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Controversial effects of *Calendula officinalis* L. on Biochemical and Pathological Factors of Nephropathy in Diabetic Wistar Rats

Iraj Salehi [1]; Shirin Moradkhani [2]; Amaneh Mohammadi Roushandeh [3]; Hamideh Nazari [4]; Alireza Pouyandeh Ravan [1,*]

[1]Neurophysiology Research Center, Hamadan University of Medical Sciences, Hamadan, IR Iran
[2]Department of Pharmacognosy and Pharmaceutical Biotechnology, School of Pharmacy, Hamadan University of Medical Sciences, Hamadan, IR Iran
[3]Department of Anatomical Sciences, Medicine Faculty, Hamadan University of Medical Sciences, Hamadan, IR Iran
[4]Islamic Azad University of Hamadan, Hamadan, IR Iran

*Corresponding author: Alireza Pouyandeh Ravan, Neurophysiology Research Center, Hamadan University of Medical Sciences, Hamadan, IR Iran. Tel: +98-8138381037, Fax: +98-8138381017, E-mail: a.r.pouyandeh.ravan@gmail.com; a.pouyandeh@umsha.ac.ir

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Background: Chronic hyperglycemia leads to microvascular and macrovascular complications such as diabetic nephropathy. Medicinal plants are good sources for finding new therapeutic chemicals to improve diabetes and relieve its symptoms.
Objectives: The purpose of the present study was to evaluate the effect of the hydroalcoholic extract (300 mg/kg) of *Calendula officinalis* (marigold) on blood biochemical profiles and histopathological changes in kidney of streptozotocin-induced diabetic rats.
Materials and Methods: Twenty male Wistar rats were divided to four groups: Normal control (NC), diabetic control (DC), normal *C. officinalis* (N+CO) 300 mg/kg, and diabetic *C. officinalis* (D+CO) 300 mg/kg. The rats were treated for a period of 13 weeks. Diabetes was induced by Streptozocin injection, intraperitoneally. Level of glucose, urea, creatinine and also total anti-oxidant capacity, malondialdehyde, total oxidant status in serum and histological alterations in the kidney were analyzed.
Results: Level of serum glucose, urea, creatinine, malondialdehyde and total oxidant status were increased in diabetic rats, whereas, total anti-oxidant capacity was decreased compared to the control animals. Also, histological findings confirmed the absence of integrity in glomerulus and mass infiltration in kidney tissue in diabetic rats compared to the normal controls. *Calendula officinalis* extract had no effect on blood glucose, but it decreased blood urea nitrogen and creatinine, total oxidant status and malondialdehyde while it increased total anti-oxidant capacity in the diabetic extract-treated group when compared to diabetic rats. *Calendula officinalis* could not prevent nephropathy changes in the diabetic rats.
Conclusions: Therefore, our results suggest that although administration of 300 mg/kg of *Calendula officinalis* extract showed salutary effects on anti-oxidant profile, yet its protective effects on anti-diabetic and regenerative properties on nephropathy were ambiguous and require more investigations.

Keywords: Diabetic Nephropathy; Hyperglycemia; Oxidative Stress; Streptozocin; *Calendula officinalis*

1. Background

Prevalence of diabetes and its associated complications for affected patients has increased dramatically (1). Chronic increase in plasma glucose level or hyperglycemia leads to microvascular and macrovascular complications such as diabetic nephropathy, accelerated atherosclerosis, neuropathy and retinopathy (2). Diabetic nephropathy (DN), as one of the critical problems of long term diabetes mellitus is the most common cause of end stage renal disease (ESRD) and it is inevitable for patients with this complication to be on dialysis (2-5). Approximately this problem has become a serious challenge to public healthcare system due to the very expensive and prohibitive cost of renal surgery and transplantation in all societies, even in developed countries (3, 6). Based on previous studies it has been shown that in the United States DN accounts for 35% of patients with ESRD (6). In DN, several pathological and histological changes can be observed in kidney tissues including glomerular hyperplasia or hypertrophy, mesangial expansion and basement membrane thickening (7). It is supposed that several mechanisms originated from hyperglycemia such as advanced glycation end product formation, aldose reductase pathway, increased protein kinase C activity, over-activation of poly ADP-ribose polymerase inflammation, and oxidative stress are involved in the pathogenesis of DN and its complications (8, 9). Increasing evidence suggests that there is a close connection between hyperglycemia, oxidative stress and diabetic complications, especially DN (5, 9, 10). Scientific studies suggest that antioxidants have favorable effects on experimental models of diabetes and human kidney (10). Although angiotensin-converting enzyme (ACE) inhibi-

tors and angiotensin II receptor blockers can ameliorate the progression of DN, there is no effective and specific treatment (11, 12). Medicinal plants are good sources for finding new therapeutic chemicals (3). Several studies have been carried out on the anti-diabetic and renoprotective effects of various plants, which resulted in appropriate and acceptable outcomes (3, 13, 14). *Calendula officinalis* L. (CO) commonly known as Marigold belongs to the Asteraceae (daisies) family and grows throughout Europe and North America. The yellow flowers of marigold either fresh or dried are traditionally used as tea and spices while the extracts of this plant are beneficial in making herbal medicines such as tinctures, ointments and creams (15, 16). As an indigenous herbal medicine it is believed that flowers of CO have anti-inflammatory properties, while according to various studies, extracts of CO also have anti-oxidant, anti-fungal, anti-edema and wound healing properties (15, 17). The main constituents of CO including flavonoids, steroids, triterpenoids, phenolic acids and carotenes, and derived components such as faradiol, rutin, caffeic acid and chlorogenic acid have biological activities in the body (16).

2. Objectives

The aim of the present study was to investigate the effect of CO hydroalcoholic extract (300 mg/kg/day) on blood biochemical profiles and histopathological changes in kidney tissue of rats with diabetes induced by streptozotocin. A number of these factors were studied previously at lower extract concentrations (18).

3. Materials and Methods

Streptozocin (STZ), sodium chloride, sodium citrate, sodium acetate, acetic acid, tripyridyl-S-triazine (TPTZ), iron (III) chloride, iron (II) sulfate, thiobarbituric acid, phosphotungstic acid, n-butanol, tetraethoxypropane, xylenol orange, glycerol, iron (II) chloride tetrahydrate and o-dianisidine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Ethanol, formaldehyde, hematoxylin, eosin and ether were obtained from Merck (Darmstadt, Germany). Glucose, blood urea nitrogen (BUN), and creatinine (Cr) chemical kits were purchased from Pars Azmoon Inc. (Tehran, Iran).

3.1. Animals and Experimental Design

Twenty male Wistar rats (180 - 220 g) were obtained from the Pasteur institute of Iran and housed in a wire-bottomed cage under standard environmental conditions (23 ± 1°C, 55 ± 5% humidity and a 12-hour light: 12-hour dark cycle) with free access to food and water.

The animals were divided to four groups (n: 5):

Normal control (NC): the non-diabetic group that was gavaged with normal saline.

Diabetic control (DC): the diabetic group that received STZ and was gavaged with normal saline.

Normal *C. officinalis (*N + CO): the non-diabetic group that was gavaged with *C. officinalis* extract (300 mg/kg). The dose was selected based on previous studies (19, 20). In preliminary studies, this dose had no negative effects on animal behavior.

Diabetic *C. officinalis (*D + CO): the diabetic group that received STZ and was gavaged with *C. officinalis* extract (300 mg/kg).

Streptozocin was dissolved in citrate buffer 0.1 M (pH 4.0). After a week of adaptation, the STZ solution was injected intraperitoneally at a concentration of 60 mg/kg/body weight following overnight fasting. Four days after injection, the diabetic animals were confirmed by measurement of tail blood glucose level using an Accu-check glucometer (21). Rats with glucose level of more than 200 mg/dL were considered diabetic (22). The animals in the NC and N + CO groups were gavaged with normal saline and those in the DC and D + CO groups were gavaged with normal saline and CO respectively for 13 weeks.

3.2. Herbal Plant Extraction

Calendula officinalis L. was purchased from Avicenna herbal center (Hamadan, Iran) in March 2013 and was authenticated by the department of pharmacognosy, faculty of Pharmacy, Hamadan university of medical sciences, Iran. Extraction was performed by maceration according to a previously described method (23). Briefly, the plant was allowed dry in darkness. Then it was milled (300 g) and macerated in 1000 mL of ethanol (80%) at room temperature, three times, each time was three days in duration. After filtration, the solution was evaporated in a rotary vacuum evaporation system to yield a waxy mass and was kept in dark vials at - 20°C until the time of the experiments.

3.3. Biochemical Analysis

At the end of the experiments, the animals were anesthetized (by diethyl ether) and blood samples were collected from their hearts and allowed to clot for 20 minutes at room temperature and then centrifuged at 10000 rpm for ten minutes. Next, the serum was separated and kept at -80°C until subsequent analysis. Level of glucose, creatinine, and blood urea nitrogen (BUN) in serum was measured by the Hitachi 911 chemical auto analyzer (Germany), based on glucose oxidase, Jaffe and enzymatic urease-glutamate-dehydrogenase (GLDH), respectively, according to the protocol by Pars Azmoon Inc. (Tehran, Iran). Measurement of total anti-oxidant capacity (TAC) was performed by the photometric method of ferric reducing ability of plasma (FRAP) according to descriptions by Ghahremanitamadon et al. with some modifications (24). In this method ferric to ferrous ion (Fe^{2+}) reduction induced by anti-oxidants at low pH caused a colored ferrous-tripyridyltriazine complex. The absorbance of the blue colored complex was measured at 593 nm using the JENWAY 6105 UV/Vis spectrophotometer (United

Kingdom) (25). We used malondialdehyde (MDA) measurement as an index of lipid peroxidation as described previously (26). Malondialdehyde is one of the several lipid peroxidation end products. At low pH and elevated temperature, MDA readily reacted with 2-thiobarbituric acid (TBA), which can be detected at 532 nm (27). Total oxidant status (TOS) was analyzed using the oxidation of ferrous ion-o-dianisidine complex to ferric ion in the presence of various oxidant species. Glycerol molecules, which were abundantly present in the reaction medium, enhanced oxidation reaction and finally xylenol orange made a colored complex with the ferric ion in an acidic medium. The produced color was measured at 650 nm with the JENWAY 6105 UV/V spectrophotometer (United Kingdom) (28, 29).

3.4. Histological Studies

The animals were sacrificed and their kidneys were removed and soaked in 10% formalin. After tissue processing, the tissues were embedded in paraffin and cut in 5 μm sections. The sections were stained with hematoxylin and eosin. The morphology of the kidney tissue was studied under a light microscope and the diameter of the Bowman space was measured using the motic version II software.

3.5. Statistical Analysis

The values were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) and post-hoc Tukey's test using the SPSS software version 22. P values of less than 0.05 were considered significant.

4. Results

4.1. Blood Indices

4.1.1. Effects of Calendula officinalis L. Extract (300 mg/kg) on Blood Glucose Level

Serum glucose level in the untreated diabetic rats in comparison to untreated normal rats (increased significantly (P = 0.000). There were not any significant differences between diabetic rats gavaged with CO and the untreated diabetic rats. (P = 0.835). It indicates that the extract was not effective to reduce glucose level in the diabetic rats. Also, Treatment with CO hydroalcoholic extract in the normal non-diabetic rats significantly increased the serum glucose level when compared to untreated normal animals (P = 0.000), (Table 1).

4.1.2. Effects of Calendula officinalis L. Extract (300 mg/kg) on Blood Urea Nitrogen Level

Serum BUN level was significantly elevated in the untreated diabetic rats in comparison to the untreated normal rats (P = 0.000). Treatment of the diabetic group with CO led to a significant decrease of BUN level in comparison with the untreated diabetic group (P = 0.000). Administration of CO extract in the normal non-diabetic rats significantly increased serum BUN level (P = 0.000) in comparison with the untreated normal group (Table 1).

4.1.3. Effects of Calendula officinalis L. Extract (300 mg/kg) on Blood Creatinine Level

Induction of diabetes resulted in a significant increase in the level of serum Cr in the diabetic control group compared to the normal group (P = 0.000). The level of serum Cr in CO gavaged diabetic rats compared with the diabetic control group showed a significant decrease (P = 0.000). Based on the results and statistical analysis between the normal group and normal rats gavaged with CO, it was observed that CO hydroalcoholic extract in healthy non-diabetic animals had no significant effect on the level of serum Cr (P = 0.908), (Table 1).

4.1.4. Effects of Calendula officinalis L. Extract (300 mg/kg) on Blood Total Anti-oxidant Capacity

The results indicated that serum TAC in the untreated diabetic group was significantly lower than that of the normal group. Compared to the untreated diabetic group, CO significantly increased serum TAC in the diabetic rats (P = 0.000). Comparison of the CO treated normal rats with the normal group showed the hydroalcoholic extract of CO caused a significant increase of TAC in the serum of normal non-diabetic rats (P = 0.000), (Table 2).

Table 1. Effects of *Calendula officinalis* L. Extract (300 mg/kg) on Blood Glucose, Blood Urea Nitrogen and Creatinine Level [a,b]

Groups	Blood Glucose, mg/dL	BUN, mg/dL	Cr, mg/dL
NC	138.80 ± 1.28	40.40 ± 1.50	0.592 ± 0.013
DC	627.60 ± 6.37 [c]	110.60 ± 1.28 [c]	0.916 ± 0.025 [c]
D + CO	635.20 ± 10.77 [c]	86.60 ± 3.47 [c,d]	0.704 ± 0.005 [c,d]
N + CO	205.80 ± 2.35 [c,d]	57.80 ± 2.47 [c,d]	0.606 ± 0.006 [d]

[a] Abbreviations: BUN, blood urea nitrogen; Cr: creatinine; DC: diabetic control; D + CO: diabetic *C. officinalis* (300 mg/kg); NC, normal control; and N + CO, normal *C. officinalis* (300 mg/kg).
[b] Data are expressed as means ± SEM.
[c] P < 0.05 compared to NC.
[d] P < 0.05 compared to DC.

Table 2. Effects of *Calendula officinalis* L. Extract (300 mg/kg) on Blood Total Anti-oxidant Capacity, Malondialdehyde and Total Oxidant Status [a,b]

Groups	TAC, mmol/mL	MDA, μmol/mL	TOS, μmol/mL
NC	0.221 ± 0.111	0.34 ± 0.02	2.44 ± 0.10
DC	0.151 ± 0.008 [c]	1.24 ± 0.10 [c,a]	3.28 ± 0.16 [c,d]
D + CO	0.345 ± 0.008 [c,d]	0.82 ± 0.03 [c,d]	1.66 ± 0.07 [c,d]
N + CO	0.408 ± 0.007 [c,d]	0.57 ± 0.01 [c,d]	1.85 ± 0.08 [c,d]

[a] Abbreviations: DC, diabetic control; D + CO: diabetic *C. officinalis* (300 mg/kg); MDA, malondialdehyde; NC, normal control; N + CO, normal *C. officinalis* (300 mg/kg); TAC, total anti-oxidant capacity; and TOS: total oxidant status.
[b] Data are expressed as means ± SEM.
[c] P < 0.05 compared to NC.
[d] P <0.05 compared to DC.

4.1.5. Effects of Calendula officinalis L. Extract (300 mg/kg) on Blood Malondialdehyde Level

The diabetic control rats showed a significant increase in the level of MDA when compared to the normal control rats (P = 0.000). Oral administration of CO to diabetic rats effectively reduced MDA level (P = 0.00). Comparing the normal control group with normal animals treated with CO showed that this extract caused no significant changes in the level of MDA in non-diabetic healthy animals (P = 0.06), (Table 2).

4.1.6. Effects of Calendula officinalis L. Extract (300 mg/kg) on Blood Total Oxidant Status

Serum TOS in the untreated diabetic rats was significantly higher than that of the control normal group (P = 0.000). Accordingly, CO treatment in diabetic animals could significantly reduce serum TOS in comparison with the untreated diabetic animals (P = 0.000). *Calendula officinalis* L. extract consumption could decrease serum TOS of the normal non-diabetic animals when compared to the control normal group (P = 0.007), (Table 2).

4.2. Histological Findings

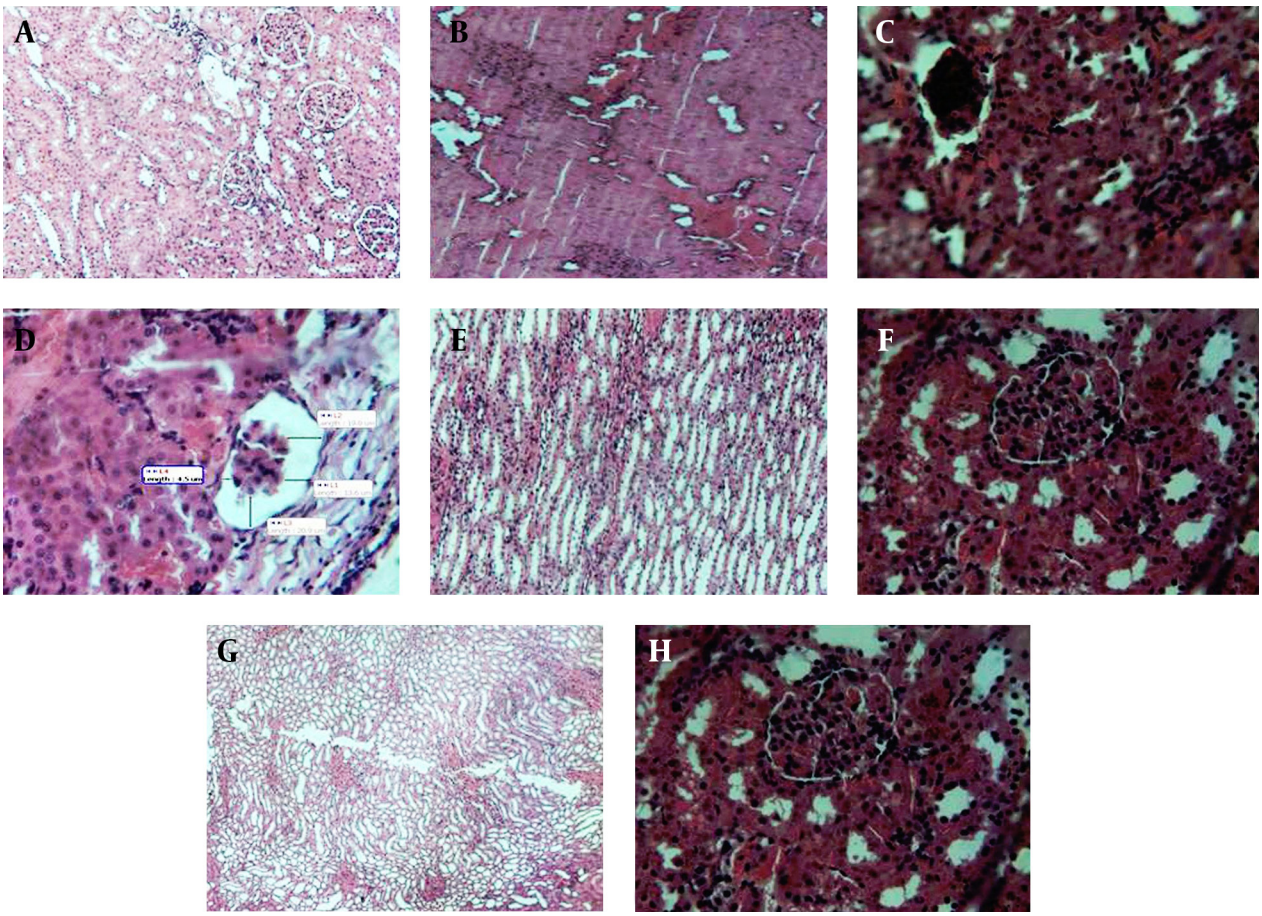
In the normal group, Bowman's space of the glomerulus was normal. The distal and proximal tubules were normal in appearance. No infiltration or cell disarrangement was observed in the tissue (A). In the diabetic group there was mass infiltration into the tissue (B). There was no integrity in the glomerulus. In a few areas, Bowman's space was increased (C) in spite of statistical analysis representing decreased Bowman's space. The glomerulus cells were arranged irregularly in the capsule as well (D). In the non-diabetic group that received the extract, there was infiltration in the glomerulus as well as in the loop of Henle and collecting tubules (E). The lumen in proximal tubules was enlarged (F). The diabetic animals that received the extract showed infiltration in their collecting tubules. Also blood cells appeared in the Bowman's capsule. The lumen of the proximal tubules was enlarged (G and H), (Figure 1).

Diameter of Bowman's space in the untreated diabetic rats decreased significantly in comparison with the normal control rats (P = 0.000). The diameter of the capsule did not show significant changes in the diabetic animals receiving CO as compared to the untreated diabetic rats (P = 0.603). A comparison between the normal animals treated with CO and the normal non-diabetic group showed that oral administration of the extract caused a significant decrease in Bowman's capsule diameter in the gavaged group (P = 0.015), (Table 3).

5. Discussion

In the present study STZ-induced diabetes in rats, which received no extract, led to increased glucose, urea and creatinine of blood. Thus induction of diabetes by STZ was successful. Expectedly, imbalance of oxidant/antioxidant system in diabetes caused oxidative stress and consequently, elevated level of MDA and TOS, and decreased TAC in the blood (26, 30). The diabetic model, which was induced by STZ, also caused pathological changes in the kidney tissue. Diabetic nephropathy (DN) is a multi-factorial condition, in which chronic hyperglycemia and oxidative stress play crucial roles for its occurrence (3, 31). Chronic elevation of blood glucose concentration in diabetes mellitus is involved in the formation of advanced glycation end-products (AGEs), the mitochondrial production of free radicals and finally in cell death and impaired renal function (31, 32). The elevated levels of serum creatinine and urea are considered as one of the DN indices (33). Based on our results, the diabetic rats, which received CO extract, had no significant changes in their blood glucose in comparison with the diabetic control rats. On the other hand, the normal animals treated with CO showed increased serum glucose in comparison with the normal group; therefore, this extract at 300 mg/kg had hyperglycemic effects. Despite reviewing numerous scientific databases, we could not find any documented and acceptable studies in relation to the effect of CO on diabetes and its complications. Only one relatively reliable case was found about alloxan-induced diabetic rats. In that study after oral administration of 80%

Figure 1. Histological Configuration of Kidneys Tissues in Different Groups



Histological sections of the kidney in control group (A) with normal appearance; B, C and D parts represent blood infiltration, cells disturbance and increase in bowman space in diabetic group. Blood infiltration and enlargement in proximal tubules were observed in Henle tubules in the group receiving only the extract (E - F). The diabetic group received the extract showed blood infiltration in glomerulus and between tubules and enlargement in proximal tubules (G - H).

Table 3. Effects of *Calendula officinalis* L. Extract on Diameter of Bowman's Capsules [a,b,c]

Groups	NC	DC	D + CO	N + CO
Diameter of Bowman's capsules (nm)	6.13 ± 0.35	4.44 ± 0.22 [c]	4.92 ± 0.25 [c]	4.92 ± 0.23 [c]

[a] Abbreviations: DC, diabetic control; D + CO, diabetic *C. officinalis*; N + CO, normal *C. officinalis* (300 mg/kg); and NC, normal control.
[b] $P < 0.05$ compared to NC.
[c] Data are expressed as means ± SEM.

hydroalcoholic extract of CO to the diabetic rats, at three different concentrations (25, 50 and 100 mg/kg) for 42 days, the results showed that at a dose of 100 mg/kg body weight, a significant reduction in blood glucose, urine sugar and serum lipids occurred (18). Several factors such as type of extraction, administration dose of extract, and treatment duration may be effective in the differences between results of our study and the mentioned report. Although serum levels of BUN in the normal non-diabetic rats, which received CO, increased yet Cr showed no significant changes, because serum creatinine remains within the reference range until notable renal dysfunction has occurred. Although increased serum creatinine generally equates with impaired kidney function, a normal serum creatinine does not necessarily imply normal kidney function (34). This extract had favorable effects on the diabetic rats, which received CO extract, and caused a significant decrease in the level of BUN and Cr in comparison to DC. In other similar studies on different plants, such favorable effects have been reported (3, 30). The present study showed that STZ made pathological changes in the kidney tissue, which was inconsistent with the results

of Nirmala et al. (35). In their study using STZ caused no significant changes. This may be due to differences in the races of the used rats as well as difference in the duration of experiments. They sacrificed animals 30 days after diabetization, while in the present study the period between the induction of diabetes and the animals' life termination was 91 days. The use of STZ in another study was followed by degenerative changes in proximal tubules that were partially consistent with our findings (36). It has been shown that STZ produces free radicals, which can cause damage to the kidney tissue along with diabetes (37). Also, the extract alone induced pathological changes in the kidney that was probably due to toxins as well as the excessive duration of the treatment period. According to the obtained results, CO could not stop or reduce tissue complications and damages resulting from STZ in the kidney. Studies showed that the CO plant contains different components such as triterpenoids (saponins), flavonoids, coumarins, quinones, volatile oil, carotenoids and amino acids (38). The adverse effect of this extract on undesirable increase of blood BUN as well as on the kidney tissue in the normal rats receiving this extract could imply the toxicity of this plant. Of course the findings in this regard are controversial, since some papers have shown no toxicity for this plant (20), while other studies suggested either low (17) or significant toxicity (39). By increasing TOS and MDA, and decreasing TAC in the serum of untreated diabetic rats, it was indicated that oxidant levels as well as lipid peroxidation increased in these animals. However, CO treatment caused a decrease of MDA and TOS and increase of TAC in the diabetic animals that received the extract. These results indicated that CO in the diabetic rats decreased the oxidative stress resulting from hyperglycemia. Although pathological changes were observed in renal tissues of the diabetic rats treated with CO, the serum levels of BUN and Cr reduced significantly.

Overall, chronic hyperglycemia, oxidative stress, STZ-induced toxicity and the possible toxic effect of CO extract were all involved in the damage to kidney tissue and inappropriate blood biochemical changes in diabetic animals. The results of the present study demonstrate favorable effects of the extract on decreasing oxidative stress, BUN and Cr levels of diabetic animals that received the extract in comparison to diabetic control animals. Because of the attenuated oxidative stress in these animals, it can be supposed that the role of oxidative stress as a tissue-destructive factor was decreased. It is possible that use of different doses may have different effects. The authors suggest that the deleterious effects on kidney tissue need additional studies.

In conclusion, although the *Calendula officinalis* extract (300 mg/kg/day) did not have an appropriate impact on chronic hyperglycemia and kidney tissue complications, yet it showed protective effects on oxidative stress, which is probably beneficial in lowering BUN and Cr levels in diabetic animals.

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Authors' Contributions

Iraj Salehi: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, administrative, technical and material support, and study supervision. Alireza Pouyandeh Ravan: analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, and administrative, technical and material support. Shirin Moradkhani; Amaneh Mohammadi Roushandeh; and Hamideh Nazari: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, and administrative, technical and material support.

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