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Research Article

The Effects of *Urtica dioica* and *Lamium album* Extracts on the Expression Level of Cyclooxygenase-2 and Caspase-3 in the Liver and Kidney of Streptozotocin-Induced Diabetic Rats

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ABSTRACT

Background: Diabetes seems to be associated with increased inflammation and induced apoptosis in several tissues. *Urtica dioica* and *Lamium album* have shown to possess a variety of beneficial properties like anti-inflammatory effects. In this experimental study, we tried to evaluate the effects of *U. dioica* and *L. album* extracts on the expression level of cyclooxygenase-2 (COX-2; as an inflammation marker) and caspase-3 (CASP-3; as an apoptotic marker) in the liver and kidney tissues of diabetic rats.

Methods: Thirty-two male Wistar rats were randomly allocated to four groups: normal control, diabetic control, diabetic treated with *U. dioica* (100 mg/kg/daily), and diabetic treated with *L. album* (100 mg/kg/daily) for 28 days. At the end of the study, liver and kidney tissues were harvested and mRNA expression level of COX-2 and CASP-3 was determined by real-time PCR technique. Also, serum glucose was measured.

Results: Liver COX-2 mRNA in diabetic rats was significantly higher than normal control rats (P=0.02). However, *U. dioica* and *L. album* caused significant decrease in mRNA expression of liver COX-2 in diabetic rats (P=0.015 and P=0.03, respectively). Also, in diabetic rats treated with both extracts, serum glucose was remarkably lower than diabetic control rats (P<0.0001 and P<0.01, respectively).

Conclusion: It appears that *U. dioica* and *L. album* might decrease liver damage by decreasing the inflammatory effects of COX-2 in streptozocin-induced diabetic rats. Since these plant extracts may influence diabetes by several mechanisms, further research in this field is warranted.

Introduction

Diabetes mellitus (DM) is one of the most common metabolic disorders defined by chronic hyperglycemia. DM is the outcome of insufficiency in insulin secretion or action which leads to main complications such as anomalies in the metabolism of carbohydrate, lipid, and protein. These anomalies might cause damage to several important organs like pancreas, kidney, and liver.[1,2]

Two isoforms of cyclooxygenase (COX) have been determined. COX-2, the inducible isoform, is little measurable in the normal tissues, however, is upregulated during inflammatory disorders leading to prostaglandins (PGs) release.[3] It has been reported that diabetes is associated with increased inflammation.[4,5] Also, it has been indicated that inflammation might cause apoptosis by activating caspases such as caspase-3 (CASP-3).[6,7] In both intrinsic and extrinsic pathways of apoptosis, CASP-3 operates as a common product.[8]

According to an estimation of the World Health Organization, the global number of patients with diabetes would be raised to around 370 million by the year 2030.[9] Therefore, researching for powerful therapeutic approaches with low adverse effects is still appreciated. *Considerable attention* has been focused on the use of medicinal herbs and traditional treatments.[10-13]

Urtica dioica, also called stinging nettle, is shown to have a variety of activities such as anti-inflammatory (through COX-1 and COX-2 inhibition),[14] antioxidant,[15,16] and antilipidemic[17] actions. *Lamium album* (non-stinging nettle) has been reported to present some useful properties including antiproliferative, anti-inflammatory, antioxidant, and free radical scavenging effects.[18-21]

The importance of biochemical pathways involved in diabetes development or progression is obvious, there is no report regarding the potential effects of *L. album* on the gene expression of COX-2 (as a marker of inflammation) and CASP-3 (as a marker of apoptosis) in the diabetic tissues. So, in the present study, by using real-

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time PCR technique, the effects of *L. album* and *U. dioica* extracts on COX-2 and CASP-3 from the liver and kidney tissues were evaluated in sterptozotocin induced diabetic rats.

Methods and Materials

Animals

Seven-week-old adult male Wistar rats (250-300 g) were obtained from breeding and maintaining laboratory animal center of Guilan University of Medical Sciences. The animals were adapted to the normal laboratory conditions (12 h light: 12 h dark cycles at 22-26 ºC) for one week before conducting tests with free access to their suitable water and diet. All experiments were done in line with the internationally accepted principles for laboratory animal use and care as established in the US guidelines (NIH publication #85-23, revised in 1985). All procedures for this experimental study were accepted by the ethics committee of Guilan University of Medical Sciences (Rasht, Iran) (Approval No. 5930069503).

Diabetes induction

After overnight fasting, the rats were treated with a single intraperitoneal (IP) injection of STZ (60 mg/kg body weight freshly prepared in sodium citrate buffer, pH 4.5)[22] (Sigma-Aldrich Diagnostic Ltd, USA, PubChem CID: 29327). Normal rats received the equal volume of sodium citrate buffer. Diabetic rats were approved after 3 days by evaluating fasting blood sugar (FBS) by glucometer (Accu chek, Roche, Germany), and animals with FBS ≥300 mg/dl were recognized as diabetic animals.[10,22]

Plant material

Aerial portions of *U. dioica* (Herbarium number: 156HGUM) and *L. album* (Herbarium number: 202HGUM) were collected around Rasht city (Guilan province) during spring 2016 and the species were confirmed at the herbarium unit of Pharmacognosy Department, Pharmacy Faculty (Guilan University of Medical Sciences).

Plant extraction and its antioxidant capacity

After drying the aerial parts of both nettles in the shade, preparation of the plant extracts was performed as previously described.[12] Polyphenols were extracted from the powdered nettles as stated by the modified method of Zhang et al[23] and Zheng et al.[24] The total phenolic content of both plant extracts was identified using the Folin-Ciocalteu reagent[25] with Gallic acid as standard and the results were expressed as mg gallic acid (GAL)/g plant extract (Table 1).

Table 1. Total phenolic and flavonoids contents of *U. dioica* and *L. album* extracts.

Plant Extract	Phenolic content (mg GAL/g)	Flavonoids content (mg QE/g)
U. dioica	0.21	1.96
L. album	0.61	2.10

Total flavonoid content was assessed using aluminum chloride ($AlCl_3$)[26] with Quercetin as standard and the results were expressed as mg quercetin (QE)/g plant extract (Table 1).

Study Design

All rats were randomly separated into four groups containing eight rats in each group as follows:

Group 1: normal control rats treated daily with sodium citrate buffer; Group 2: diabetic control rats treated daily with citrate buffer; Group 3: Diabetic rats treated daily with the hydroalcoholic extract of *U. dioica* at a dose of 100 mg/kg; Group 4: Diabetic rats treated daily with the hydroalcoholic extract of *L. album* at a dose of 100 mg/kg. Treatment was initiated three days after diabetes induction via IP injection and all rats were kept for 28 days on their relevant treatments.[12] On the 28th day of the treatment, liver and kidney tissues were obtained, stored in liquid nitrogen until evaluation of COX-2 and CASP-3 mRNA expression by real-time PCR. Also, FBS was measured at day 0, day 14, and day 28.

RNA extraction

Approximately100 mg of the liver and kidney tissues were minced into the small pieces and added to 1 ml YTzol Pure RNA solution (YEKTA TAJHIZ AZMA, Iran). Then, total RNA was extracted according to the manufacturer procedure. The purity of the extracted RNA was determined through assessing the absorbance at 260/280 nm using the Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., USA).

DNase I treatment

DNase I is an endonuclease that digests single and double-stranded DNA. For the preparation of DNA-free RNA prior to real-time PCR, the extracted RNA was treated with RNase-free DNase I according to the manufacturer protocol (Thermo Fisher Scientific Inc., USA). Briefly, 1 µL RNase-free DNase I per µg RNA in a 10-µL reaction mixture was used, then, incubated at 37 °C for 30 min and subsequently 1 µL EDTA (50 mM) was added and incubated at 65 °C for 10 min.

Complementary DNA (cDNA) synthesis

Single strand cDNA was synthesized from 1 µg of the total RNA in a 20-µL reaction mixture using HyperScriptTM First-strand Synthesis Kit (GeneAll, South Korea). Briefly, 1 µg RNA, 1 µl Oligo dT primer (50 µM), 1 µl dNTPs (10 mM) was added into a 0.2 ml nuclease-free microtube, and then reached to 14 µl using nuclease-free water and mildly mixed. Then, it was incubated at 65 °C for 5 min and immediately placed on ice for at least 1 min. Following a small spinning, the mixture was pipetted into the reaction tube containing 2 µl of RTase reaction buffer (10x), 2 µl of 0.1 M DTT, 1 µl of HyperScript^{TM} Reverse Transcriptase (200 U/µl) and 1µl of ZymAll^{TM} RNase Inhibitor. After brief centrifugation, microtubes were incubated at 55 ºC for 60 min. Termination of the reaction was done by incubating

at 85 °C for 5 min. Finally the samples were placed on ice for a while, and then preserved in -20 ºC until needed.

Real-Time PCR

The mRNA expression levels of COX-2 and CASP-3 were analyzed using real-time PCR with the ABI instrument (StepOne™, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β2-microglobulin (B2M) genes were considered as reference genes for normalization of COX-2 and CASP-3 expression levels. The PCR primers were designed by Primer3web (version 4.0.0), and were synthesized by GenFanAvaran Co.; Tehran, Iran. The specificity of the designed primers for the selected genes was checked using the Primer-BLAST system available at the National Center for Biotechnology Information (NCBI). The sequences and product sizes of the designed primers are given in Table 2.

The reaction mixture contained 1 µl of each primer, 4 µl of diluted cDNA, 10 µl of SYBR Green Master Mix (YEKTA TAJHIZ AZMA, Iran), and 4µl nuclease-free water. The amplification protocol was initially planned at 95 °C for 10 min, then continued with 40 cycles in two steps (95 °C for 10 s, 60 °C for 60 s), and finally terminated with one cycle at 72 °C for 5 min. Each amplification product was analyzed with a dissociation curve to confirm no non-specific bands or primer dimer formation. At the end, the differences between mRNA expression of reference and test samples were calculated, and the relative mRNA expressions of COX-2 and CASP-3 were calculated using $2^{-\Delta\Delta CT}$ method.[27,28] Each sample was run in triplicate.

Statistical analysis

Data are presented as mean ± SD. Inter-group comparisons were performed using the one-way analysis of variance (ANOVA) followed by post hoc Tukey's test. In each experimental group, repeated measure ANOVA was used to compare serum glucose level among different times. For all tests, $P<0.05$ was considered as statistically significant. Data were analyzed using SPSS software version 22.

Results

Evaluation of relative mRNA expression of COX-2 in the liver and kidney of different experimental groups

According to the real-time PCR results, the expression of B2M gene was more stable than GAPDH gene in the samples extracted from the liver tissues, therefore, B2M was selected as the reference gene. In contrast, for the samples obtained from the kidney tissues, GAPDH was selected as the reference gene.

The mRNA expression of COX-2 in the liver of diabetic rats was significantly higher than that of normal control rats (P=0.02). However, hydroalcoholic extract of *U. dioica* and *L. album* caused significant decrease in mRNA expression of liver COX-2 in diabetic rats (P=0.015 and P= 0.03, respectively) (Figure 1).

Table 2. Sequences of gene-specific primers.

Primer name	Sequence (5' ⟶ 3')	Nucleotide count	product size
GAPDH-F	CCACAGTCCATGCCATCACT	20	101
GAPDH-R	TGCAGGGATGATGTTCTGGG	20	
B2M-F	AATTCACACCCACCGAGACC	20	94
B2M-R	TACATGTCTCGGTCCCAGGT	20	
COX-2-F	ATGATCTACCCTCCCCACGT	20	119
COX-2-R	ACTCTGTTGTGCTCCCGAAG	20	
CASP-3-F	GCTGGACTGCGGTATTGAGA	20	142
CASP-3-R	CCATGACCCGTCCCTTGAAT	20	

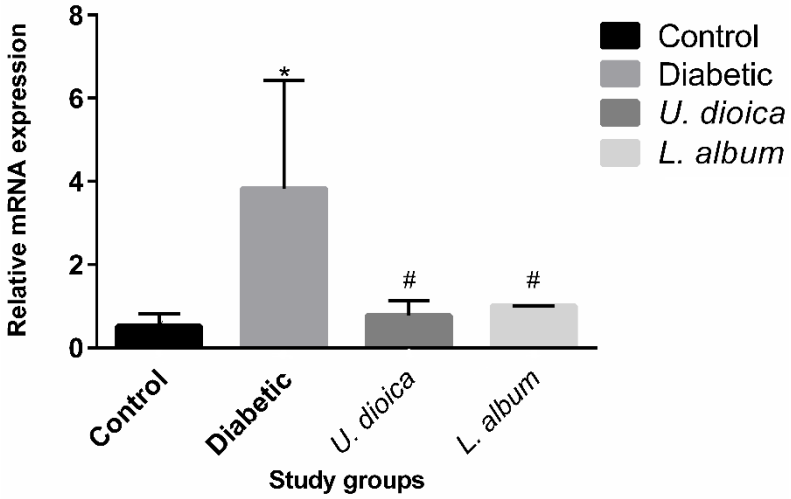

Figure 1. Relative mRNA expression of COX-2 in the liver of different experimental groups. Values are presented as mean±SD.* P<0.05 by comparison with normal control rats; #P<0.05 by comparison with diabetic rats.

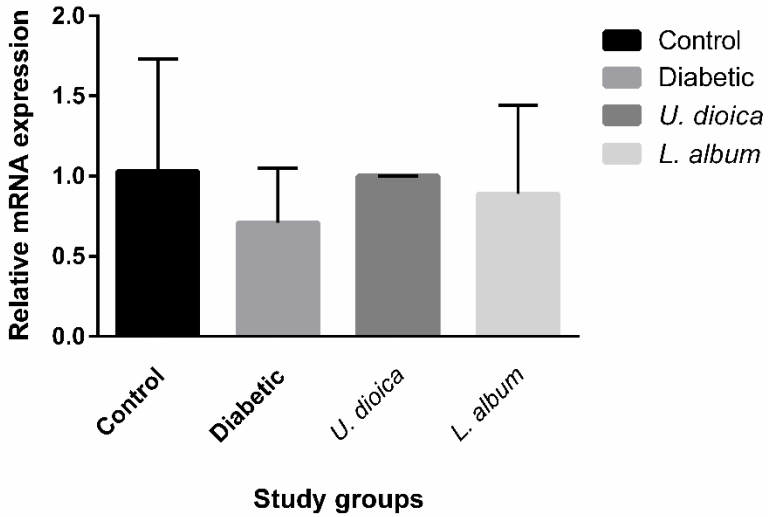

Figure 2. Relative mRNA expression of CASP-3 in the liver of different experimental groups. Values are presented as mean±SD.

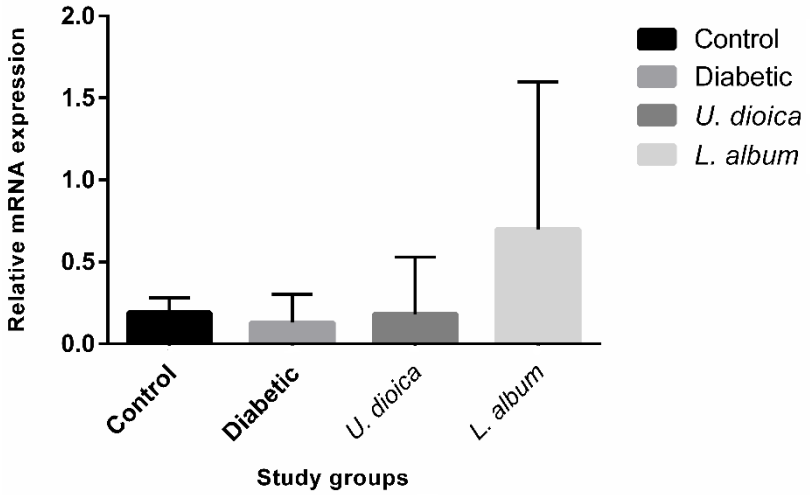

Figure 3. Relative mRNA expression of CASP-3 in the kidney of different experimental groups. Values are presented as mean±SD.

There was a significant increase in the mRNA level of liver COX-2 in diabetic rats treated with *L. album* as compared to normal rats (P=0.01). No significant difference was observed in the expression level of COX-2 mRNA in the liver between diabetic rats treated with *U. dioica* and *L. album* (P=0.2) (Figure 1).

The expression level of COX-2 mRNA in the kidney did not differ significantly between normal control rats and diabetic control rats. Also, there was no significant difference in mRNA expression of kidney COX-2 between diabetic control rats and diabetic rats treated with *U. dioica* and *L. album* separately (data not shown).

Evaluation of mRNA expression of CASP-3 in the liver and kidney of different experimental groups

The mRNA expression of CASP-3 was similar in normal control rats and diabetic control rats in both liver and kidney tissues (P=0.43 and P=0.7, respectively) (Figure 2 and Figure 3, respectively). Also, for the liver and kidney tissues separately, the expression level of CASP-3 mRNA did not differ significantly between diabetic control and diabetic groups under each of the plant extract treatments (Figure 2 and Figure 3, respectively).

Table 3. Comparison of serum glucose levels in different experimental groups.

Groups	Serum glucose* (mg/dl)		
	day 0	day 14	day 28
Normal Control	100±12	94±11	103±10
Diabetic Control	495±48 [a]	472±107 [a]	499±27 [a]
Diabetic + *U. dioica*	431±83 [a]	224±16 [a,b,d]	144±121 [b,d]
Diabetic + *L. album*	461±110 [a]	257±16 [a,c,d]	176±146 [c,d]

*Values are given as mean± SD
[a] P < 0.0001 by comparison with normal control rats
[b] P < 0.0001 by comparison with diabetic control rats
[c] P < 0. 01 by comparison with diabetic control rats
[d] P < 0. 01 by comparison with the day 0

Comparison of serum glucose levels during four weeks in different experimental groups

Serum glucose level significantly increased in diabetic control rats in comparison with normal control rats (P<0.0001). In diabetic rats treated with *U. dioica* and *L. album* extracts, serum glucose level was remarkably lower than diabetic control rats (P<0.0001and P<0.01,

respectively) (Table 3). In *U. dioica* and *L. album* groups, the level of serum glucose showed significant decrease on the day 14 and the day 28 when compared with the day 0 ($P<0.01$) (Table 1).

Discussion

For the first time, we studied the effect of *L. album* on mRNA expression of COX-2 and CASP-**3** in the kidney and liver of diabetic rats. We found that the expression level of COX-2 in the liver of diabetic rats was significantly higher than that of normal control rats. *U. dioica* and *L. album* extracts improved the inflammatory reaction to diabetes in the liver, since indicated by markedly reduction in the expression level of COX-2 mRNA in this tissue. As mentioned earlier, COX-2 is an important enzyme involved in the biosynthesis of prostaglandins, and is related to inflammatory responses.[29] It is indicated that the induction of COX-2 following an inflammatory reaction could lead to toxic impacts on the liver.[30] The present finding was partly in line with that of our previous report[12] in which *U. dioica* and *L. album exhibited hepatoprotective property in diabetic rats, however, only the serum level of hepatic marker enzymes were evaluated. Also, we recently found that the U. dioica and L. album extracts could increase serum concentration of insulin-like growth factor 1 (IGF-1) in diabetic rats.*[31] *Also,* in the study performed by García-Mediavilla et al,[30] two flavonoids, called quercetin and kaempferol, showed significant decrease in the level of COX-2 mRNA in Chang liver cells, which could be due to their anti-inflammatory properties. Our findings were similar to those found by the study of Lee et al. in which the expression level of COX-2 in the liver of diabetic mice was significantly higher than that of normal control mice.[32] The expression level of COX-2 in the liver of diabetic mice treated with persicarin (an important flavonoid element separated from *O. javanica*) was remarkably lower than that of diabetic mice. It was concluded that persicarin reduced the inflammation related to COX-2 expression in the liver.[32]

Komers et al. found[33] that the expression level of COX-2 protein in the renal cortex of diabetic rats was significantly higher than that of normal animals. The principal role of the diabetes condition at the beginning of this abnormality was suggested.[33] However, in our study, the expression level of COX-2 mRNA in the whole kidney was determined and showed no significant difference in diabetic versus nondiabetic control rats. In the present study, the expression level of COX-2 mRNA in the kidney did not differ considerably between diabetic control and diabetic groups under each of the plant extract treatments. In the study conducted by Golalipour et al,[34] *U. dioica* leaves extract didn't exhibit any beneficial effect on morphometric changes of the kidney in STZ-induced diabetic rats. Our finding was somewhat similar to that found by the study of Yar et al[35] in which the expression level of COX-2 mRNA in rat kidney between the diabetic group and the diabetic group treated with resveratrol (RSV; as an anti-inflammatory compound) was not significantly different. It was suggested that combination therapies with other antidiabetic agents might be an attempt to change the expression of COX-2. It is likely that the use of higher concentrations or longer-term treatment of our plants extracts can be more useful than the present therapeutic procedure.

In the present study, there was no significant difference in the expression level of CASP-3 in the liver between diabetic control rats and diabetic animals treated with each of the two extracts. In one study conducted by Giribabu et al,[36] the effect of *Vitis Vinifera* seed extract (VVSE) on CASP-3 in the liver of STZ-nicotinamide induced diabetic rats was evaluated. VVSE induced significantly decrease in CASP-3 of the liver in diabetic rats. It was suggested that VVSE through anti-inflammatory effect might be effective in decreasing apoptosis of the liver in diabetic rats. In that study, CASP-3 in the liver was examined by immunohistochemistry, real-time PCR, and western blotting and three different doses of the extract were used. However in the present study, protein quantification of cleaved CASP-3 was not examined and only one dose of the extracts was used. Kumar et al.[37] evaluated the effect of *Houttuynia cordata Thunb* extract on the expression level of CASP-3 in the liver of STZ-induced diabetic rats. *H. cordata* did not induce any significant change on the level of CASP-3 in the liver, indicating that the extract might not be effective on diabetes-induced apoptosis in the liver. It was concluded that further studies are needed in this field.

Arjumand et al[38] showed that two doses of rutin (a potent flavonoid) were potential protective against apoptosis induced by cisplatin in the kidney of Wistar rats. In that study, CASP-3 expression in the kidney as a marker of apoptosis in tubular cells was evaluated by immunohistochemical method. Ibrahim et al[39] revealed that three doses of strawberry leaves extract caused significant decrease in CASP-3 activity in the kidney of diabetic rats. It was suggested that this finding might be attributed to declining renal reactive oxygen species (ROS) after administration of strawberry leaves extract. However, in our study**,** *U. dioica* and *L .album* extracts did not exhibit any considerably change on the expression level of CASP-3 in the kidney of diabetic rats. As mentioned earlier, the level of CASP-3 mRNA in the kidney was evaluated and as one limitation we didn't assess CASP-3 activity.

As we know, liver is among the most important tissues involved in biomolecules metabolism such as maintenance of glucose homeostasis, and the association between liver abnormality and DM has been indicated.[40] In the present study, in line with our previous studies,[12,31] *U. dioica* and *L. album* improved abnormal metabolism in the liver by reducing hyperglycemia.[32] It could be mentioned again that the presence of polyphenols and flavonoids in *U. dioica* and *L. album* extracts has been determined using phytochemical analysis[19]. The hypoglycemic influence of both plant extracts might be partly due to the polyphenol compounds.[41,42]

Our study had some limitations; 1) We didn't assess the

enzymatic activity of CASP-3 and COX-2. 2) The histologic characteristics of the livers and kidneys were not evaluated using light microscopy. Additional studies exploring the potential effects of different doses of these plant extracts on other inflammatory or apoptotic markers together with protein quantification of the cleaved CASP-3 in several tissues are warranted.

Conclusion

It appears that *U. dioica* and *L. album* might decrease liver damage by decreasing the inflammatory effects of COX-2 in streptozotocin–induced diabetic rats. Since these plant extracts may influence diabetes by several mechanisms, further research in this field might be regarded as an additional support for their helpful impacts on diabetes.

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Conflict of interests

The authors claim that there is no conflict of interest.

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