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Original article

Attenuation of Diabetic Nephropathy by Carvacrol through Anti-oxidative Effects in Alloxan-Induced Diabetic Rats

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Abstract

Background and Objectives: Diabetes, a common metabolic disorder, is prevalent in many countries. Nephropathy is a main debate's side effect. Role of oxidative stress is well known in induction of diabetic nephropathy while carvacrol is a potent anti-oxidant that might attenuate oxidative stress. The aim of this study was to explore the effect of carvacrol in decreasing nephropathy-induced oxidative damage in diabetic rats. **Methods:** Thirty five Wistar rats (200-250 g) were divided to 7 groups. The rats received alloxan (i.p., 200 mg/kg) for induction of diabetes. After one week, fasting blood sugar (FBS) was assessed and the rats with FBS>250 mg/dL were considered as diabetic. Three weeks after alloxan injection, the blood urea (BUN) and creatinine (Cr) were determined for confirmation of inducing nephropathy. Then, the animals were treated with carvacrol for one week. Finally, they were anesthetized and blood was collected from animal's heart for calculation of BUN and Cr. Furthermore, the kidneys were for oxidative stress markers such as glutathione capacity, protein carbonyl, lipid peroxidation and catalase activity. **Results:** Our results showed that glutathione level and catalase activity significantly increased after treatment with carvacrol. Same results were found in rats that received vitamin E. Also, lipid peroxidation, protein carbonyl content, BUN and Cr levels significantly decreased after treatment with carvacrol in comparison with diabetic rats. **Conclusion:** Our results showed that carvacrol improved nephropathy-induced oxidative damage similar to vitamin E. Therefore, it may be suggested that carvacrol can be suggested as a useful supplement in decreasing diabetic complaints along with anti-diabetic drugs.

Keywords: alloxan; carvacrol; diabetes; nephropathy; oxidative stress

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Introduction

Diabetes mellitus (DM) is defined as a chronic disease caused by reduction in the level of insulin secretion or insulin insufficiency [1]. The type 2 diabetes is the most typical form of DM which is the cause of about 90 percent of diabetic cases. It has been previously called non-insulin-dependent diabetes or NIDDM [2]. The number of people with diabetes has been predicted to rise to 642 million people by 2040 [3].

Diabetic hyperglycemia produces reactive oxygen species (ROS) , which sequentially cause oxidative stress [4]. Oxidative stress is the imbalance between the rate of ROS generation and the protective effects of antioxidants [5]. which seems important in the development of

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diabetic complications [6]. Many studies on the experimental animals and diabetic patients have confirmed that hyperglycemia resulted in progress of nephropathy [7,8].

Nephropathy is one of the most common micro vascular complications in diabetes mellitus which is observed in more than one third of the diabetic patients [9]. Furthermore, hyperglycemia plays significant roles in the development of vascular complications such as nephropathy [10]. Hyperglycemia in diabetes leads to mitochondrial dysfunction and increases the reactive free radicals; then causes DNA damage, which results in apoptotic cell death. Hyperglycemia also causes oxidative stress, increases glutathione (GSH) oxidation and lipid peroxidation [7]. Finally, hyperglycemia induces oxidative stress in diabetic nephrons and results in activation of multiple biochemical pathways that lead to renal cell death, increased albuminuria, and renal dysfunction [7]. Oxidative stress pathways are the main sources of damage and are potential therapeutic targets in diabetic nephropathy [11].

Using medicinal herbs have been found to be efficacious, economical, and safe in treatment and preventing diabetic complications in developing countries. One of the proposed therapies to reduce the oxidative stress induced by free radicals in diabetic nephropathy is the use of herbal antioxidant. Carvacrol (5-isopropyl 2-methyl phenol) is a known monoterpene in essential oil of aromatic plants such az *Origanum vulgare* (oregano) and *Thymus vulgaris* (thyme), that has recently become noticeable for its substantial pharmacological properties [12].

Many studies have shown that carvacrol has anti-inflammatory effects through inhibition of cyclooxygenase enzyme [13]. It is also responsible for the biological activity of Labiatae family including antibacterial, anti-mutagenic, anti-genotoxic, analgesic, antispasmodic, and angiogenesis effects [13]. Carvacrol leads to the reduction of blood cholesterol, the amount of free fatty acid and sugar in plasma. Recently, anti-oxidant effect of carvacrol has been observed in many studies. It has been shown that carvacrol has similar powerful antioxidant effects in comparison to ascorbic acid, butyl hydroxy toluene, and vitamin E [12]; therefore, carvacrol can be applied as an antioxidant in oxidative pathologic circumstances.

The present study has tried to explore the protective effects of carvacrol against oxidative induced nephropathy in diabetic rats. Furthermore, the effects of carvacrol were compared with vitamin E as a potent anti-oxidant. To the best of our knowledge, this is the first report on nephroprotective effects of carvacrol in alloxane-induced diabetic nephropathy.

Material and Methods

Ethical considerations

All experimental procedures were approved by the ethical standards of ethic committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran (registration code: 4850, 2017).

The method of inducing diabetes

Two weeks after adaptation of the rats with the environment, diabetes was induced by injection of alloxan. Alloxan was dissolved in normal saline and injected with intraperitoneal injection (200 mg/kg). Blood glucose was measured before and one week after intraperitoneal injection of alloxan via taking a drop of blood from tails of rats, and rats with a blood glucose level higher than 250 mg/dL were considered as diabetic [14]. Three weeks after injection of alloxan, diabetic rats were selected and their urea and creatinine levels were measured and nephropathy was confirmed.

Animal treatment

Thirty five Wistar male rats with an average weight of 200-250 g were used. The animals were placed in separate standard cages and 12 h light-12 h darkness and access to adequate water and food at a temperature of 22 ± 2 °C. After the approval of animal neuropathy, the animals were divided in 7 groups and according to previous studies, 5 rats were assigned to each group: Dia group (diabetic rats receiving only normal saline), Dia+ solvent group (diabetic rats which received 5% of DMSO), Dia+ Car 25 (diabetic rats which have received 25 mg/kg of carvacrol, Dia+ Car 50 (diabetic rats which have received 50 mg/kg of carvacrol, Dia+ Car 100 (diabetic rats which have received 100 mg/kg of carvacrol, Dia+ Vit E 400 (diabetic rats which have received 400 mg/kg of vitamin E, Dia+ Vit E 600 (diabetic rats which have received 600 mg/kg of

vitamin E). All treatments were applied as peritoneal injection for one week [14-16].
After these processes, the animals were anesthetized by ketamine (80 mg/kg) and xylazine (5 mg/kg). For biochemical studies, about 2 mL of the whole blood was collected from the retro-orbital plexus of each mouse and the serums of samples were extracted by centrifuge [5]; furthermore, surgery was performed and kidney tissues were removed. The kidney tissues were washed with cold mannitol solution and homogenized by handle homogenizer with cold normal saline. After that, some homogeneous tissues were centrifuged with the speed rate of 2000g for 10 min, then the supernatant was collected.

Measurement of blood urea and creatinine
Blood urea nitrogen (BUN) and creatinine (Cr) are blood markers of the kidney damage that increase in renal insufficiency. In this project, after the animals were anesthetized, blood was collected from the heart (2 mL) of the rats, and after removing the serums, BUN and Cr levels were determined by commercial reagents (BUN detection kit's Cat Number: 1400029, Cr detection kit's Cat number: 1400009, Parsazmoon CO. Iran).

Measurement of lipid peroxidation
The lipid peroxidation product (malondialdehyde (MDA)) was quantified by the thiobarbituric acid (TBA) based on the method used by Ahangar et al. Briefly, 0.25 mL phosphoric acid (0.05 M) was added to 0.2 mL of kidney tissue homogenate with the addition of 0.3 mL 0.2% thiobarbituric acid. All samples were placed in boiling water for 30 min. At the end, the tubes were shifted to an ice-bath and 0.4 mL *n*-butanol was added to each tube. Then, they were centrifuged at 3500 rpm for 10 min. The amount of MDA formed in each sample was assessed through measuring the absorbance of the supernatant at 532 nm with an ELISA reader (Tecan, Austria). Tetramethoxypropane was used as the standard [17].

Measurement of protein carbonyl
Protein carbonyl was measured using a 2, 4-dinitrophenyl-hydrazine (DNPH) reagent. After identifying the tissue protein, 500 µL of trichloroacetic acid (20% w/v) was added to 250 µg of the sample, and was placed at 4 °C for 15 min. Then, the deposited proteins were centrifuged at 6500 g for 10 min; the supernatant was discarded. The lower sediment was completely dispersed in 500 µL of NaOH (0.1 M) and dissolved in 500 µL of 10 mM DNPH and HCl 2 M was added to the samples. Also a blank was provided by adding 500 µL of 2 M HCl without DNPH to a protein sample. Then, the samples were incubated for about 30 min at room temperature away from light and 500 µL of trichloroacetic acid (20% w / v) was added afterwards. The protein sediment was collected at 6500 g for 10 min and the supernatant was discarded. The lower sediments was combined ethanol:ethyl acetate (1:1) and centrifuged again at 6500 g for 10 min to remove the surface solution. The deposited sediment of final the protein was dispersed in 200 µL of guanine hydrochloride solution. The amount of protein carbonyl was measured by reading absorbance at 365 nm [18].

Measurement of glutathione levels
Glutathione (GSH) concentration was determined by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as the indicator by spectrophotometry method. Briefly, tissue homogenates were deprotonated with trichloroacetic acid (TCA) by centrifugation. Then 0.1 mL of tissue homogenates was added to 0.1mol/L phosphate buffer and 0.04% DTNB in a total volume of 3.0 mL (pH=7.4). Then, the developed yellow color was read at 412 nm using a spectrophotometer (UV-1601 PC, Shimadzu, Japan). A standard curve was drawn using different specified concentrations of GSH solution. With the help of this standard curve, the GSH content was calculated [19].

Measurement of catalase activity
In another part of this study, considering the importance of catalase activity in response to oxidative stress, we examined the activity of the catalase enzyme in the kidney tissue samples of the experimental groups. After preparation of tissue homogeneity, the activity rate of catalase enzyme was calculated using Catalase activity (CAT) Assay kit (ZellBio, Germany).

Measurement of protein concentration
Protein content was determined in tissues with

Bradford method. Bovine serum albumin was used as the standard and homogenate samples were mixed with coomassie blue, and after 10 minutes, absorbance was determined at 595nm by spectrophotometer [5].

Statistical analysis

The results have been presented as Mean±SD. All statistical analyses were performed using the SPSS software, version 16 (2007, Chicago, SPSS Inc.). The graphs were plotted by GraphPad Prism software (version 5, 2010). Assays were performed in at least, triplicate and the mean was used for statistical analysis. Statistical significance was determined using the one-way ANOVA test, followed by the post hoc Tukey test. Statistical significance was set at $p<0.05$.

Results and Discussion

In the present study, the effect of carvacrol on diabetic nephropathy in male rats induced by alloxan was investigated.

According to table 1, diabetic rats had a raised level in serum levels of BUN and Cr which can be indicators of kidney damage. Administration of carvacrol could significantly ameliorate these parameters in comparison with diabetic group (table 1).

Table 1. The effects of carvacrol on serum level of BUN and creatinine

Animal groups	Creatinine (mg/dL)	BUN (mg/dL)
Diabetic+ solvent	2.2±0.10	160.7±11.72
Diabetic	2.2±0.10	162±14
Diabetic+ Car 25	1.93±0.15	141.3±5.03
Diabetic+ Car 50	1.87±0. 06*	136.7±6.11
Diabetic+ Car 100	1.47±0.11***	113±9.85***
Diabetic+ Vit E 400	1.37±0.15***	110±9.16***
Diabetic+ Vit E 600	1.3±0.10***	102.7±4.16***

The results were expressed as mean±SD; Car 25: 25 mg/kg of carvacrol; Car 50: 50 mg/kg carvacrol; Car 100: 100 mg/kg carvacrol; Vit E 400: 400 mg/kg vitamin E; Vit E 600: 600 mg/kg of vitamin E; *significantly different from diabetic group ($p<0.05$); ***significantly different from diabetic group ($p<0.001$)

Recent clinical studies have shown that hyperglycemia is a main cause of progression and development of nephrotoxicity and high mortality rate [20]. Furthermore, different studies have investigated the effect of diabetes on the biochemical parameters of BUN and Cr (as important markers for the glomerular filtration rate) and pathological damage [21]. In a study conducted by Ahmedvand et al. in 2016 on the protective effect of carvacrol on gentamicin-induced nephropathy in rats, it was observed that carvacrol had a considerable effect on the reduction of urea [22]. These studies were consistent with the present study and we observed a decrease in the amount of BUN and Cr in rats that had nephropathy and treatment with carvacrol and vitamin E improved BUN and Cr level. The decrease in BUN and Cr levels can be due to the antioxidant effects of these compounds. Also, in studies with other antioxidants such as essential oil of garlic, essential oil of olive leaf and etc. a decrease in BUN and Cr levels was reported [22].

In diabetic patients, oxidative stress plays a key role in the pathogenesis of vascular complications [14]. There are numerous studies that have shown the role of oxidative stress in diabetes-related nephropathy [8,23].

Lipid peroxidation is a main result of oxidative stress that was assessed by measuring the amount of malondialdehyde (MDA) as a byproduct of oxidative damage in kidney tissue. As shown in figure 1, treatment with carvacrol for one week led to a significant decrease in diabetes-induced lipid oxidation in the kidney tissue of diabetic rats. Similarly, vitamin E significantly reduced the amount of lipid peroxidation (figure 1).

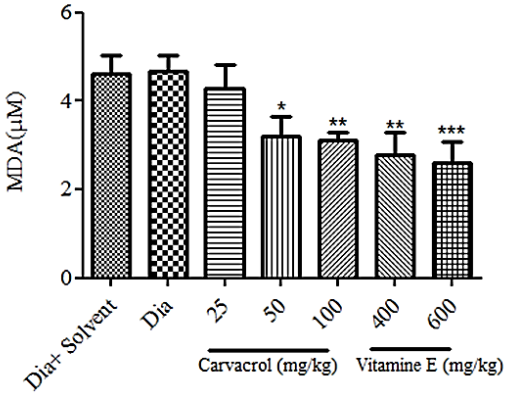

Figure 1. The effects of carvacrol on lipid peroxidation in kidney tissue of diabetic rats; the results obtained from three times of repeated experiment were reported as mean±SD. *significantly different from diabetic group ($p<0.05$); **significantly different from diabetic group ($P<0.01$); ***significantly different from diabetic group ($P<0.001$)

Lipid peroxidation and increase of ROS can cause nephropathy by various mechanisms. One of the possible mechanisms is the increase in the activity of the phospholipase enzyme A_2 (PLA2). This enzyme produces prostacyclin I_2 and

thromboxane I_2. Prostacyclin I_2 is vascular vasodilator but thromboxane I_2 is vasoconstrictor, and the balance between these, protects the normal tune of the vessels. Oxidative damage in diabetic's patients alters the activity of PLA2 that leads to the increases in thromboxane I_2 and decreases in prostacyclin I_2. These alterations can be dangerous for kidney and could induce nephropathy [24,25].

Protein carbonyl is a good marker for oxidative damage of proteins [26]. ROS can attack to proteins and oxidize the amino acid side of the protein bonds and create additional carbonyl groups (protein carbonyl) that influence the protein function [27]. According to the findings of this study, the level of protein carbonyl was higher in the diabetic group compared to the treatment groups and prescribing carvacrol in the diabetic group decreased the amount of carbonyl protein compared to the diabetic group, in a way that, the concentrations of 50 and 100 mg/kg of carvacrol significantly reduced the amount of protein carbonyl ($p<0.05$). Also, in the diabetic group receiving vitamin E, similar effects were observed compared to carvacrol (figure 2).

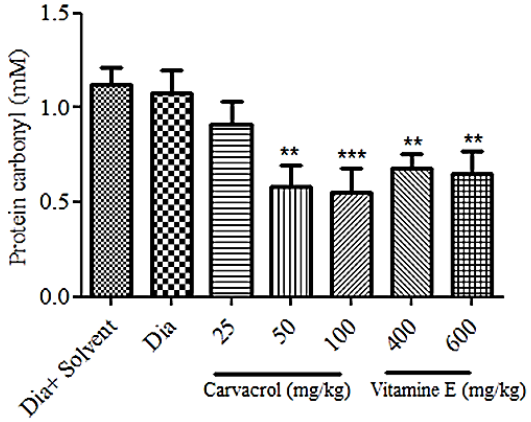

Figure 2. The effects of carvacrol on the level of protein carbonyl in kidney tissue of diabetic rats; the results obtained after three times of repetition were reported as mean ± SD. **significantly different from diabetic group ($p<0.01$); ***significantly different from diabetic group ($p<0.001$)

Glutathione (GSH) is one of the most important non-enzymatic antioxidants defense in tissues which plays an important role in neutralizing ROS [5]. According to the results of the present study, mean concentration of GSH in the diabetic rats was 75.5 µM and in the diabetic group receiving carvacrol concentrations were 89.25, 100.92 and 113.83 µM, respectively. However, carvacrol had dose dependently and significantly increased the levels of glutathione. In the diabetic group which had received a dose of 600 mg/kg of vitamin E, glutathione levels had also increased significantly compared to the diabetic group (figure 3).

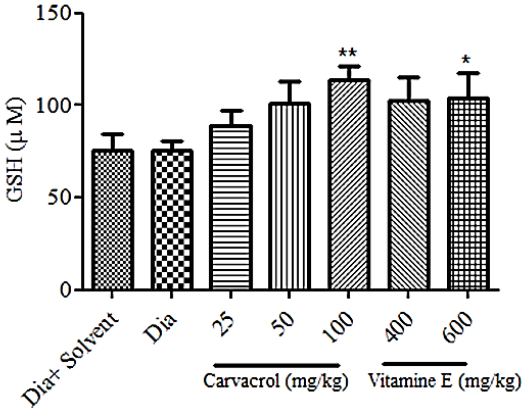

Figure 3. The Effect of carvacrol on glutathione level in kidney tissue of diabetic rats; the results obtained through three times of repetition were reported as mean ± SD; *significantly different from diabetic group ($p<0.05$); **significantly different from diabetic group ($p<0.01$)

Similarly, in a study conducted by Maboob et al. on diabetic males and females in India, raising of MDA and reduction of glutathione content were shown in both male and female groups of type 2 diabetes compared to non-diabetic group and it was concluded that the change in these two factors may occur much earlier than the emergence of secondary complications of type 2 diabetes [28].

One of the cellular responses to oxidative stress is the increase of antioxidant enzymes and increase of expression of their mRNA gene [29]. Catalase is an antioxidant enzyme that plays oxidation protective role in the body. In the present study, the level of activity of catalase was lower in the diabetic group compared to the carvacrol group. As it has been shown in figure 4, the activity of catalase in the diabetic group has decreased, and prescribing carvacrol has significantly increased the activity of this enzyme in diabetic group receiving carvacrol compared to the control group ($p<0.05$) and the effect of vitamin E on the activity level of catalase enzyme in diabetic rats was approximately the same for the group receiving high-concentration of carvacrol (figure 4).

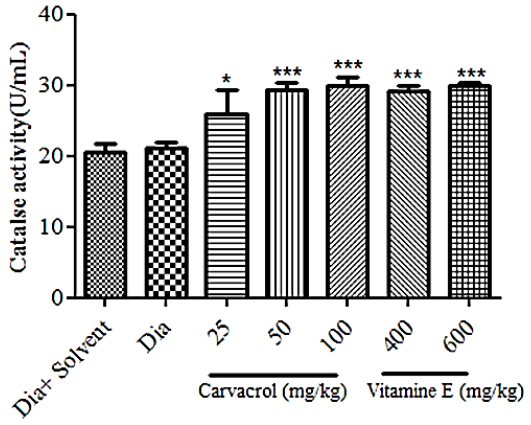

Figure 4. The effects of carvacrol on catalase activity in kidney tissue of diabetic rats; the results obtained through three times of repetition were reported as mean±SD. **significantly different from diabetic group ($p<0.01$); ***significantly different from diabetic group ($p<0.001$)

Samarqandian et al. found that carvacrol reduced the MDA level and glutathione oxidation; furthermore, the activity of catalase enzyme increased after treatment by carvacrol [12]. These results were in accordance with our findings.

In our study, carvacrol showed acceptable antioxidant features in comparison with vitamin E. the results of this study indicated that prescribing carvacrol for a week could improve the nephropathy caused by diabetes. In fact, our findings showed that oxidative stress could develop nephropathy in diabetic rats and using of carvacrol attenuated the oxidative damage and improved diabetic nephropathy. Thus, after further investigations, it can be applied as a supplementary treatment for diabetes complications along with other medicines applied to lower blood glucose.

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Author contributions

Each author participated sufficiently in the work to take public responsibility for appropriate portions of the content. Hamid Reza Jamshidi, Mohammad Shokrzadeh and Fatemeh Shaki conceived and planned the experiments. Ehsan Zamani designed the methods and Zahra Zeinabady performed them with support from Mohammad Shokrzadeh. Fatemeh Shaki analyzed the data. Hamid Reza Jamshidi wrote the manuscripts. The revising of manuscript was done by Zahra Zeinabady and Ehsan Zamani.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

FBS: Fasting blood sugar; BUN: Blood urea; Cr: creatinine; DM: diabetes mellitus; ROS: reactive oxygen species; GSH: glutathione; MDA: malondialdehyde; DTNB: 5,5'-dithiobis-2-nitrobenzoic acid; DNPH: 2, 4-dinitrophenyl-hydrazine