Investigating the protective effect of the methanolic extract of Salvia multicaulis on renal ischemia-reperfusion injuries in rats

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ABSTRACT

In this research the protective effects of the methanolic extract of S. multicaulis was evaluated in renal ischaemia-reperfusion injuries in rats. 42 male rats were divided into 6 groups. In the treatment groups 1-3, before causing ischemia in the kidneys, rats received 50, 100, and 150 mg/kg/day doses of the extract orally for 20 days, then ischaemia was created. In the evaluation of urea and creatinine factor, rosmarinic acid and extract dose of 150 mg/kg/day had a significant effect in reducing these two factors.

Keywords: Salvia multicaulis; rosmarinic acid; renal ischaemia-reperfusion

INTRODUCTION

Ischemia means complete or partial interruption of blood flow to an organ or tissue [1, 2]. Among the causes of ischemia, the following can be mentioned: thrombosis, hypovolemia, organ transplantation, myocardial infarction, atherosclerosis, sickle cell disease,
Kidney ischaemia-reperfusion injury is a condition that can cause negative consequences such as a decrease in glomerular filtration rate (GERD), decrease in tubular reabsorption of sodium and potassium, decrease in renal blood flow, high blood pressure, and acute and chronic renal failure (AKI). Ischemic kidney injury can be the result of several conditions or causes. The causes of this damage can be kidney transplant, partial nephrectomy, renal artery revascularization, trauma, hydronephrosis, shock, sepsis, and non-emergency urology surgery [4-6]. Reperfusion which means the return of blood flow to the tissue or in other words, the restart of oxygen supply, may be more harmful than ischemic damage [2,3]. Reperfusion acts as a double-edged sword and can lead to kidney damage. Because the generated Reactive Oxygen Species (ROS) cause endothelial destruction, increase capillary permeability, cause tissue edema, necrosis, apoptosis, activation of adhesion molecules, multi-organ disorder, the release of cytokines, and cause a systemic inflammatory response [7-11]. Renal ischaemia-reperfusion injury can lead to kidney failure or dysfunction, which is a common cause of acute renal failure that is associated with a wide range of mortality. Also, one of the important concerns at the time of kidney transplantation is ischaemia-reperfusion in the transplanted tissue and subsequent rejection of the transplant [9-12]. Reactive oxygen species can cause oxidative damage to cellular macromolecules such as membrane lipids, proteins and nucleic acids and produce other free radicals of active species [13-16]. As a result, the use of antioxidants is one of the most important treatment strategies to reduce ischaemia-reperfusion injury. The mechanism of action of these antioxidants is to prevent oxidative stress by collecting free radicals and neutralizing them. Since medicinal plants create secondary metabolites that have various medicinal uses such as antioxidant and antibacterial, they have received much attention in recent years.

Flavonoids are a group of polyphenolic compounds in various herbs, including plants of the mint family (Lamiaceae), which can inhibit ROS, lipid peroxidation and have anti-inflammatory effects [17-19]. Salvia genus, grows all over the world, is known in Iran as ‘Maryam Goli’ and belongsto the Lamiaceae family. This genus has about 1000 species that are...
Ghasemian et al. scattered in warm temper regions of the world, and it has 58 species in Iran, of which about 17 species are endemic [20-22]. The Salvia multicaulis is one of the species of Salvia. In the aerial parts of Salvia, there are compounds such as rosmarinic acid, quercetin, vanillin, catechin and chlorogenic acid, which make these plants have high antioxidant power. In recent years, various investigations have been reported about the beneficial effects of natural polyphenolic compounds on ischemic reperfusion. Therefore, the purpose of this research is to evaluate the protective effects of the methanolic extract of the aerial parts of S. multicaulis in the damage caused by renal ischaemia-reperfusion in rats [22-27].

MATERIALS AND METHODS

Chemicals

Rosmarinic acid powder was from Sigma Company, ketamine and xylazine were from Rotex Medica Company, dimethyl sulfoxide, paraffin, xylene, hydrochloric acid, aluminum potassium sulfate, thio barbituric acid, potassium phosphate and hydrogen peroxide, deionized water, normal saline, mercury oxide, formalin 10 %, hematoxylin, eosin powder, tris buffer and sodium azide were from Merck Company and ethanol was from Iran.

Hematoxylin eosin staining method was used, which requires the following three solutions: Hematoxylin solution, eosin solution and acid alcohol solution, first, 50 g of aluminum potassium sulfate was added to 500 ml of distilled water in a flask with a suitable capacity, and then it was completely dissolved on the heat of the flame, then the solution containing 2.5 g of hematoxylin powder, which was in 50 ml of absolute alcohol was added to the contents of the above flask and then it was heated to the boiling point, and after the contents of the flask boiled, it was removed from the heat and after 20 seconds and a slight decrease in the temperature of the solution, red mercury oxide was added to the solution and stirred for 10 to 20 seconds by shaking (Table 1).

The flask was immediately cooled under water, and finally, for every 100 mL of this dye, 0.5 mL of glacial acetic acid was added, and as the last step, the dye was passed through a sieve and the hematoxylin solution was ready for use. To prepare the eosin solution, 80 mL of 96 % alcohol was diluted with 20 mL of distilled water and 1 gr of eosin powder was added to it, and
Finally, 0.5 g of glacial acetic acid was added to it. To prepare an acid-alcohol solution, for every 0.5 mL of hydrochloric acid, 100 mL of 70% alcohol was needed, and hydrochloric acid was added to the alcohol (Table 1).

**Plant material**

In this study, the aerial part of *S. multicaulis* plant was collected in April 2021 from the Oshtorankuh mountains of Lorestan and was identified by a botanist and dried in the shade and then crushed using a laboratory mill.

**Extraction**

400 g of dried plant aerial powder was soaked with 1000 mL of methanol for 24 h and then filtered (This operation was performed 3 times and each time for 24 h). The product obtained from filtration was distilled at room temperature (25°C) using a rotary evaporator under a hood and the remaining extract was collected. In order to completely remove the remaining methanol solvent from the methanolic extract, the extract was subjected to a freeze-dryer. The obtained extract was subjected to a freeze-dryer for 24 h to dry as well as possible. Then the necessary amount of the extract was dissolved in distilled water and it was used orally in rats according to the defined dosage.

**Tissue processing**

In the tissue processing process, dehydration, clarification and paraffin penetration into the tissue are done. Before tissue processing, to perform the dehydration step as best as possible, each kidney was divided into three parts and placed in special porous molds, and then tissue processing was performed.

**Molding**

German Merck paraffin with a melting point in the range of 57-60°C was used for molding. Paraffin was placed in an incubator with a temperature set at 60°C. After melting, it was poured into special molds. Then the tissues were placed inside the paraffin with forceps in such a way that the maximum possible information can be obtained from the tissue.

**Sectioning and staining**

Slee Mainz cut 5062 manual microtomes was used for sectioning. The tissues were prepared for cutting after the molding stage. Each template was placed in a special place in the microtome, and after ensuring the stability of the tissue block and the
distance from the microtome blade; the blade was placed in its place and fixed.

**Laboratory animals and grouping**

The obtained extract was subjected to a freeze dryer for 24 h to dry as well as possible. Then the necessary amount of the extract was dissolved in distilled water and it was used orally in rats according to the defined dosage. For the study, 42 healthy wistar male desert mice, weighing 220-250 g, were obtained from Pasteur Institute. The rats were kept in the conditions of 12 h of light and 12 h of darkness and at a temperature of about 25 °C and suitable humidity for 20 days in the animal house of Lorestan University of Medical Sciences located in Kamalvand and were randomly divided into 6 groups as follows (Each group included 7 rats).

1- Treatment group 1: In this group, before the creation of ischemia in the kidneys, rats were given 50 mg/kg/day of methanolic extract orally with specific doses for 20 days, and then ischaemia was created in them.

2- Treatment group 2: In this group, before the creation of ischaemia in the kidneys, rats were administered 100 mg/kg/day of the extract orally with specific doses for 20 days, and then ischaemia was created in them.

3- Treatment group 3: In this group, rats were given 150 mg/kg/day of the extract orally with specific doses for 20 days before ischemia was caused in the kidneys, and then ischaemia was caused in them.

4- Treatment group 4: In this group, before causing ischemia in the kidneys, rats were injected intraperitoneally with rosmarinic acid dissolved in DMSO and normal saline at the rate of 125.0 mg/kg daily for 20 days, and then they developed ischaemia.

5- Sham group: a group in which no ischemia was induced and no drug was injected.

6- Control group: a group in which ischemia was induced but no medicine was injected.

**Induction of ischaemia reperfusion**

The stages of induction of ischaemia-reperfusion are as follows: First, the exact location of the surgery was determined on the side of the rats, and then the hair in the area was shaved using a razor and sterilized with betadine. By opening the side and discarding the fats, the kidneys were found and the renal pedicles were identified. After the operation, the umbilical vessels of the
kidney were closed on both sides with a thread with gentle pressure for 45 min (at this time, the kidney turned white, and after confirming the pallor, ischemia was performed). After 45 min of ischemia, the thread knot was untied and the browning of the kidney was followed again (confirmation of reperfusion is by the reddening of the kidneys). During 45 min of ischemia, viscera and kidneys were supported with sterile moist and warm normal saline gas. After making sure that the blood flow was established, all the layers of the cut area of the animal were sewn by silk thread.

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*Data analysis*

SPSS software and Mann-Whitney U test were used to analyze the study data. ANOVA and Tukey tests were used to check parametric data, and the Kruskal-Wallis test was used to check non-parametric data. Also, to measure Tubular necrosis, Eosinophilic casts and Leukocyte infiltration were used by the Carmelo method (Table 2).

**Table 1:** Hematoxylin-eosin staining was used in this study

<table>
<thead>
<tr>
<th>Stage</th>
<th>Processing stage</th>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dehydration</td>
<td>Alcohol 70 %</td>
<td>1.5 h</td>
</tr>
<tr>
<td>2</td>
<td>Dehydration</td>
<td>Alcohol 80 %</td>
<td>1.5 h</td>
</tr>
<tr>
<td>3</td>
<td>Dehydration</td>
<td>Alcohol 96 %</td>
<td>1.5 h</td>
</tr>
<tr>
<td>4</td>
<td>Dehydration</td>
<td>Alcohol 96 %</td>
<td>1.5 h</td>
</tr>
<tr>
<td>5</td>
<td>Clarification</td>
<td>Xylose</td>
<td>45 min</td>
</tr>
<tr>
<td>6</td>
<td>Clarification</td>
<td>Xylose</td>
<td>45 min</td>
</tr>
<tr>
<td>7</td>
<td>Blending</td>
<td>Paraffin</td>
<td>1.5 h</td>
</tr>
<tr>
<td>8</td>
<td>Blending</td>
<td>Paraffin</td>
<td>1.5 h</td>
</tr>
</tbody>
</table>
**Table 2.** Carmelo’s method was used for measurement of tubular necrosis, eosinophilic casts and leukocyte infiltration as follows

<table>
<thead>
<tr>
<th>Damage Level</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>No damage</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>1 unicellular, patchy isolated</td>
</tr>
<tr>
<td>Moderate</td>
<td>2 damage less than 25%</td>
</tr>
<tr>
<td>Severe</td>
<td>3 damage between 25-50%</td>
</tr>
<tr>
<td>Very severe</td>
<td>4 more than 50% damage</td>
</tr>
</tbody>
</table>

**RESULTS**

Examining kidney functional factors: Urea and serum creatinine are also considered functional indicators of a kidney which were measured using the relevant assay kit with the autoanalyzer device. Checking and determining the amount of malondialdehyde: In the examination of the MDA factor, it was found that the ROZ, MC100 and MC150 groups’ showed a significant decrease compared to the control group, and the MDA serum level in the MC150 and ROZ groups showed a significant decrease compared to the MC50 group (Figure 1).

The amount of MDA factor in the serum sample, Sham group: the group that did not receive any medication. MC50: a group receiving the methanolic extract of *S. multicaulis* plant with a concentration of 50 mg/kg/day, which was treated with p<0.05, MC100: The group receiving the methanolic extract of *S. multicaulis* with a concentration of 100 mg/kg/day, MC150: The group receiving the methanolic extract of *S. multicaulis* with a concentration of 150 mg/kg/day, with p<0.001 compared to the control group, which is shown in the diagram with three stars, shows a significant decrease, ROZ group: the group receiving daily 0.125 mg/kg/day of rosmarinic acid (standard substance) dissolved in distilled water and DMSO (in the form of intraperitoneal injection) which with p<0.001 , that is shown in the diagram with three stars, shows a significant decrease compared to...
the control group. Checking and determining the amount of urea: In the examination of the serum urea factor, the average urea level in terms of mmol/L in the sham group was 17.611 ± 1.28 and the average of this parameter in the control group rats that were only subjected to ischaemia-reperfusion without drugs was 42.643 ± 2.250 (Figure 2). The mean and level of urea in the ROZ and MC150 groups show a significant decrease compared to the control group (both with p<0.05).

Sham: the group that did not receive any medicine and did not get ischaemia, control group: the group that only got ischemia and did not receive any medicine. MC50: The group receiving the methanolic extract of *S. multicaulis* with a concentration of 50 mg/kg/day, which was treated with p<0.05, MC100: The group receiving the methanolic extract of *S. multicaulis* with a concentration of 100 mg/kg/day, MC150: The group receiving the methanolic extract of *S. multicaulis* with a concentration of 150 mg/kg/day, with p< 0.001 compared to the control group, that is shown in the diagram with three stars, shows a significant decrease, ROZ group: the group receiving daily 0.125 mg/kg/day of rosmarinic acid (standard substance) dissolved in distilled water and DMSO (in the form of intraperitoneal injection) which with p<0.001, that is shown in the diagram with three stars, shows a significant decrease compared to the control group. In the examination of the creatinine factor (Figure 3), which also indicates the level of kidney function, the average creatinine levels show a significant decrease compared to the control group, and the serum average of this biomarker in the ROZ group shows a clear decrease compared to the MC50 group (p<0.05).

Sham: the group that did not receive any medication and did not get ischaemia, control group: the group that only got ischemia and did not receive any medication. MC50: the group receiving the methanolic extract of *S. multicaulis* with a concentration of 50 mg/kg/day, MC100: the group receiving the methanolic extract of *S. multicaulis* with a concentration of 100 mg/kg/day, which was treated with p<0.05, MC150: the group receiving the methanolic extract of *S. multicaulis* with a concentration of 150 mg/kg/day, which shows a significant decrease with p<0.001 compared to the...
Ghasemian et al. control group, that is shown in the diagram with three stars, ROZ group: the group receiving daily mg/kg 0.125 kg/day of rosmarinic acid (standard substance) dissolved in distilled water and DMSO (in the form of intraperitoneal injection),

Methanolic extract of Salvia multicaulis which shows a significant decrease with p<0.001 compared to the control group, that is shown in the diagram with two stars.

Figure 1. Comparison chart of MDA serum level in the study groups. Significant decrease compared to the control group with p-value<0.001.
Figure 2. Comparison chart of urea serum level in the study groups. Significant decrease compared to the control group with p-value<0.001.

Figure 3. Comparison chart of creatinine serum levels in the study groups. Significant decrease compared to the control group with p-value<0.001.
DISCUSSION

Hypoxia and ischemia of kidney tissue can occur for reasons such as heart failure and renal artery embolism; this ischemia can cause severe destruction of kidney tissue and endogenous and exogenous toxic metabolites [13]. Ischemia-reperfusion injury often results in free radical formation reactive oxygen species and inflammation within hours. Excessive production of oxidants and proinflammatory mediators leads to multiple organ dysfunctions. Flavonoids are polyphenolic compounds with various pharmacological properties and act as scavengers of free radicals by OH groups in their molecular structure. Especially, the Lamiaceae family includes a large number of plants that are well known for their antioxidant properties. The results of previous research showed that using *Salvia* floral extract before surgical procedures to reduce oxidative renal tissue damage and prevent severe deterioration of renal function. As a result, considering that *Salvia multicaulis* was one of the plant species belonging to the *Lamiaceae* family from Lorestan province, this plant was selected for investigation. The use of *S. multicaulis* plant extract was effective and helpful to prevent ischemic damage and help to heal the damaged tissues [16].

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Pretreatment of laboratory animals using antioxidants and also the use of laboratory animals that were manipulated genetically, the expression of antioxidant enzymes is increased in them, indicating a reduction in the amount of damage resulting from ischaemia-reperfusion [12,28-30]. Based on the studies, ROS have a high destructive effect on kidney and heart cells and their function, which can be prevented by using pre-treatment with antioxidants to prevent these destructive effects during ischemia-reperfusion [31]. Malondialdehyde is active and highly reactive and is produced in the human body from the peroxidation of unsaturated fatty acids. In the current study, the average levels of MDA in the rats of the rosmarinic acid and MC150 and MC100 groups show a significant decrease compared to the control group, which indicates the positive effect of the methanolic extract of *S. multicaulis* on the average lipid peroxidation, also in the comparison between the groups. Rosmarinic acid and other treatment groups show a significant decrease in the level of malondialdehyde factor in the rosmarinic acid group, which indicates the better performance of rosmarinic acid in reducing lipid peroxidation compared to the methanolic extract of *S. multicaulis*. Also, other studies have shown that CAT and
SOD enzymes have a positive pretreatment effect in ischaemia-reperfusion models [32-39]. Furthermore, diterpenoids [40], norditerpenoids [41], triterpenoids [40,42], abietane diterpenoids [43] and flavonoids [44] were reported from the *S. multicaulis* roots and aerial parts extracts. Recently, in 2021 Barhoumi and colleagues investigated the aqueous methanol and butanol extracts of *S. multicaulis* from Jordan area that resulted in the extraction and identification of 17 pure compounds including one new abietane diterpene derivative named as 2,20-dihydroxyferruginol and 16 known compounds like methyl palmitate, ethyl palmitate, palmitic acid, 4-hydroxy-3-(methoxyacetophenone), β-sitosterol, betulin, betulinic acid, oleanolic acid, fleuryinol B, cirsimaritin, maslinic acid, apigenin, β-sitosteryl glucoside, luteolin, apeginin-7-O-glyco-side and luteolin-7-O-glucoside [45]. Previous phytochemical researches on *S. multicaulis* mainly reported the chemical composition of its volatile oil [46-49], and on its biological properties like antioxidant, antimicrobial, wound healing, cytotoxic and antiangiogenic activities [24,50-53].

In the present study, the average levels of MDA in the rats of the rosmarinic acid and MC150 and MC100 groups were significantly reduced compared to the control group shows that it shows the positive effect of the methanolic extract of *S. multicaulis* on the average lipid peroxidation [32]. In this research, we were looking for compounds with antioxidant properties. Compounds such as polyphenolic structures like flavonoids, which are polar compounds, are more soluble and according to previous reports presented in a methanolic extract, which is a polar extract. As a result, we choose the methanolic extract for this test. There are many reports of the effects of MDA in the treatment groups with the methanolic extract of *S. multicaulis*, which increases with increasing the therapeutic dose that confirms the dose-dependent therapeutic effect in reducing lipid peroxidation [32]. Studies have shown that the levels of Serum creatinine (Scr) and urea nitrogen (BUN) in rats with renal ischemia-reperfusion were much higher than in rats in the sham group, which means that kidney disorders occur after ischaemia-reperfusion. Also, pretreatment with ethanolic extract of *S. miltiorrhiza* reduces the increase in serum creatinine and blood urea nitrogen caused by ischaemia-reperfusion surgery. Therefore,
Ghasemian et al. pretreatment with ethanol extract of *S. miltiorrhiza* plant has been useful in preventing ischemia-reperfusion disorder [54-56]. In the present study, it is estimated that doses of 150 mg/kg/day and 100 mg/kg/day of the methanol extract from the *S. multicaulis* are the most effective and useful doses of this plant.

**CONCLUSION**

Overall, from the results of this study, it could be concluded that doses of 150 mg/kg/day and 100 mg/kg/day of the methanolic extract of *Salvia multicaulis* are the most effective and useful doses of this plant. The use of these doses caused a significant and maximum decrease in eosinophilic cast, leukocyte infiltration, tubular necrosis, urea and creatinine and also increased the levels of antioxidants investigated in this study. Considering that the maximum dose used in this study showed its desired effects, in order to investigate the effect of higher doses as well as the toxic dose of this extract, more studies should be conducted in this field.

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