C for the analysis of malondialdehyde (MDA), and glutathione (GSH) levels, myeloperoxidase (MPO) activity, and to determine apoptotic protein expression levels.

*2.6. The Assays of GSH level, MDA level, and MPO Activity*

Tissue samples of the liver and kidney were homogenized in ice-cold using 150 mM KCl for the determination of the levels of GSH and MDA (expressed as µg/g and nmol/g; respectively) [26], whereas the Hillegeas Method was used in the determination of the activity of MPO (expressed as U/g) [27]. The absorbances of colors obtained from GSH, MDA and MPO results were measured at 412, 532, and 460 nm respectively using a UV-Vis spectrophotometer (VersaMax, Molecular Device, Sunnyvale, USA).

*2.7. Immunoblotting Analysis*

Frozen tissues were homogenized, and then centrifuged at 2000 x g for 10 min, finally incubated with 0.5 mM DTT, 1 % glycerol, 0.1 mM EDTA, 10 mM Tris-HCl, protease inhibitors and 0.05 % Triton X-100 for 60 min. Lowry method was used to the determination of the protein amount in tissues [28]. Samples containing 100 µg of protein (for both tissues) were loaded on gel electrophoresis and transferred onto nitrocellulose membranes (Schleicher and Schuell, 0.45 m, Germany) at 80 V for 70

min. Following incubation overnight with primary antibodies [bcl-2 (1:200 cat. no: sc-7382) or bax (1:100 cat. no: sc-20067)] at +4 °C, all the membranes were incubated with alkaline phosphatase-conjugated rabbit monoclonal anti-goat IgG secondary antibodies (1:1500) for 1 h. Bio-Rad Molecular Analyst software (free edition, www.totallab.com) was used densitometric analysis of the membranes [29,30]. Molecular weights for bcl-2, bax, and β-actin (was used for standardization in all membranes, (1:100 dilution, cat. no: sc-130657) are 26, 23, and 43 kDa, respectively.

*2.8. Statistically Analysis*

Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). All data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was employed to compare multiple groups followed by Bonferroni's multiple comparison tests to determine the differences between multiple groups. The values of p <0.05 were regarded as significant.

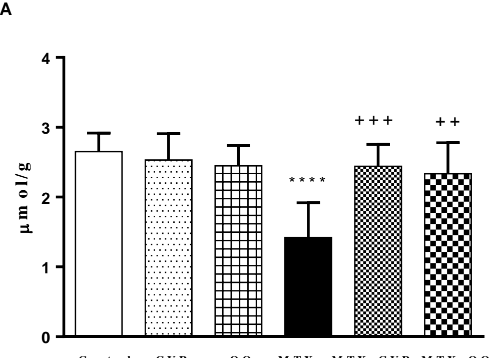
## 3. Results and Discussion

*3.1. Chemical Composition of the O. onites-EO*

The content of *O. onites*-EO obtained by steam distillation was determined by GC-FID and GC-MS. Results are given in Table 1. As shown in Table 1, carvacrol (78.4 %) was found to be a major component of the oil followed by γ-terpinene (6.9 %) and *p*-cymene (4.1 %) respectively.

*3.2. The evaluation GSH, MDA Levels, and MPO Activity in Tissues*

Biochemical parameters such as MDA, GSH, and MPO were measured in liver and kidney tissues to evaluate the efficacy of CVR and *O. onites*-EO against MTX-induced hepatotoxicity. The GSH levels of the liver and kidney significantly reduced in MTX group than the control group (p <0.0001, t=5.690, df=40; p<0.001, t=4.648, df=40; respectively). The treatment of CVR and *O. onites*-EO significantly increased liver and kidney GSH levels in MTX+CVR and MTX+*O. onites* groups compared to the MTX group (p <0.001, t=4.717; p <0.01, t=4.229, df=40 for liver; p <0.01, t=3.914; p <0.01, t=3.757, df=40 for kidney) (Figure 1 A and B).



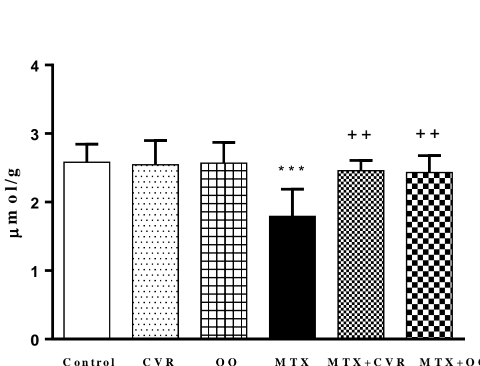




**Figure 1**. Glutathione levels (μmol/g) in liver (A), and kidney (B) tissues of control, carvacrol, *O. Onites*-EO, methotrexate, methotrexate + carvacrol and methotrexate + *Origanum onites* groups. Each group consists of six rats. All data was expressed as mean ± SD and the group of data were compared with one-way ANOVA followed by Bonferroni's multiple comparison test.

*** The data was significant when compared with control group (p<0.001), **** The data was significant when compared with control group (p<0.0001), ++ The data was significant when compared with MTX group (p<0.01) +++ The data was significant when compared with MTX group (p<0.001), MTX: methotrexate, CVR: carvacrol, OO: *Origanum onites oil*

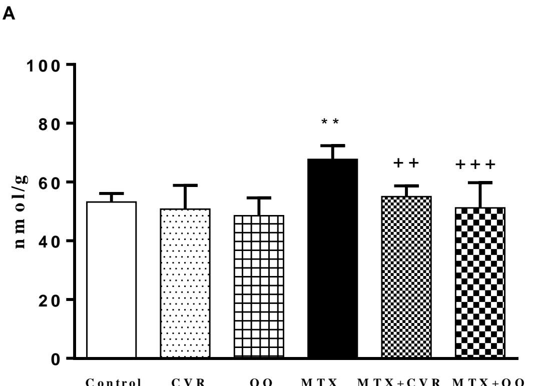
**Table 1.** Essential oil composition of *Origanum onites* L.

| LRI | RRI | Compound | Relative amounts (%) |
|---|---|---|---|
| 1020 | 1032 | α–Pinene | 0.4 |
| 1024 | 1035 | α-Thujene | 1.1 |
| 1072 | 1076 | Camphene | 0.3 |
| 1119 | 1118 | β–Terpinene | 0.1 |
| 1172 | 1174 | Myrcene | 0.6 |
| 1177 | 1179 | α–Phellandrene | 0.2 |
| 1191 | 1188 | α-Terpinene | 1.3 |
| 1213 | 1203 | Limonene | 0.2 |
| 1222 | 1218 | β–Phellandrene | 0.2 |
| 1260 | 1255 | γ-Terpinene | 6.9 |
| 1287 | 1280 | *p*-cymene | 4.1 |
| 1298 | 1290 | Terpinolene | 0.1 |
| 1457 | 1452 | 1-octen-3-ol | 0.1 |
| 1478 | 1474 | *trans*-Sabinene hydrate | 0.4 |
| 1555 | 1553 | Linalool | 1.4 |
| 1564 | 1556 | *cis*-Sabinene hydrate | 0.2 |
| 1569 | 1565 | Linalyl acetate | 0.1 |
| 1624 | 1609 | Terpinene-4-ol | 0.7 |
| 1628 | 1612 | β-Caryophyllene | 0.5 |
| 1638 | 1628 | Aromadendrene | 0.1 |
| 1717 | 1706 | α-Terpineol | 0.2 |
| 1728 | 1719 | Borneol | 0.4 |
| 1748 | 1741 | β-Bisabolene | 1.1 |
| 1770 | 1751 | Carvone | t |
| 1786 | 1773 | δ–Cadinene | t |
| 1793 | 1776 | γ–Cadinene | 0.1 |
| 1899 | 1890 | Carvacryl acetate | 0.1 |
| 2033 | 2008 | Caryophyllene oxide | 0.1 |
| 2159 | 2144 | Spathulenol | 0.1 |
| 2205 | 2187 | T-Cadinol | 0.2 |
| 2210 | 2198 | Thymol | 0.2 |
| 2243 | 2239 | Carvacrol | 78.4 |
| | | **Total** | 100.0 |

LRI: The linear retention indices against the n-alkane series. RRI: relative retention indices calculated against n-alkanes on a polar column [22]. t: Trace (<0.1%)

MTX treatment increased MPO activity in liver and kidney tissues (p <0.001, t=4.881, df=40; p <0.0001, t=5.491, df=40; respectively). After CVR and *O. onites*-EO treatment, liver tissue MPO levels were significantly decreased in rats that have MTX-induced hepatotoxicity than untreated MTX group. (p <0.01, t=3.741; p <0.05, t=3.800; df=40, respectively) (Figure 3A). In kidney tissue, MPO level significantly reduced after treatments with CVR and *O. onites*-EO in MTX group when compared with untreated MTX group (p <0.05, t=3.101, df=40; p <0.05, t=3.222, df=40) (Figure 3B).

MDA levels of liver and kidney tissue were significantly higher in the MTX group than those of control groups (p <0.01, t=4.142, df=40; p<0.001, t=4.489, df=40; respectively). Additionally, significantly decreased MDA levels were measured in CVR and *O. onites*-EO treated MTX-induced hepatotoxicity group in both liver and kidney tissue (p <0.01, t=3.620, df=40; p <0.001, t=4.712, df=40 for liver; p <0.05, t=3.050, df= 40; p <0.001, t=3.020, df=40 for kidney) (Figure 2 A and B).



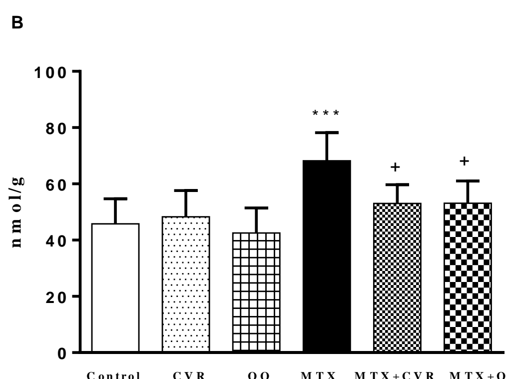
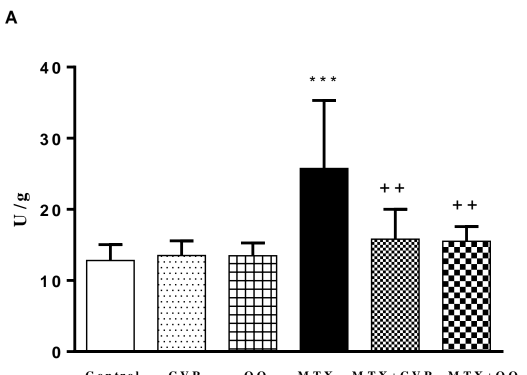




**Figure 2.** Malondialdehyde (MDA) levels in liver (A), and kidney (B) tissues of control, carvacrol, *O. onites*-EO, methotrexate, methotrexate + carvacrol and methotrexate + *Origanum onites* oil groups. Each group consists of six rats. All data was expressed as mean ± SD and group of data were compared with one-way ANOVA followed by Bonferroni's multiple comparison test.
** The data was significant when compared with control group ($p<0.01$), *** The data was significant when compared with control group ($p<0.001$), $^{+}$ The data was significant when compared with MTX group ($p<0.05$)
$^{++}$ The data was significant when compared with MTX group ($p<0.01$), $^{+++}$ The data was significant when compared with MTX group ($p<0.001$), MTX: methotrexate, CVR: carvacrol, OO: *Origanum onites oil*



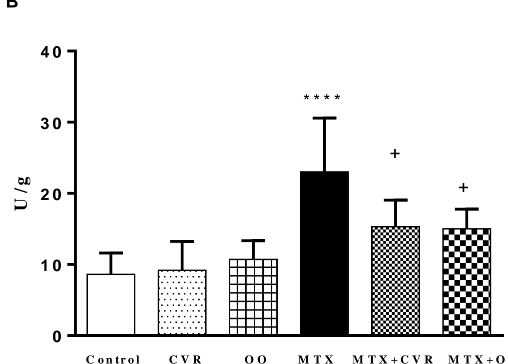



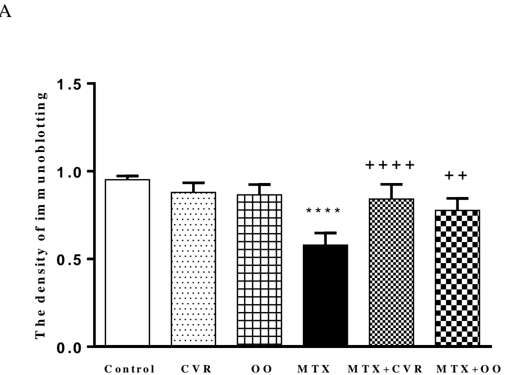

**Figure 3.** Myeloperoxidase (MPO) activity in liver (A), and kidney (B) tissues of control carvacrol, *O. onites*-EO, methotrexate, methotrexate + carvacrol and methotrexate + *Origanum onites* oil groups. Each group consists of six rats. All data was expressed as mean ± SD and group of data were compared with one-way ANOVA followed by Bonferroni's multiple comparison test.
*** The data was significant when compared with control group ($p<0.001$), **** The data was significant when compared with control group ($p<0.0001$), $^{+}$ The data was significant when compared with MTX group ($p<0.05$)
$^{++}$ The data was significant when compared with MTX group ($p<0.01$), MTX: methotrexate, CVR: carvacrol, OO: *Origanum onites oil*

### *3.3. Immunoblot Analysis*

Western blotting was performed to investigate the impacts of CRV and *O. onites*-EO treatments on the expression levels of bcl-2 and bax protein in the liver and kidney tissues of MTX-induced hepatorenal oxidative injury rats (Figure 5). MTX treatment caused a statistically significant increase in the expression level of bax in comparison with the control groups in liver and kidney tissues. The

expression level of bax was significantly increased in the MTX group when compared with the MTX+CVR and MTX+ *O. onites* groups. On the other hand, in the MTX-induced hepatorenal oxidative injury rats, the levels of bcl-2 were significantly decreased in comparison with the control, MTX+CVR, and MTX+ *O. onites* groups. Moreover, according to Figure 4, there was no significant difference between the control, CRV and *O. onites*-EO groups when both expressions level was compared in both tissues ($p>0.05$). A comparison of the level of the bcl-2/bax ratio indicated that in the MTX group, it was significantly decreased in comparison with the control group ($p<0.0001$, $t=8.464$, $df=20$ for liver, $p<0.001$, $t=5.766$, $df=20$ for kidney). Moreover, MTX group bcl-2/bax ratio was significantly decreased in comparison with the MTX+CVR, and MTX+*O. onites* groups ($p<0.0001$, $t=5.965$, $df=20$; $p<0.01$, $t=4.488$, $df=20$ for liver; $p<0.01$, $t=4.382$, $df= 20$, $p<0.01$, $t=3.863$, $df= 20$ for kidney).

A

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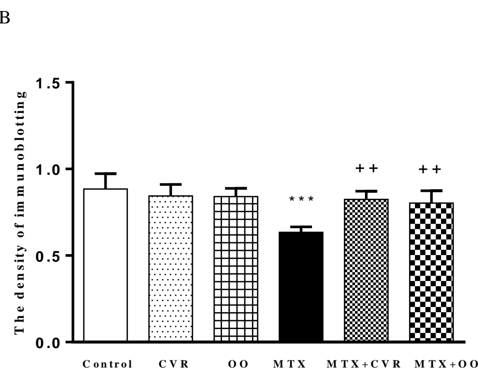


**Figure 4.** The expression levels of bcl-2 / bax ratio after carvacrol and *O. onites*-EO treatments in MTX-induced hepatorenal oxidative injury rat model in liver (A) and kidney (B) tissues.
*** The data was significant when compared with control group ($p<0.001$), **** The data was significant when compared with control group ($p<0.0001$), ++ The data was significant when compared with MTX group ($p<0.01$)
++++ The data was significant when compared with MTX group ($p<0.0001$), MTX: methotrexate, CVR: carvacrol, OO: *Origanum onites oil*

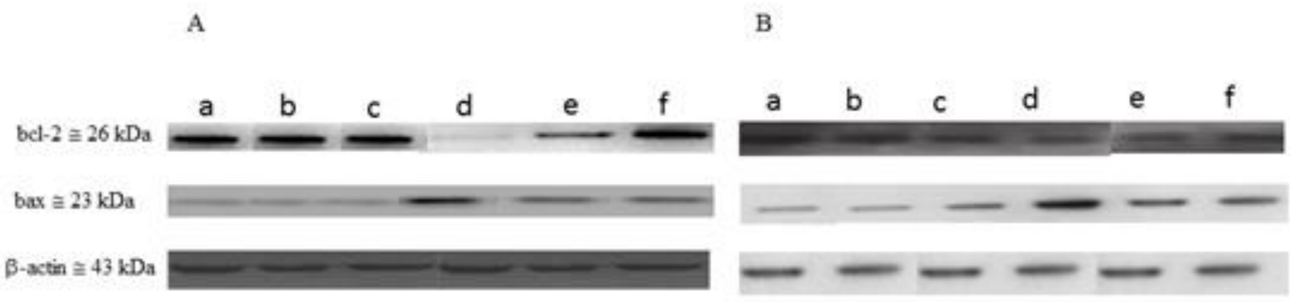


**Figure 5.** Effect of carvacrol and *O. onites*-EO treatment on bcl-2 and bax expression in liver (A) and kidney (B) tissues in MTX-induced hepatorenal oxidative injury rat model. Western blots were obtained using the antibodies directed against bcl-2 and bax. Lanes and abbreviations are as follows: **lane a**, control group; **lane b**, carvacrol group; **lane c**, *O. onites* group; **lane d**, methotrexate group**; lane e**, methotrexate+ carvacrol group; **lane f**, methotrexate + *O. onites* oil group.
The immunoblots indicate the bcl-2 (≅ 26 kDa), and bax (≅ 23 kDa) levels (duplicated). β-actin (≅ 43 kDa) was used to normalize the amount of protein loaded in each lane.

In recent years, many researchers have focused their efforts on antioxidant and hepatoprotective properties of O. onites-EO and carvacrol. To date, there is no study investigating the effects of O. onites-EO and carvacrol with respect to antioxidant and apoptotic activities in MTX-induced hepatorenal oxidative injury rat model. Therefore, the antioxidant and protective properties of carvacrol and *O. onites*-EO against liver and kidney tissues in MTX-induced hepatorenal toxicity in rats were investigated for the first time in the present study.

Mitochondrial function was impaired and reactive species were increased by MTX treatment. Excess amount of oxidative stress causes damage in tissues and organs. GSH is a non-protein sulfhydryl compound that protects the cell from oxidative injury and inhibiting lipid peroxidation. It is known that the oxidative degradation of lipids containing fatty acids defined as lipid peroxidation causes damage to the cell membrane and generates a number of secondary products such as MDA [8,31]. Canbek et al. [2008] reported that carvacrol had protective effect on liver tissue by increasing GSH and decreasing MDA levels [32]. In accordance with the literature, in the control animals exposed to MTX-induced hepatorenal toxicity, there was a considerable increase in the liver, and kidney MDA levels, indicating stress-induced lipid peroxidation. Also, they showed that GSH levels decreased with an excess amount of oxidative stress caused by MTX treatment [31]. It has been indicated that carvacrol increases total antioxidant capacity levels *in vivo* and *in vitro* studies [31]. Aristatile et al. [2015] reported that CVR protects human peripheral lymphocytes against lipid peroxidation by reducing the rate of oxidative stress [33]. Carvacrol including essential oils such as *O. onites* have antioxidant and hepatoprotective activities [9,34]. In the current study, the MDA levels significantly decreased in MTX-induced hepatorenal toxic rat's liver and kidney tissues after treated with carvacrol and *O. onites*-EO. Additionally, GSH levels significantly elevated in rat’s kidney and liver tissues after treated with CVR and *O. onites*-EO. These data suggest that both *O. onites*-EO and carvacrol can inhibit oxidative stress and has protective properties in MTX-induced hepatorenal toxic rat's through obstructed production of MDA and increased GSH levels.

Myeloperoxidase is a pro-inflammatory enzyme, mainly produced by activated neutrophils [35]. Many organ injuries mediated by neutrophils have been reported in various experimental animal models such as liver and kidney [36-38]. Result from tissue injury, local upregulation of inflammatory mediators and chemical/drug-induced oxidative stress such as MTX in liver and kidney can increase neutrophils and, also MPO enzyme level [35]. Considering our results, the levels of MPO significantly decreased in both carvacrol and *O. onites*-EO treated MTX-induced hepatorenal toxic rats' liver and kidney tissues. It may therefore be concluded that both carvacrol and *O. onites*-EO has protective effects against relevant inflammatory mechanisms, including neutrophils and MPO enzyme.

Bcl-2 is an outer mitochondrial membrane anti-apoptotic protein that has a prominent role in the regulation of apoptosis. Bax, bcl-2 homologue, is a cytosolic protein which displays a pro-apoptotic function. Bcl-2 prevents bax from releasing cytochrome c in mitochondria, thus decreases apoptotic machinery activation in cells. It has been preferred that the ratio of bcl-2 to bax may govern the apoptotic stimuli sensitivity of cells [39]. Al-Fatlawi showed that low dose of carvacrol treatment significant increase in bcl-2 gene mRNA level however high dose of carvacrol decreased bcl-2 gene mRNA transcript and increased bax gene transcript in HepG2 and SiHa cells [40]. On the other hand, Sadeghzadeh et al. [2018] demonstrated that CVR treatment increased bcl-2 and bcl-xL mRNA when compared with control group in a rat model of aortic banding-induced cardiac hypertrophy [41]. We have found no data on the effects of *O. onites*-EO on bcl-2 and bax protein levels in the literature. Thus, this is the first study of the potential effect of *O. onites*-EO and carvacrol bcl-2/bax protein expression ratio in MTX-induced hepatorenal toxic rats' liver and kidney tissues. Our results showed that CVR and *O. onites*-EO treatment increased bcl-2 protein level and decreased bax protein level. The result inferred that carvacrol and *O. onites*-EO treatment may prevent apoptosis in MTX-induced hepatorenal toxic rats' liver and kidney tissues via the increased intensity ratio of bcl-2/bax proteins.

In conclusion, we identified an active chemical component of *O. onites*-EO in this study. Our results suggest that *O. onites*-EO rich in carvacrol and carvacrol have protective effect in MTX-induced hepatorenal toxic rats' liver and kidney tissues by decreasing oxidative stress and apoptosis.

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