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# Pretreatment Effect of D-Limonene on Oxidative Stress Induced by Renal Ischemia-Reperfusion Injury in Rats

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## ABSTRACT

**Background and Objective:** Reactive oxygen species are the main factors involved in kidney damage during renal ischemia-reperfusion (RIR). Since D-limonene has antioxidant, anti-diabetic, anti-apoptotic, and lipid peroxidation effects, it prevents mitochondrial dysfunction and inhibits ROS, this study was conducted to evaluate the effects of pretreatment with D-limonene on oxidative stress and antioxidant activity in RIR injury.

**Methods:** In this experimental study, 24 male Wistar rats were randomly divided into 3 groups: control, RIR (ischemia was induced by clamping of renal pedicles for 45 minutes and reperfusion was considered 24 hours after ischemia), and RIR+D-limonene (100 mg/kg by oral gavage for 12 days). Serum and kidney were used to evaluate malondialdehyde (MDA), myeloperoxidase (MPO), paraoxonase1 (PON1), glutathione (GSH), catalase (CAT), glutathione peroxidase (GPX), and nitric oxide (NO).

**Findings:** Serum and renal levels of MDA ([18.2±98.77 vs. 9.21±1.77] and [19.85±3.39 vs. 9.84±1.65]) and MPO ([67.25±32.67 vs. 40.21±6.1] and [18.44±2.86 vs. 10.42±1.68]) and serum level of NO (31.3±36.1 vs. 27.88±2.6) significantly increased in the RIR group compared with the control group ($p<0.05$). Serum and kidney levels of GSH, activities of CAT and GPX in serum and kidney, and serum activity of PON1 significantly decreased in the RIR group compared with the control group ($p<0.05$). Pretreatment with D-limonene could significantly ameliorate serum and renal levels of MDA, serum levels of GSH and NO, and serum activity of CAT in rats pretreated with D-limonene in comparison with RIR rats ($p<0.05$).

**Conclusion:** This study indicated that pretreatment with D-limonene could ameliorate RIR injuries in rats through its antioxidant activities.

**Keywords:** *D-Limonene, Renal Ischemia-Reperfusion, Oxidative Stress, Rats.*

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## Introduction

Ischemia is one of the most common damages usually induced by the reduction in blood circulation. After ischemia, anoxia, food shortage, and the stop of energy production will be happening in vascular beds of tissues (1). Renal ischemia is an important event usually occurring after kidney transplantation, heminephrectomy, correction of suprarenal aneurysm, and hemorrhagic shock. Reperfusion is essential for the survival of tissues, but it may lead to more damage known as ischemia-reperfusion injuries (IRI) (2). During ischemia, vascular endothelium promotes the production of reactive oxygen species (ROS) and chemotactic agents (3). Neutrophils migrate to the ischemic region and ROS, cytokines, myeloperoxidase (MPO) and other mediators lead to tissue damage (4). According to previous studies, ROS causes toxic effects in IRI-induced injuries such as protein oxidation, DNA damage, lipid peroxidation, and apoptosis (5, 6).

Enzymatic and non-enzymatic antioxidants are the main defense systems against damage caused by free radicals (7, 8). In addition, it has been demonstrated that the reperfusion phase suppresses endogenous antioxidant enzymes and activates inducible nitric oxide synthetase (iNOS) (9). Many natural antioxidants, such as selenium, rutin (5, 10), nobiletin and glutathione, have been indicated to ameliorate renal ischemia-reperfusion (RIR) damages (11, 12). These natural antioxidants were able to reduce oxidative stress and partially restored renal function after RIR. Chemical drugs have many side effects compared to natural antioxidants (13). Therefore, it is still important to find new natural antioxidants with beneficial effects on RIR, because natural antioxidants have no side effects and are presented as a good alternative treatment for diseases related to oxidative stress (14, 15).

D-limonene (Figure 1), also known as 1-methyl-4- (1-methylethenyl) cyclohexene, is a natural cyclic monoterpene with the smell of lemon and is considered the main compound in the essences of orange, grapefruit, cherry, and mint (16). D-limonene has a proven impact on different types of cancer (17). Also, it possesses antioxidant, anti-diabetic, anti-apoptotic, and lipid peroxidation inhibitory effects, prevents mitochondrial dysfunction, and inhibits ROS (18, 19). Published research has shown different biological activities of D-limonene, including hypolipidemic and immunomodulatory activities and hepatoprotective and dermatoprotective effects (20-22). In our previous study, we showed that D-limonene could ameliorate acute kidney injury following gentamicin administration (22). Additionally, it has been demonstrated that D-limonene alleviated gallstone- and doxorubicin-induced renal damages (23). To the best of our knowledge, there are no studies on the effects of D-limonene pretreatment on RIR in rats. Therefore, the present study was conducted to evaluate the possible protective effect of D-limonene on RIR in rats.

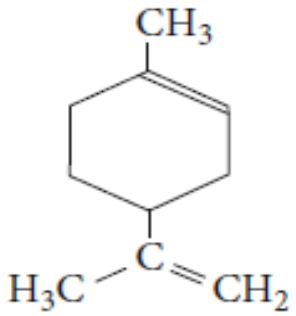


**Figure 1. Chemical structure of D-limonene (24).**

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## Methods

All of the experimental protocols in this study were approved by the ethics committee of Lorestan university of medical science with ethics code IR.LUMS.REC.1400.033. In this experimental study, 24 male Wistar rats (180-200 gr) were taken from Razi Herbal Medicine Research Center, Lorestan, Iran. These animals were kept in a room with a temperature of 24±1 °C and a 12-hour light/dark cycle in the animal lab of Razi Herbal Medicines Research Center. During the study, the animals had access to sufficient food and water.

**Design of the experiment:** The rats were randomly divided into 3 equal groups (n=8): group 1 (healthy control); group 2 (RIR): these animals underwent RIR and received normal saline (1 mL/kg) every day by oral gavage. Group 3 underwent RIR and received D-limonene (100 mg/kg) every day by oral gavage (23). Pretreatment began 12 days before RIR induction on a daily basis. Induction of ischemia was performed under anesthesia with Ketamine HCl 50 mg/kg and Xylazine 5 mg/kg by intraperitoneal injection (5). The abdominal surface was shaved and immediately scrubbed. Then, the abdominal cavity of rat was opened by a midline incision. After that, the right and left pedicles were separated carefully and occluded by appropriate clamps for 45 minutes. After 45 minutes of ischemia, clamps were removed for induction of reperfusion. Reperfusion period was 24 hours in our experiment. The control group underwent IR surgery similar to the RIR group but RIR was not induced. D-limonene (≥95%) was purchased from Sigma-Aldrich Company.

**Biochemical analysis:** After the reperfusion, under anesthesia with Ketamine HCl (50 mg/kg, IP injection) and Xylazine (5 mg/kg, IP injection), the blood samples were taken from left ventricle of rats and were allowed to clot in room temperature for 20 min. The samples were then centrifuged at 3000 rpm for 15 min. After that, the serum samples were separated and preserved at -70 °C for serum biochemical evaluations. Kidneys were removed from body and left kidney was used for renal biochemical measurements. The kidneys were homogenized with saline buffer phosphate (PBS). Then, they were centrifuged with 5000 rpm at 4 °C for 15 min, and the supernatant was used for renal biochemical measurements.

**Measurement of oxidative stress markers:** Malondialdehyde (MDA) levels in serum and kidney, as an index of lipid peroxidation, were evaluated by the thiobarbituric acid (TBA) method (24). The absorbance was measured spectrophotometrically at 540 nm wavelength and MDA content was reported as nM MDA/mg-pr.

Assessment of glutathione (GSH) contents in serum and kidney was performed spectrophotometrically at 412 nm, by Shimadzu spectrophotometer (Tokyo, Japan), based on the Ellman method (25). The Sinha method was used for measurement of serum and renal Catalase (CAT) activities (5). The reaction started after adding 20 μL of sample to hydrogen peroxide (2 mL, 30 mM) and potassium phosphate buffer (pH 7.0, 50 mM). Catalase activity was reported as the amount of hydrogen peroxide consumed per unit time (min) per ml of serum or gram of tissue.

Glutathione peroxidase (GPX) activity in serum and kidney were measured based on the method by Flohé et al. (26). A mixture containing samples, hydrogen peroxide and tert-butyl hydroperoxide was prepared. Using the ELISA reader, the samples were read at 420 nm.

Paraoxonase1 (PON1) activity in serum was assessed by utilizing paraoxon as a substrate and measured spectrophotometrically based on absorbance at 412 nm due to 4-nitrophenol production. For measurement of the enzymatic activity, 50 mL of serum was added to 1 mL of 100 mM tris-HCl buffer (containing 2

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mM CaCl2 and 5 mM paraoxon, pH=8.0) at 25 °C. The rate of 4-nitrophenol production was evaluated at 412 nm. For calculation of enzymatic activity, molar extinction coefficient of 17,000 Mcm was considered (27).

MPO activity of serum and kidney based on hydrogen peroxide caused oxidation of O-dianisidine dihydrochloride at 450 nm (28). In summary, a mixture with a final volume of 3 ml including sample (10 µl), 0.3 ml of phosphate buffer (0.01 M at 6 pH), 0.3 ml of hydrogen peroxide (0.01 M and 0.5 ml) and O-dianisidine (0.02 M) was transferred to a 1-cm long cuvette. The sample was added in the last step of mixing and the changes in the adsorption of the mixture were measured for 10 minutes at 460 nm. The unit of MPO activity was reported as unit/mg protein.

Nitrite is the final product of nitric oxide (NO), which is evaluated to measure serum NO. In our study, the method of Cheraghi et al. was used to measure nitrite (29).

Statistical analysis was performed using SPSS software and LSD tests were used to compare different groups and One-way ANOVA also used and $p<0.05$ was considered significant.

## Results

**Effect of D-limonene on biochemical parameters in RIR model:**

Serum MDA level in the RIR group significantly increased compared with the control group (18.98±2.77 vs. 9.21±1.77). D-limonene could significantly reduce serum MDA level in the pretreated group compared with the RIR group (Table 1).

**Table 1. Effects of D-limonene on serum MDA (µmol/mg protein), serum GSH (µmol/mg protein), serum CAT (U/mg protein), serum GPX (U/mg protein), serum MPO (U/mg protein) and serum PON1 (U/min) levels in study groups**

| Groups / Parameter | Control | RIR | RIR+D-limonene | p-value |
|---|---|---|---|---|
| CAT | 157.83±38.53[a] | 84.70±13.95[b] | 126.21±29.42[c] | <0.001 |
| GPX | 54.40±6.76[a] | 34.52±6.27[b] | 39.86±2.27[b] | 0.006 |
| GSH | 25.86±5.7[a] | 10.24±2.04[b] | 14.59±1.26[c] | <0.001 |
| MDA | 9.21±1.77[a] | 18.98±2.77[b] | 10.16±2.42[c] | <0.001 |
| PON1 | 78.95± 8.74[a] | 38.90±20.40[b] | 50.46±23.86[b] | <0.001 |
| MPO | 40.21±6.1[a] | 67.32±25.67[b] | 58.99±26.41[b] | <0.001 |
| NO | 17.23±2.73[a] | 31.36±3.1[b] | 27.88±2.60[c] | <0.001 |

**Different letters in each row (a, b and c) show significant difference (p<0.05).**

The level of renal MDA significantly increased in RIR rats compared with control rats (19.85±3.39 vs. 9.84±1.65). The level of renal MDA significantly decreased in the group pretreated with D-limonene compared with the RIR group (Table 2).

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**Table 2. Effects of D-limonene on renal MDA (µmol/mg protein) and renal GSH levels (µmol/mg protein), renal CAT (U/mg protein), renal GPX (U/mg protein), and renal MPO activities (U/mg protein) in study groups**

| Groups / Parameter | Control | RIR | RIR+D-limonene | p-value |
|---|---|---|---|---|
| CAT | 97.46±23.29[a] | 39.71±13.67[b] | 54.27±11.44[b] | <0.001 |
| GPX | 207.03±32.12[a] | 136.50±18.58[b] | 146.57±19.68[b] | <0.001 |
| GSH | 27.34±4.93[a] | 13.75±3.49[b] | 14.67±1.77[b] | <0.001 |
| MDA | 9.84±1.65[a] | 19.85±3.39[b] | 11.11±1.75[c] | <0.001 |
| MPO | 10.42±1.68[a] | 18.44±2.86[b] | 15.65±0.75[b] | <0.001 |

**Different letters in each row (a, b and c) show significant difference (p<0.05).**

Serum GSH level in the RIR group significantly decreased in comparison with the control group (10.24±2.04 vs. 25.86±5.7) (Table 1). In the pretreated group with D-limonene, serum GSH level significantly elevated compared with the RIR group (Table 1). The level of renal GSH significantly decreased in RIR rats compared with the control rats (13.75±3.49 vs. 27.34±4.93). D-limonene could enhance the renal level of GSH compared with the RIR group (14.67±1.77 vs. 13.75±3.49), but was not statistically significant (Table 2).

The serum CAT activity significantly decreased in the RIR group compared to the control group (84.70±13.95 vs. 157.83±38.53). The activity of serum CAT significantly increased in the pretreated group in comparison with the RIR group (Table 1). The activity of renal CAT significantly decreased in the RIR group compared with the control group (39.71±13.67 vs. 97.46±23.29). In the pretreated group with D-limonene, the increase in the activity of renal CAT was observed compared with the RIR group, but it was not statistically significant (Table 2).

The serum GPX activity in the RIR group significantly decreased compared with the control group (34.52±6.27 VS. 54.40±6.76). D-limonene could increase serum GPX activity in the pretreated group compared with the RIR group, but it was not statistically significant (Table 1). The activity of renal GPX significantly decreased in the RIR group compared with the control group (136.50±18.58 vs. 207.03±32.12). The renal GPX activity increased in the pretreated group with D-limonene compared with the RIR group, but it was not statistically significant (Table 2).

The activity of the serum PON1 significantly decreased in the RIR rats compared with control rats (38.90±20.40 vs. 78.95±8.74). However, pretreatment with D-limonene enhanced serum PON1 activity in pretreated rats compared with RIR rats, but it was not statistically significant (Table 1).

Serum NO level was observed to be significantly higher in the RIR group in comparison with the control group (31.36±3.1 vs. 17.13±2.73). Pretreatment with D-limonene could significantly decrease NO levels in the pretreated RIR group compared with the RIR group (Table 1).

The serum activity of MPO significantly increased in the RIR group in comparison with the control group (67.32±25.67 vs. 40.21±6.1). Serum MPO activity decreased in the pretreated group compared with the RIR group, but it was not significant (Table 1). Renal MPO activity significantly increased in the RIR group compared with the control group (18.44±2.86 vs. 10.24±1.68). D-limonene reduced renal MPO activity compared with the RIR group, but it was not significant (Table 2).

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## Discussion

The results of this study revealed that pretreatment with D-limonene could decrease RIR injuries in rats through its antioxidant and anti-inflammatory activities. Serum and renal MDA levels significantly increased in the RIR group compared with the control group, whereas the level of GSH and the activities of CAT, GPX, and PON1 significantly decreased in the RIR group compared with the control group. Pretreatment with D-limonene could significantly improve serum and renal levels of MDA, serum level of GSH, and serum CAT activity compared with RIR rats. MDA and antioxidant enzymes such as GPX and CAT are considered as markers for the determination of oxidative stress status. In accordance with the present study, many studies indicated that RIR induced lipid peroxidation and decreased the activities of antioxidant enzymes (10). Previous studies showed that natural antioxidant such as selenium (8), oleuropein (30), vitamin E (31), pycnogenol (32), and melatonin (33) could enhance antioxidant status and mitigate lipid peroxidation. In a research, Murali *et al.* indicated that D-limonene could mitigate lipid peroxidation and increase CAT activity in streptozotocin-induced diabetic rats (34). In other study, Santiago *et al.* showed that D-limonene could reduce oxidative stress-induced blood pressure in high-fat diet and Nω-Nitro-L-Arginine Methyl Ester. They showed that D-limonene significantly increased GSH level and GPX activity in treated group compared with untreated group (35). The use of D-limonene as a natural antioxidant could exert beneficial effects on RIR injuries through its antioxidant activities.

MPO is an enzyme associated with the oxidative stress and inflammation. It is released by neutrophils during inflammatory process. MPO catalyzes the production of HOCl as a cytotoxic agent (36). In this study, RIR significantly enhanced serum and renal MPO activities compared with the control group. The anti-inflammatory effects of D-limonene were previously reported (37). Many studies showed that natural antioxidants such as vitamin E (38), garlic oil (39) and apocynin (40) can significantly reduce MPO activity and inflammation. In our study, D-limonene reduced MPO activity, but it was not significant. This difference between our study and other studies may be associated with the treatment dose or the method or the period of antioxidant administration. Our study also showed that the level of NO significantly elevated in the RIR group in comparison to the control group. Pretreatment with D-limonene could significantly reduce NO level compared with the RIR group. It has been demonstrated that NO acts as an antioxidant and prooxidant. It is used for the determination of oxidative stress status (41). NO in physiological conditions is necessary for vascular function. However, NO in abnormal cases reacts with ROS and results in peroxynitrite formation as a cytotoxic agent (42). Some researchers showed that natural antioxidants such as selenium, vitamin E (43), and garlic oil (39) could mitigate NO level in pathological condition. Moreover, Sarika *et al.* showed that D-limonene played a role in the reduction of NO levels in the mice that experienced memory loss after receiving aluminium chloride (44). Our results and other researches indicated that natural antioxidants can decrease the level of NO. So, the use of natural antioxidants such as D-limonene with beneficial effects on NO level can reduce the complications of RIR that are associated with oxidative stress and inflammation.

This study indicated that pretreatment with D-limonene had beneficial effects on oxidative stress including MDA, GSH, and NO levels, and CAT activity. These effects can be related to the anti-inflammatory and antioxidant properties of D-limonene as previously reported (33). In our study, the detailed mechanisms of antioxidant functions of D-limonene were not fully demonstrated. We suggest more cellular and molecular investigations on the effect of pretreatment with D-limonene on damages caused by RIR.

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**Conflicts of interest:** The authors declared no competing interests.
**Ethical considerations:** Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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