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Synergism effects of pioglitazone and *Urtica dioica* extract in streptozotocin-induced nephropathy via attenuation of oxidative stress

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ABSTRACT

Objective(s): Hyperglycemia promotes oxidative stress that plays a crucial role in the pathogenesis of Diabetic nephropathy (DN). In this study, we investigated the synergism effects of hydroalcoholic extract of *Urtica dioica* and pioglitazone (PIO) on the prevention of DN in streptozotocin induced-diabetic mice.

Materials and Methods: Forty-two mice were divided into six groups as follows: non-diabetic control group, DMSO group (as solvent), diabetic group and four treatment groups which received *U. dioica*, pioglitazone, *U. dioica* plus pioglitazone and vitE. Diabetes was induced by a single dose of streptozotocin (STZ) (200 mg/kg body wt, IP) diluted in citrate buffer (pH= 4.6). After 4 weeks treatment, all animals were anaesthetized and blood was collected for serum urea and creatinine levels assessment in plasma and kidney tissue were excised for evaluation of oxidative stress markers.

Results: Treatment with *U. dioica* significantly inhibited increase in serum urea and creatinine in plasma that were observed in diabetic mice. Furthermore, the elevated level of oxidative stress markers (glutathione oxidation, lipid peroxidation (LPO), protein carbonyl) in renal supernatant of diabetic mice was inhibited by *U. dioica* treatment. Interestingly, *U. dioica* promoted beneficial effects of PIO in reducing STZ-induced hyperglycemia, renal damage and oxidative stress markers.

Conclusion: Our findings showed that PIO plus *U. dioica* have synergism protective effects against STZ-induced nephropathy that can be a candidate as a therapeutic approach in order to treatment of DN.

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Introduction

Diabetes mellitus (DM) is a chronic and progressive metabolic disease which is described with hyperglycemia ensuing impaired insulin secretion and insulin resistance that leads to hyperglycemia (1). The prevalence of type 2 diabetes is rapidly growing with various complications. One of the most important difficulties of this metabolic disease is diabetic nephropathy, which is believed as the main cause of end-stage renal failure (2). However, the main mechanisms of the pathogenesis of Type 2 diabetes still remains to be elucidated, but it is shown that oxidative stress is involved in the progression of Type 2 diabetes, which leading to increased lipid peroxidation and DNA damage (3). Also impairment in anti-oxidant protection systems creates a condition known as oxidative stress (4). Oxidative stress (OS) is the result of disequilibrium between increased production and decreased anti-oxidant capacity of cell (5). OS not only takes part in the cluster of procedures that eventually leads to impaired glucose metabolism, insulin resistance and diabetes, but also is important in the development of diabetes-related complications. OS plays an important role in both micro and macro vascular complications of diabetes, including retinopathy, nephropathy, cerebro- and cardiovascular and peripheral vascular diseases (6). With spare of their production pathway, reactive oxygen species (ROS) consequently, cause damage to cellular proteins, lipids and DNA (7). Hyperglycemia is believed to boost ROS production directly through generation of high amounts of OH free radicals resulting from glucose autoxidation (8). With regard to the role of OS in diabetes, many studies focused on anti-oxidants, especially in herbal medicine to reduce the complications. Plant have different compounds with various biological effects that make it possible to search for natural anti-hyperglycemic agents with minor side effects (9). *Urtica dioica* is belonging to the plant family Urticaceae which is used in the world as a herbal medicine (10). The blood sugar

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lowering effectof this medicinal plant has been reported previously (11, 12). Also, agents with anti-oxidant effects widely used in traditional natural treatments for diabetes (13). Pioglitazone (PIO) is a member of the thiazolidinedione category that used as synthetic ligands for peroxisome proliferator-activated receptor-γ (PPAR-γ) (14). The anti-hyperglycemic effect of PIO is related to its ability to enhance insulin sensitivity, which increases the efficacy of insulin Dose-related improvements in hyperglycemia, hyperinsulinemia, and hypertriglyceridemia have been indicated in animal models of type 2 DM after administration of PIO (10). With respect to weak management of diabetic nephropathy (DN) with common therapeutic approach, and known role of hyperglycemia-induced oxidative stress in pathogenesis of DN, in this study, we evaluated the synergism efficacy of pioglitazone in combination with *U. dioica* on inhibition of DN in streptozotocin- induced diabetic mice.

Materials and Methods

Plant Extraction

U. dioica was collected around the city of Sari, Iran and identified by a Faculty of the Department of Pharmacognosy, Mazandaran University of Medical Sciences. The plants were dried in the shade and ground to powder by an electric grinder. The extraction was prepared using Soxhlet method at most 50 °C temperature using 70% ethanol and 30% water. The mixture was filtered with Whatman filter paper (No 1), and the filtrate was centrifuged at 3000 rpm for 20 min. The supernatant was evaporated at limited temperature, and the extract powder was kept at 4 ˚C until used.

Animal treatment

Male Albino mice (25±2 g) were purchased from Laboratory Animals Research Center, Mazandaran University of Medical Sciences, Sari, Iran. Animals were housed in an air-conditioned room with controlled temperature of 22±2 °C and maintained on a 12:12 hr light cycle with free access to food and water. All experiments were done according to the ethical protocols approved by the Committee of Animal Experimentation of Mazandaran University of Medical Sciences.

Experimental design

Forty-two mice were divided into 6 groups as follows: non-diabetic control group, DMSO group (as a solvent), diabetic group and 4 treatment groups, which received *U. dioica*, PIO, *U. dioica* plus PIO and vitE (as known anti-oxidant). Diabetes in male Swiss albino mice was induced by a single dose of intraperitoneal injection of streptozotocin (200 mg/kg body wt, IP) diluted in citrate buffer (pH= 4.6). One week after STZ administration, blood was taken from the lateral veins of the tail and blood glucose was measured by a glucometer using glucose oxidase method. The mice whose blood glucose rates were above 200 mg/dl were accepted as diabetic. The day on which hyperglycemia had been confirmed was considered as day 0. Diabetes was symptomatically confirmed by the presence of hyperglycemia, polyuria, polydipsia and weight loss in the following weeks. Serum glucose concentration and body weight were monitored at the start and the end of the study. Also, any type of insulin didn't use during study in diabetic animals. After 4 weeks treatment, all animals were anaesthetized and blood was collected for serum urea and creatinine levels assessment in plasma and kidney tissue was excised on ice and was homogenized in phosphate buffered saline (5 times of tissue volume), the homogenate was centrifuged at 800 × g for 10 min at 4°C to separate the nuclear and cellular body debris. The supernatant was obtained by centrifugation at 10,500×g for 20 min for assessment of oxidative stress markers.

Scavenging effect on DPPH radical

Free radical scavenging activities of herbal extracts was determined by using a stable DPPH radical (15). Three concentration of hydroalcoholic extract was prepared (250, 500 and 1000 mg/ml) and well mixed with 4 ml of methanol and 0.4 mM of DPPH solution. The mixture was kept at room temperature for 30 min and then the absorbance was measured at 517 nm. The scavenging effect was derived following equation:

DPPH scavenging %= $[1/(A_{517\ nm, sample} - A_{517\ nm, control})] \times 100$

Oxidative stress assay

Determination of ROS

To determine the amount of ROS generation, dichlorofluorescin-diacetate (DCFH-DA) was used as an indicator. Briefly, 2 ml of renal supernatant (1 mg protein/ml) loaded with DCFH by incubating with this buffer for 15 min at 37 °C. Then it was monitored at 480 nm (excitation) and at 520 nm (emission) by Shimadzu RF5000U fluorescence spectrophotometer (16).

Measurement of Lipid peroxidation (LPO)

The content of MDA was determined by using the method of Zhang *et al* 2008(17). Briefly, 0.25 ml phosphoric acid (0.05 M) was added to 0.2 ml of kidney tissue supernatant with the addition of 0.3 ml 0.2% thiobarbituric acid (TBA). All the samples were placed in a water bath (100 °C) for 30 min. Then, the tubes were moved to an ice-bath and 0.4 ml n-butanol was added to each tube and was centrifuged at 3500 rpm for 10 min. The absorbance of the supernatant at 532 nm with an ELISA reader (Tecan, Rainbow Thermo, Austria)

and the content of MDA in each of the samples was estimated through standard curve using tetramethoxypropane (TEP) as standard (18).

Measurement of glutathione content

Glutathione (GSH) content was determined by DTNB as an indicator and spectrophotometer. Briefly, 0.1 M of phosphate buffers and 0.04% DTNB was added to 0.1 ml of renal supernatant in a total volume of 3.0 ml (pH 7.4). Then developed yellow color, was read at 412 nm on a spectrophotometer (UV-1601 PC, Shimadzu, Japan). GSH content was expressed as µg/mg protein (19).

Measurement of protein carbonyl

Determination of protein carbonyl was performed by the spectrophotometric method. Briefly 200 µl of kidney tissue is needed to homogenate. Samples are extracted in 500 µl of 20% (w/v) TCA. Then, samples are placed at 4 °C for 15 min. The precipitates are exposed with 500 µl of 0.2% DNPH and 500 µl of 2 NHCl for control group, and samples are incubated at room temperature for 1 hr with vortexing at 5-min intervals. Then proteins are precipitated by adding 55 µl of 100% TCA. The microtubes are centrifuged and washed three times with 1 ml of the ethanol-ethyl acetate mixture. Finally, the pellet is dissolved by adding 0.2 ml of guanidine hydrochloride (6 M). The carbonyl concentration is estimated by measurement of the absorbance at 365 nm wavelength (20).

Statistical analysis

All results are expressed as mean±SEM. Distribution of our data follows a normal pattern. Significance of difference between two groups was evaluated using unpaired and paired Student's t-test. For multiple comparisons, one-way analysis of variance (ANOVA) was used. When ANOVA showed significant difference, Tukey's *post-hoc* test was applied. Statistical significance was regarded as $P<0.05$.

Results

Before and one week after the STZ administration (day 0) the blood glucose were measured. As showed in Table 1, *U. dioica* caused significant decrease in blood glucose in comparison with diabetic control but this effect was lower than PIO. Also, PIO plus *U. dioica* showed synergism effect on downturn of blood glucose.

According to Table 2, at the end of study the weight of the diabetic control mice significantly decreased as compared with control mice ($P< 0.05$) and *U. dioica* treatment markedly inhibited decrease in the weight of the diabetic mice as compared with control diabetic group ($P<0.05$).

As shown in Table 3, diabetes induction was associated with significant ($P<0.05$) increase in serum levels of serum urea and creatinine which are indicators of kidney damage and administration of PIO and *U. dioica* prevented the elevation of serum urea and creatinine in diabetic mice. Also simultaneous treatment with PIO and *U. dioica* showed better effect than PIO alone.

Table1. Effect of *Urtica dioica* extracts on blood glucose levels in Streptozocin-induced diabetic mice (mg/dl)

Groups	Before streptozotocin	Day 0	Day 30
Control	86±4.1	87±7.06	91±6.3
Diabetic	85±6	289±21[a]	310±22[a]
DMSO	91±7.2	88±6	90±5.5
D+U	87±5.4[b]	268±18[b]	188±11[b]
D+Pio	88±3.9[b]	281±20[b]	168±13.7[b]
D+Pio+U	87±6.04[b]	280±19.6[b]	150±10.5[b]

Values represented as mean± SEM (n=6). [a]$P<0.05$ compared with control mice, [b] $P<0.05$ compared with diabetic mice

Table2. Effect of *Urtica dioica* extracts on body weight in streptozotocin-induced diabetic mice (g)

Groups	Day 0	Day 30
Control	26±2.08	35±2.8
Diabetic	25.6±2	21±1.6 a
DMSO	26.1±3	33±2.5
D+U	25.8±2.05	30±2.36 b
D+Pio	26±2.09	29.5±2.7 b
D+Pio+U	24.8±2.56	32.3±2.58 b

Values represented as mean± SEM (n=6). [a]$P<0.05$ compared with control mice, [b] $P<0.05$ compared with diabetic mice

Table 3. Effect of *Urtica dioica* extracts on serum urea and Creatinine levels in streptozotocin-induced diabetic mice (mg/dl)

Groups	Serum urea (mg/dl)	Creatinine (mg/dl)
Control	24±3	0.5±0.03
Diabetic	73±9[a]	1.2±0.08[a]
DMSO	25.1±1.4	0.56±0.04
D+UD	50.2±8[b]	0.85±0.1[b]
D+Pio	38±4[b]	0.66±0.03[b]
D+Pio+UD	30±6.3[b]	0.61±0.05[b]

Values represented as mean±SEM (n=6). [a]$P<0.05$ compared with control mice, [b] $P<0.05$ compared with diabetic mice

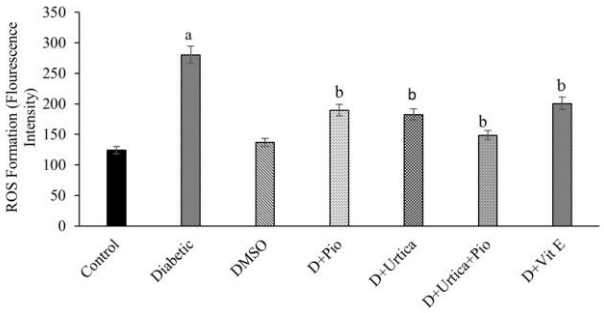

Figure 1. Effect of *Urtica dioica* and pioglitazone (Pio) on diabetes-induced ROS formation in kidney tissue. Values represented as mean±SEM (n=6). [a]*P*<0.05 compared with control mice, [b]*P*<0.05 compared with diabetic mice

It is showed in Figure 1 that ROS formation significantly was increased in diabetic mice (*P*<0.05), and markedly were decreased after treatment with *U. dioica* and PIO (*P*<0.05). Simultaneous administration of PIO and *U. dioica* causes more reduction in ROS formation.

Elevation of MDA and protein carbonyl is accepted as an important marker for oxidative stress. MDA (In Figure 2) and protein carbonyl level (in Figure 3) was increased in diabetic mice in comparison with control group (*P*<0.05). Furthermore, PIO and *U. dioica* treatment showed more inhibition against LPO and protein carbonyl than PIO.

The GSH levels (as the main intracellular anti-oxidant) in diabetic mice decreased as compared to control mice. Treatment with *U. dioica* showed effects like PIO in inhibition of GSH oxidation in diabetic mice that significantly (*P*<0.05) inhibited GSH oxidation in diabetic mice which is showed in Figure 4.

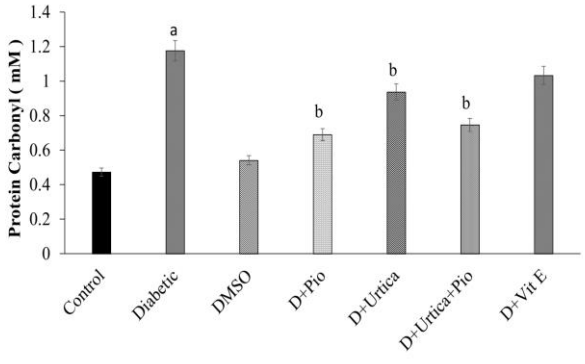

Figure 3. Effect of *Urtica dioica* and pioglitazone (PIO) on protein carbonyl level in kidney of diabetic mice. Values represented as mean± SEM (n=6). [a]*P*<0.05 compared with control mice,[b]*P*<0.05 compared with diabetic mice

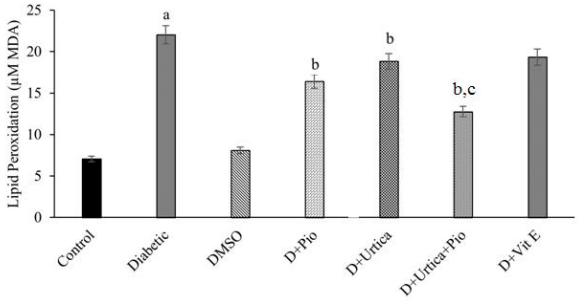

Figure 2. Effect of *Urtica dioica* and pioglitazone (Pio) on Diabetes-induced lipid peroxidation in kidney tissue. Values represented as mean± SEM (n=6). [a]*P*<0.05 compared with control mice, [b]*P*<0.05 compared with diabetic mice.[c]*P*<0.05 compared with PIO group

DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a stable free radical which its absorbance at 517 nm use for studying the effects of trapping free radicals. Anti-oxidants have ability to donor protons to free radicals and reduce the absorbance that is a yardstick for measurement of trapping free radicals. Capacity of scavenging free radicals by DPPH method shown in Figure 5. Inhibition at concentrations of 250, 500 and 1000µg/ml, respectively, 21, 40 and 77% was determined. Butylated hydroxyl toluene (BHT) also known as a synthetic anti-oxidant prepared at the same concentration and the inhibitory effect was determined which was 29, 49 and 87% respectively.

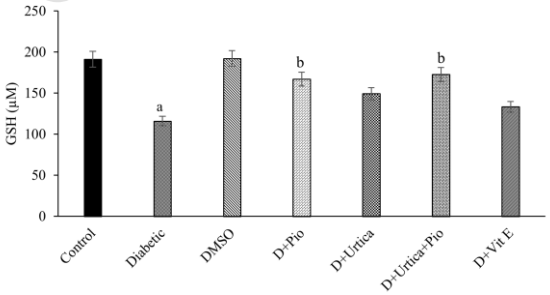

Figure 4. Effect of *Urtica dioica* and pioglitazone (PIO) on diabetes-induced GSH oxidation in kidney tissue. Values represented as mean±SEM (n=6). [a]*P*<0.05 compared with control mice,[b] *P*<0.05 compared with diabetic mice

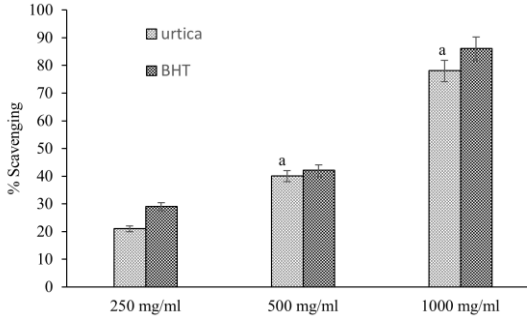

Figure 5. the mean percentage of DPPH free radical scavenging and anti-oxidant BHT by different concentrations of *Urtica dioica* extract

Discussion

Nowadays, diabetic nephropathy considered as a long-term complication of diabetes and in despite of the current treatments for lowering blood glucose and blood pressure, many diabetic patients are still experience developing kidney failure (21). In this study, we showed the potential benefits of *U. dioica* in attenuation of the kidney damage and the decreased oxidative stress which observed in the diabetic kidney tissue. We also exhibited that *U. dioica* seems to have synergism effect with pioglitazone by lowering and improving the oxidative stress status in the kidney via the scavenging of ROS.

We used STZ-induced diabetes mice as a relevant example of endogenous chronic oxidative stress and hyperglycemia. We Showed STZ administration a diabetic state characterized by hyperglycemia. STZ- induced nephropathy was confirmed by an increased serum concentration of in serum urea and creatinine. Also, STZ increased ROS formation, lipid peroxidation, protein carbonyl level and decrease in GSH concentration that was consistent with the previous studies (22, 23).

Increasing prevalence of Type 2 diabetes and subsequent complication of this disorder through of the world, provided needing to new therapeutic approaches based on pathogenesis of this disorder (24, 25). In fact, current therapeutic protocols could not achieve tight glycemic control in diabetic patients. Moreover, oxidative stress was suggested as one of the main processes in the pathogenesis of diabetes complications (26). It has been shown that elevation of blood glucose level in diabetic patients could lead to induction of ROS generation in both humans and animals (27). Our results confirmed the imbalance of the ROS production and anti-oxidant system in kidney tissue in comparison to control mice that was parallel to elevation of serum urea and creatinine in serum of diabetic mice. These results confirmed the previous studies that reported oxidative stress occurred due to hyperglycemia and also hyperglycemia was considered as the major risk factor for development of diabetic nephropathy (22). Therefore, in addition to strict glycemic control, using of anti-oxidant may be a useful approach to amelioration of pathologic consequences of hyper-glycemia. Use of anti-oxidants has been increased in the management of diabetes side effects over the last few years (28, 29). Recently it has been shown that anti-oxidant effects of many natural and synthetic compounds were effective for protection against diabetic nephropathy (30, 31). However, it seems that using a natural product alone cannot be suitable for management of DN. Therefore, in this study we used hydroalcoholic extract of *U. dioica* in combination with a common chemical medicine, pioglitazone, for lowering blood glucose and DN in the model of STZ- induced diabetes. According to Ilhami Gülçinl (2004) study, *U. dioica* had powerful anti-oxidant activity. The 50, 100 and 250 µg amounts of water extract of *U. dioica* showed 39, 66 and 98% inhibition on peroxidation of linoleic acid emulsion, respectively, while 60 µg/ml of α-tocopherol, exhibited only 30% inhibition (32). *U. dioica* is known for its useful effects in lowering blood sugar in the traditional medicine (12, 32). We observed that high serum levels of glucose, creatinine and serum urea in diabetic mice improved after treatment with *U. dioica*. Although effect of *U. dioica* was lower than PIO but showed synergism effects in lowering both blood glucose and nephrotoxicity markers with PIO. On the other hand, increased levels of ROS, LPO and protein carbonyl in the diabetic group are consistent with previous reports that showed increased oxidative stress during diabetic nephropathy (23, 28). These parameters significantly (P<0.05) decreased in diabetic mice that received *U. dioica*. Indeed, administration of vit E showed lower protective effect than *U. dioica* on amelioration of DN that showed both anti-oxidant and also lowering blood glucose effects of *U. dioica* are contributed in its protective effects.

So, it can be suggested that anti-oxidant effects of *U. dioica* besides its lowering effects on blood glucose may be helpful for improvement of DN as adjuvant therapy with common therapeutic protocols.

Conclusion

Our results showed that *U. dioica* has protective effects against DN via reducing oxidative stress and blood glucose. Therefore, anti-oxidant features of *U. dioica* make it an attractive candidate as complementary therapy beside other blood glucose-lowering drugs for diabetic complications.

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