The protective effect of carvacrol on acetaminophen-induced renal damage in male rats

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Abstract
Background  Acetaminophen overdose causes renal injury via oxidative stress and apoptosis induction. Carvacrol has several pharmacological properties such as antioxidant, anti-inflammation and anti-apoptotic effect. The aim of this study was to determine the protective effect of carvacrol on acetaminophen-induced renal damage in rats.

Methods and results  Forty male Wistar rats were randomly divided to five groups (n = 8) including control, carvacrol 10 mg/kg, acetaminophen, acetaminophen + carvacrol 5 mg/kg, and acetaminophen + carvacrol 10 mg/kg. Animals received a single dose of acetaminophen (500 mg/kg), then were treated with carvacrol for 1 week (daily). Afterwards, renal blood flow (RBF), mean arterial pressure, renal perfusion pressure, renal vascular resistance (RVR), blood urea nitrogen (BUN), and serum creatinine were measured. Also, malondialdehyde (MDA) concentration, glutathione peroxidase (GPx) and superoxide dismutase (SOD) activity levels were measured in the kidney tissue. Hematoxylin and eosin method was used for histological assessment. The Western blotting analysis was used to determine the Bax, Bcl-2 and cleaved caspase-3 proteins expression level in the kidney tissue. Carvacrol (10 mg/kg) significantly increased the RBF, GPx and SOD activities and also reduced the RVR, serum creatinine, BUN, and MDA in the acetaminophen + carvacrol 10 mg/kg group versus acetaminophen group (P < 0.05). Also, carvacrol significantly decreased the cleaved caspase-3, Bax proteins expression level, and kidney tissue damage score in the acetaminophen + carvacrol 10 mg/kg group versus acetaminophen group (P < 0.05).

Conclusions  This study showed that carvacrol can attenuate the acetaminophen induced acute kidney damage via suppressing oxidative stress and apoptosis biochemical factors.

Keywords  Acetaminophen · Oxidative stress · Apoptosis · Carvacrol · Antioxidant · Protective effect

Introduction

Currently, acetaminophen (paracetamol) is widely applied as a febrifuge and analgesic drug worldwide, but it induces kidney injury at high doses administration [1]. So that, acetaminophen overdose causes renal tubular damage and uremia in animals and humans [2] and it induces renal injury at least in 1–2% of patients [3]. It has been shown that phenacetin (phenacetin’s ether is cleaved to leave acetaminophen, which is the clinically relevant analgesic) has a nephrotoxic effect at high dose administration [2]. Exceeding acetaminophen consumption (more than maximum medication dose) is one of the most common causes of liver and renal damage worldwide [3]. Although the mechanisms involved in renal injury following high dose acetaminophen administration are not well understood, it has been shown that N-acetylcysteine can be used to treat acetaminophen-induced hepatotoxicity. N-acetylcysteine increases the hepatic glutathione stores.
but is not able to protect the kidneys against acetaminophen overdose administration [4]. The molecular mechanism of nephrotoxicity is probably different from hepatotoxicity [2]. It has been shown that, taking high doses of acetaminophen weakens the kidneys’ antioxidant system and causes acute kidney damage [5]. Moreover, it has been reported that acetaminophen induces apoptosis by activating the caspase cascade [2]. Today there is a tendency to use natural products for the treatment of various disorders in several experimental and clinical trials investigations [6–9]. In addition to their protective effects, they reduce the risk of side effects caused by the use of chemical drugs such as nephrotoxicity [10, 11]. Along with classic antioxidants in plants, phenolic compounds have been identified as important antioxidants [12]. Carvacrol is a main component of essential oils of phenolic compounds. Carvacrol is a monoterpenic phenol and exists in various plants such as Origanum vulgare and Thymus vulgaris [13]. Carvacrol has various biological properties such as antioxidant, antibacterial, antifungal, anti-cancer, anti-inflammatory, antispasmodic, and vasodilator [14]. In addition, it has been shown that by increasing free radicals in the body, an imbalance is created between oxidants and antioxidants, which is defined as oxidative stress, leading to oxidative damage [15]. So, previous studies have shown that antioxidant compounds can protect the damage of various body tissues by scavenging the harmful free radicals in oxidative stress situation [6–8, 16]. In this regard, it has been observed that carvacrol can effectively neutralize free radicals such as peroxyl radicals, superoxide radicals, and hydrogen peroxide [17]. Carvacrol has renoprotective effect via its phenolic hydroxyl group in renal ischemia–reperfusion model [18]. Since acetaminophen overdose administration can induce acute renal damage, accordingly, this study was designed to evaluate the protective effect of carvacrol on acetaminophen-induced renal damage in male rats.

**Experimental groups and protocol**

After the adaptation period (1 week), rats were randomly assigned to five groups (n = 8 per group) including:

Group 1: (control/vehicle): animals in this group received vehicle (1% tween 80 in normal saline; 0.5 ml) intraperitoneally (i.p.) to eliminate the role of daily i.p. injection [19].

Group 2: (carvacrol): animals in this group received carvacrol (10 mg/kg) (Sigma, USA) for 1 week (daily, i.p.) [20].

Group 3: (acetaminophen): this group received acetaminophen (500 mg/kg) (Sigma, USA) in a single dose by i.p. injection [3].

Group 4: (acetaminophen + carvacrol 5 mg/kg): this group initially received a single dose of acetaminophen (500 mg/kg, i.p.). Then rats were treated with carvacrol (5 mg/kg, i.p.) 1 h later for 1 week (daily).

Group 5: (acetaminophen + carvacrol 10 mg/kg): this group initially received a single dose of acetaminophen (500 mg/kg, i.p.). Then rats were treated with carvacrol (10 mg/kg, i.p.) 1 h later for 1 week (daily) [20].

A single dose of acetaminophen (500 mg/kg, i.p.) was used in this study [3]. In the treatment groups, rats first received high-dose of acetaminophen and then were treated with carvacrol (5 or 10 mg/kg, i.p.) for 1 week [20]. The animals in group 2 received only carvacrol (10 mg/kg) without acetaminophen. The acetaminophen and carvacrol were dissolved in 1% tween 80 in normal saline solution [19]. Finally, 24-h after the last intervention, the rats were weighed and anesthetized with urethane (1.7 g/kg, i.p.) (Sigma, USA) to measure the hemodynamic parameters.

**Hemodynamic parameters measurement**

At first, the rats’ trachea was intubated via polyethylene tube (Microtube Extrusions, Australia) to facilitate breathing [21]. Then, two polyethylene tubes were used for catheterizing the left carotid and femoral arteries [22]. Mean arterial pressure (MAP) and renal perfusion pressure (RPP) were assessed via these catheters jointed to two transducers linked to PowerLab hardware (ADInstruments, Australia) and lab chart software. Also, the left renal artery was exposed and renal blood flow (RBF), as perfusion unit (PU), was measured by help of a laser-Doppler perfusion monitoring instrument (DRT4, Moor Instruments, UK) [23]. The hemodynamic parameters were recorded for 30 min, then the last 5 min of recording time were used for analysis [24]. RPP to RBF ratio was...
applied for the calculation of the renal vascular resistance (RVR) (mm Hg/perfusion units) indicator [23]. During the measurement period, the rat’s body temperature was sustained at 37 °C via a heated platform.

**Blood urea nitrogen and creatinine measurement**

After hemodynamic parameters measurement, heart puncture was used for blood samples collection. Then rat’s blood samples were centrifuged at 6000 rpm for 20 min. The blood urea nitrogen (BUN) (Pars Azmoon, Iran, BT 3500) and creatinine (Cr) (Pars Azmoon, Iran, PA1022013) concentrations were evaluated via quantitative determination kits.

**Oxidative stress parameters assessments**

The rats were killed in deep anesthesia. Kidneys were removed. One of them was homogenized in ice-cold buffer solution and centrifuged (20 min at 6000 rpm). The supernatant was prepared for oxidative and apoptosis parameters. Some of the oxidative stress parameters like malondialdehyde (MDA) (Zellbio-Germany, ZB-MDA-A96) concentration, glutathione peroxidase (GPx) (Zellbio-Germany, ZB-GPx-A96) and superoxide dismutase (SOD) (Zellbio-Germany, ZB-MDA-A96) activity levels were measured in the kidneys using their commercial kits [25–27].

**Histopathological assessments**

The other kidney was kept in formalin (10%) for histological assessment. Then histological staining was done via hematoxylin and eosin (H&E) method. Kidney tissue damage score (KTDS) was applied for histopathological assessments. KTDS was considered based on the tubular vacuolization and dilatation, debris, hyaline cast, interstitial edema and interstitial infiltration in the kidney tissue [28, 29]. The slides were graded from zero to three based on intensity of kidney tissue damage for each sample (0–0.5 = normal, 1 = minor damage, 2 = moderate damage, 3 = severe damage) [6, 7].

**Apoptosis parameters measurement**

The immunoblotting method was done to measure the Bax, Bcl-2 and cleaved caspase-3 proteins expression level in the kidney tissue. In brief, each protein sample was separated from 12.5% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. Each membrane was incubated overnight (at temperature of 4 °C and pH 7.4) in Tris-buffered saline and Tween-20 (20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) with 5% nonfat milk. Then polyvinylidene difluoride membranes were incubated with rabbit monoclonal anti-Bax (1:1000, ab184787, Abcam, USA), and rabbit monoclonal anti-Bcl-2 (1:1000, ab196495, Abcam, USA) antibodies for 3 h at temperature of 20–22 °C. Subsequently, each blot was washed with 20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20 (three times), and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5000, ab205718, Abcam, USA) at room temperature for another hour. Subsequently, blots were detected via an enhanced chemiluminescence method. Band densitometry analysis was done by the ImageJ software. Beta (β)-actin (1:5000, ab115777, Abcam, USA) was considered as a loading control.

**Statistical analysis**

All data were statistically analyzed by GraphPad Prism version 6.01 for Windows (GraphPad Software, USA). Results are presented as mean ± SD. For comparison between the groups (in quantitative values), One-way ANOVA followed by post hoc Tukey test was used. Moreover, the Kruskal–Wallis test was used for KTDS data analysis between the groups. The null hypothesis was rejected at the level of 0.05.

**Results**

**The effect of carvacrol on body weight, renal hemodynamic and functional parameters**

Based on our results, no significant difference was seen between animal body weights at the end of the study. A single dose of acetaminophen (500 mg/kg) significantly decreased the RBF in the acetaminophen administered rats compared to the control group (P < 0.05, Table 1). Furthermore, carvacrol (10 mg/kg) could significantly increase the RBF in the acetaminophen + carvacrol 10 mg/kg group compared to acetaminophen administered rats (P < 0.05, Table 1). Moreover, acetaminophen (500 mg/kg) did not significantly increase the RVR indicator (Table 1). Moreover, the RVR was decreased in the acetaminophen + carvacrol group (10 mg/kg) versus the acetaminophen group. This means that treatment with 10 mg/kg carvacrol could reduce the RVR in the acetaminophen administrated rats (P < 0.05, Table 1). However, acetaminophen (500 mg/kg) did not significantly increase the RVR indicator (Table 1). Moreover, the RVR was decreased in the acetaminophen + carvacrol group (10 mg/kg) versus the acetaminophen group. This means that treatment with 10 mg/kg carvacrol could reduce the RVR in the acetaminophen administrated rats (P < 0.05, Table 1). However, indicators such as MAP and RPP did not change significantly in different groups (Table 1).

Our result also showed that serum creatinine (P < 0.001) and BUN (P < 0.01) increased in acetaminophen (500 mg/kg) administered animals when compared to control (Table 1). Furthermore, carvacrol (10 mg/kg) significantly decreased the serum creatinine (P < 0.001) and BUN (P < 0.05) in the acetaminophen + carvacrol 10 mg/kg group compared to acetaminophen administered rats.
Also, carvacrol at the dose of 5 mg/kg could decrease the serum creatinine level in the acetaminophen + carvacrol 5 mg/kg group (P < 0.05, Table 1).

### The effect of carvacrol on MDA concentration, GPx and SOD activities

Free radical injury (using lipid peroxidation) was evaluated after administration of acetaminophen (500 mg/kg), which was considered as MDA level. According to Fig. 1A, a single dose of acetaminophen significantly increased the kidney tissue MDA concentration in acetaminophen (500 mg/kg) administered rats rather than the control group (P < 0.05). It is notable that, MDA concentration was reduced in the acetaminophen + carvacrol group (10 mg/kg) versus the acetaminophen group. It is indicated that treatment with 10 mg/kg carvacrol could decrease the MDA level in the kidney tissue of the acetaminophen treated rats (P < 0.05, Fig. 1A).

In addition, acetaminophen (500 mg/kg) significantly decreased the GPx (P < 0.01) and SOD (P < 0.001) (two antioxidant enzymes) activity levels in the kidney tissue of acetaminophen administered rats compared to the control group (Fig. 1B, C). Treatment of the rats with carvacrol (10 mg/kg) increased the GPx and SOD activity levels in the kidney tissue of the acetaminophen + carvacrol 10 mg/kg group versus acetaminophen administered group (P < 0.05, Fig. 1B, C). Also, carvacrol at the dose of 5 mg/kg could increase the SOD activity level in the kidney tissue of the acetaminophen + carvacrol 5 mg/kg rats compared to acetaminophen administered group (P < 0.05, Fig. 1C).

### The effect of carvacrol on renal apoptosis

The results also showed that acetaminophen (500 mg/kg) markedly increases the cleaved caspase-3 (P < 0.01), Bax (P < 0.001) proteins expression level, and also decreases the Bcl-2 protein expression level (P < 0.05) in the kidney tissue of acetaminophen administered rats compared to the control (Fig. 2). Moreover, 10 mg/kg carvacrol administration could prevent the cleaved caspase-3 and Bax proteins expression levels in the kidney tissue of the acetaminophen + carvacrol 10 mg/kg animals compared to acetaminophen administered group (P < 0.05, Fig. 2A, B). As shown in Fig. 2D, carvacrol (5 and 10 mg/kg) significantly decreased the Bax:Bcl-2 ratio in the kidney tissue in the acetaminophen + carvacrol 5 mg/kg and acetaminophen + carvacrol 10 mg/kg groups compared to acetaminophen administered group (P < 0.05, Fig. 2D).

### The effect of carvacrol on kidneys tissue damage

After histopathologic analysis, no significant pathologic changes were found in the control group (Fig. 3). The hematoxylin and eosin staining results also showed that acetaminophen (500 mg/kg) administration can increase the glomerular or tubulointerstitial damage in the kidney tissue, so that severe kidney damage was seen in acetaminophen administered rats when compared with the control (Fig. 3A). Also, KTDS was significantly higher in the acetaminophen administrated group versus control (P < 0.001, Fig. 3B). The data showed that, carvacrol could significantly decrease the KTDS in the acetaminophen + carvacrol 10 mg/kg group versus acetaminophen treated group (P < 0.05, Fig. 3B).

### Table 1

The body weight, renal hemodynamic parameters values, BUN and creatinine levels according to the groups (n = 8) at the end of experiment

| Groups                        | Body weight (g) | Hemodynamic parameters | Function parameters |
|-------------------------------|----------------|------------------------|---------------------|
|                               |                | MAP (mmHg) | RPP (mmHg) | PBF (PU) | RVR (mmHg/PU) | Serum Cr (mg/dl) | BUN (mg/dl) |
| Control                       | 218 ± 3.25     | 106 ± 3.80 | 94 ± 6.18 | 155 ± 11.62 | 0.63 ± 0.07 | 0.38 ± 0.06 | 36 ± 8.21 |
| Carvacrol 10 mg/kg            | 214 ± 4.45     | 100 ± 6.39 | 95 ± 7.13 | 151 ± 12.93 | 0.65 ± 0.06 | 0.40 ± 0.08 | 38 ± 7.45 |
| Acetaminophen                 | 211 ± 4.92     | 103 ± 4.65 | 96 ± 5.54 | 123 ± 10.24* | 0.78 ± 0.10 | 1.04 ± 0.13*** | 62 ± 9.56** |
| Acetaminophen + carvacrol 5 mg/kg | 209 ± 6.13   | 104 ± 3.17 | 94 ± 3.36 | 139 ± 15.11 | 0.68 ± 0.10 | 0.81 ± 0.12# | 46 ± 9.32 |
| Acetaminophen + carvacrol 10 mg/kg | 215 ± 2.98 | 105 ± 6.18 | 89 ± 2.75 | 150 ± 12.67# | 0.59 ± 0.06# | 0.57 ± 0.11### | 42 ± 8.18# |

All statistical data were analyzed by one-way ANOVA follow-up post-hoc Tukey test. All quantitative data are shown as mean ± SD

MAP mean arterial pressure, RBF renal blood flow, RPP renal perfusion pressure, RVR renal vascular resistance, Cr creatinine, BUN blood urea nitrogen

*P < 0.05, **P < 0.01, and ***P < 0.001 compared to control

*P < 0.05, and ###P < 0.001 versus acetaminophen group
Discussion

Our result showed that a single administration of acetaminophen overdose (500 mg/kg) can decrease the RBF in rats. In this regard, it has already been reported that acetaminophen administration in a dose dependent manner induced RBF and GFR impairment in rats [30, 31]. So, this condition can affect kidney function and decrease the renal ability to concentrate urine [30]. However, acetaminophen overdose does not alter MAP, RPP, and RVR indicators significantly in our study (Table 1). In this regard, some previous studies have shown that intravenous injection of acetaminophen has an acute effect on some hemodynamic parameters (MAP, RVR, and RPP) [32, 33]. However, the data obtained in our study did not show significant changes in MAP, RVR, and RPP parameters. These findings may be due to the timeline between acetaminophen administration and hemodynamic parameter measurement (1 week later after acetaminophen administration). However, in line with our findings, some studies have shown that the effect of acetaminophen on blood pressure is transient and acetaminophen has no long-lasting effect [32]. Our study also showed that treatment with carvacrol can significantly ameliorate the RVR, and also improved the RBF in the acetaminophen administered rats (Table 1). In confirmation of this finding, it is specified that carvacrol blocks the Ca2+ influx through the membrane into the cells [34] and improves blood circulation via the reduction of vascular resistance [14]. In this regard, it has also been revealed that the vasorelaxant effect of carvacrol is mediated via endothelium transient receptor potential cation channel subfamily V member 3 (TRPV3) channels [14]. Our result additionally showed that serum creatinine and BUN increased in acetaminophen administered animals. In addition, carvacrol can significantly decrease the serum creatinine and BUN in acetaminophen administered rats that were treated with carvacrol (Table 1). Acetaminophen overdose also elevates the urea and creatinine plasma levels, a condition that is considered as drug-induced nephrotoxicity [16]. According to our study it has been determined that carvacrol administration can attenuate the serum creatinine and BUN in a renal ischemia–reperfusion model and subsequently reverses the pathological changes in the kidney [35]. The results of the present study also showed that carvacrol can prohibit the kidney tissue MDA overproduction in the acetaminophen administered rats (Fig. 1A). In line with our findings, it has been reported that, acetaminophen overdose increases the kidney and liver tissue MDA concentration as the second messenger of free radicals via depleting

Fig. 1 The serum MDA concentration, GPx and SOD activity levels as three oxidative stress parameters according to the groups (n = 8) at the end of experiment. All statistical data were analyzed by one-way ANOVA follow-up post-hoc Tukey test. All quantitative data are shown as mean±SD. *P<0.05, **P<0.01, and ***P<0.001 versus control group. #P<0.05 versus acetaminophen group. MDA malondialdehyde, SOD superoxide dismutase, GPx glutathione peroxidase
the antioxidant enzymes [36]. Furthermore, Bozkurt et al., (2014) revealed that carvacrol attenuates serum and kidney tissue MDA levels and inhibits oxidative stress via its antioxidant effect in rats subjected to methotrexate-induced renal damage [37]. Our study also showed that treatment with carvacrol increases the GPx and SOD enzyme activity in the kidney tissue of acetaminophen administrated rats (Fig. 1B, C). In this regard, Roy et al., (2015) showed that acetaminophen overdose reduces antioxidant enzyme activity such as SOD and GPx, thereby debilitating the antioxidant system and inducing renal oxidative injury [1]. On the other hand, Suganthi et al. (2013) have shown that carvacrol exerts a potent antioxidant effect via its hydroxyl group and scavenges the free radicals both in-vivo and in-vitro models [38]. Moreover, it has been reported that carvacrol inhibits oxidative stress-induced renal injury in the ischemia–reperfusion model [35]. These findings revealed that carvacrol via antixodative properties prohibited the oxidative stress induced by high dose of acetaminophen administration.

Our findings also showed that high dose acetaminophen administration can trigger the apoptosis cascade via over expression of evaluated pro-apoptotic factors such as cleaved caspase-3 and Bax proteins rather than anti-apoptotic Bcl-2 protein in the kidney tissue. (Fig. 2). Based on the results of our study, carvacrol can prevent acetaminophen-induced apoptotic biomarkers in the kidney tissue (Fig. 2). In line with the present study, it has been determined that acetaminophen can induce renal tubular cell apoptosis via activation of the caspase-9 and caspase-3 and cytochrome-c cascade mechanism [2]. However, Potočnjak and Domitrović (2016) showed that carvacrol can ameliorate cisplatin-induced renal injury via its anti-apoptotic (upregulation of Bcl-2 protein and downregulation of Bax proteins expression rate) effects [39]. Overall, the apoptosis measurement in the present study showed that treatment with carvacrol can prevent the acetaminophen induced cell death in kidney tissue.

Our results revealed that administration of a high dose of acetaminophen can lead to kidney histopathological damage
Furthermore, the data showed that carvacrol administration can ameliorate the acetaminophen induced histopathological damage (Fig. 3). Consistent with our study, it has been shown that acetaminophen overdose causes kidney injury via tubular necrosis and glomerular damages [1], which is consistent with our result of the renal histopathological change. In this regard, Gunes et al. (2017) reported that carvacrol improves kidney histology and ameliorates cyclophosphamide-induced renal damage [20]. Moreover, El-Sayed et al. (2015) showed that carvacrol attenuates cisplatin-induced renal injury via glomerular and tubular necrosis reduction [40]. So, based on this part of our findings, carvacrol therapy has protective effects against acetaminophen tissue cytotoxic effects.

**Conclusion**

Altogether, carvacrol exerts potent protective effects against acetaminophen-induced renal toxicity. This protective effect may related, at least in part, to a reduction in acetaminophen-induced oxidative stress and cellular apoptosis mechanisms. So, the cellular mechanisms underlying these effects may relate to a reduction in oxidative stress and apoptosis factors. Totally, carvacrol may be used as a kidney damage reducer after acetaminophen overdose administration in the future. However, further study is needed.

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**Author contributions** Conceived and designed the experiments: JH. Performed the experiments: AK, JH, MR, and AN. Analyzed the data: JH and MR. Contributed reagents/materials/analysis tools: EH, and JH. Wrote the paper: AN, JH and AK.

**Declarations**

**Conflict of interest** The authors declare that there are no conflicts of interest.

**Consent to participate** All authors voluntarily agreed to participate in this study.

**Consent to publish** All authors voluntarily agreed to publish the results of this study.

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![Fig. 3 The hematoxylin and eosin stained sections (magnification x100) (A) and Kidney tissue damage score (B) in all groups (n = 8) at the end of experiment. All statistical data were analyzed by Kruskal–Wallis test. Data are shown as mean ± SD. ***P < 0.001 versus control group. #P < 0.05 versus acetaminophen group. KTDS kidney tissue damage score](image)
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