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Corrective effects of *centaurium umbellatum* against diabetes-induced hepatic injury and oxidative stress in rats

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ABSTRACT

The present study investigated the effects of *Centaurium umbellatum* (CU) extract on liver oxidative stress in streptozotocin (STZ) induced diabetic rats. Animals were divided into four groups of seven each: a negative control which received saline solution; a positive control which received 200 mg/kg bw of CU; STZ diabetic rats and diabetic rats co-treated with CU (200 mg/kg bw). The activities of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) were decreased, while those of catalase (CAT) and the levels of reduced glutathione (GSH); malondialdehyde (MDA) and carbonyls (PCO) were increased in liver of STZ diabetic rats as compared to those of controls. Moreover, there was an increase in the activities of liver biomarkers like alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and γ-glutamiltransferase (γ-GT). Daily treatment with CU improved all the biochemical parameters cited above. All changes were confirmed by liver histological pictures. The results indicated that CU possessed antioxidant properties and might be used in the future as a protective agent against hepatotoxicity induced by diabetes.

KEYWORDS

Streptozotocin;
Centaurium umbellatum;
Rat;
Oxidative stress;
Liver.

INTRODUCTION

Oxidative stress plays a major role in the pathogenesis of diabetes and its associated complications[1]. Free radicals are formed disproportionately in diabetes by glucose oxidation, non-enzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins, as well as of unsaturated fats in plasma and membrane proteins[2]. The mechanism of β-cell destruction involves the secretion of cytokines, principally tumor necrosis factor (TNF)-α, interleukin (IL)-1, and interferon (IFN)-γ, as a result of the oxidative stress[3]. Diabetes provokes damages in multiple organs and induces several complications such as coronary artery disease, renal and ophthalmologic diseases that can result in the disability and mortality for diabetic patients. Liver disease as one of diabetic complications has not been well addressed, but it can be actually very significant[4]. Increasing evidence suggests that among patients with diabetes, the standardized mortality rate from

end-stage liver disease (i.e., cirrhosis) is higher than that for cardiovascular disease[5].

Although, many drugs are marketed for the management of diabetes, their continuous intake may cause harmful side effects. Therefore, many studies have been undertaken to investigate the potential of natural medicine plants for the effective treatment of diabetes. *Centaurii herba* (*Centaurium umbellatum*), belonging in Gentianaceae family, is a medicinal plant used in numerous countries combined with other plants. As preliminary phytochemical study, this medicinal plant (CU) has been reported to contain phenolic acids, natural drugs[6]. Studies in experimental animals have shown that plant mixture extract containing *Centaurium umbellatum* and other plants like *Vaccinium myrtillus* L. and *Taraxacum officinale* Web. have a variety of pharmacological functions including antihyperglycemic, free radical scavenging and antioxidant activities[7-9]. We have recently reported that leaf extracts of *Centaurium umbellatum* attenuated nephrotoxicity induced by streptozotocin in adult rats[10]. While, the protective effects of *C. umbellatum* on free radicals and antioxidant enzyme activities against oxidative stress inducing liver damage in streptozotocin-induced diabetic rats, has never been examined. Thus, to achieve this goal, the current investigation was carried out in order to study the possible hepatoprotective effects of *Centaurium umbellatum* leaf extracts in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Plants source and extracts preparation

Mature whole plants were collected in North Tunisia between May and June (2009). According to the flora of Tunisia, they were identified as *Centaurium umbellatum* by Professor Emeritus Abdelhamid Nabli, botanist at the Faculty of Sciences, University of Tunis El Manar. A voucher specimen was deposited at the Faculty of Pharmacy (Monastir, Tunisia). Leaves of *Centaurium umbellatum* were washed quickly in running water, dried in an oven at 40°C and then finely powdered in a Willey mill. The powder was extracted with distilled water (50 g powder/500 ml water) by boiling under reflux for 20 min. The decoction obtained was centrifuged, filtered, frozen at -20 °C and then lyophilised (Free Zone® Dry 4.5, USA) to yield approximately 10% (w/w) of the tansy extract, and was stored at -20 °C until used.

Animals

The experimental protocol was approved by the Local Animal Care Committee at Sfax University. All the experimental procedures were carried out in accordance with international guidelines for Care and use of laboratory animals[11].

Adult male Wistar rats weighing between 230 and 250 g were obtained from the Central Pharmacy (SIPHAT, Tunis, Tunisia). The animals were housed at 22 ± 3°C, 45–75% humidity, 12 h light–dark cycle, and kept to acclimate for 1 week before the onset of experiments. They were fed commercial standard pellet diet (SICO, Sfax, Tunisia) and provided with water *ad libitum*.

Experimental diabetes induction

The experimental animals were made diabetic with an intraperitoneal injection of streptozotocin (STZ) at a dose of 65 mg/kg bw diluted immediately before injection in citrate buffer (0.1 M, pH 4.5). Diabetes was conûrmed in the overnight-fasted rats by measuring blood glucose concentration 72 h after injection with STZ. The rats with blood glucose above 250 mg/dl were considered to be diabetic and they were used for the experiments. Treatment with plant extract was started on the third day after STZ injection and continued for 30 days.

Animals treatment

The animals were divided into four groups of seven each. They were treated as follows. Group 1: (C) negative control rats received vehicle only; group 2: (STZ) diabetic rats received STZ in a single dose (65 mg/kg bw, intraperitoneal way); group 3: (STZ + CE) CE-treated diabetic rats received by intraperitoneal way 200 mg/kg bw of CE extract; group 4: (C + CE) CE-treated rats, received only CE extract (200 mg/kg bw, intraperitoneal way).

At the end of the experiment, overnight fasted rats were sacriûced by cervical decapitation to avoid animal stress and blood was collected without heparin. Serum samples were obtained after centrifugation at 2200 × g for 15 min. Liver tissue was excised immediately from the rats. Median portions were drawn, weighed and homogenized with phosphate buffer (0.1 M, pH 7.4), centrifuged at 3000 × g and the superna-

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tants were collected. Biochemical estimations were carried out in the homogenates.

Biochemical assays

Estimation of AST, ALT, LDH and γ-GT activities

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ-glutamyltransferase (γ-GT) activities in serum, used as biochemical markers of hepatic damages, were estimated spectrophotometrically using commercial reagent kits (refs. 20043; 20047; 20012; 20022 respectively. Biomaghreb Diagnostic, Ariana. Tunisia).

Assay of lipid peroxidation

Lipid peroxidation in the liver tissue was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) which were expressed in terms of malondialdehyde content according to Draper and Hadley method[12]. Briefly, aliquots of liver homogenates were mixed with 0.5 ml of 30% TCA and centrifuged at 4000 × g for 10 min.1 ml of thiobarbituric acid reagent (TBA, 0.67%) was added to 1 ml of supernatant and heated at 95°C for 15 min. The mixture was then cooled and was measured for absorbance at 532 nm. The MDA values were calculated using 1,1,3,3-tetraethoxypropane as the standard and expressed as nmol of MDA/g of tissue.

Assay of protein carbonyl

Protein oxidation was determined based on the reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazone, as described by Reznick and Packer[13]. Samples were read at 370 nm and carbonyl content was calculated using the molar absorption coefficient for aliphatic hydrazones (22.000 M^{-1} cm^{-1}) and expressed as nmol carbonyl/mg protein.

Measurement of reduced glutathione (GSH)

Hepatic GSH content was determined by Ellman's method[14], modified by Jollow *et al*.[15] based on the development of a yellow colour when DTNB is added to compounds containing sulfhydryl groups. Briefly, 3 ml of sulfosalicylic acid (4%) were added to 500 µl of homogenate tissues for deproteinisation. The mixture was centrifuged at 2500 × g for 15 min. Then Ellman's reagent was added to 500 µl of supernatant. The absorbance was measured at 412 nm after 10 min. Total GSH content was expressed as mg/g of tissue.

Assay of antioxidant enzymes

The catalase (CAT) activity was determined according to the Aebi method[16]. The rate of H_2O_2 decomposition was followed by monitoring absorption at 240 nm. One unit of CAT activity is defined as the amount of enzymes required to decompose 1 µmol of hydrogen peroxide in 1 min. The enzyme activity was expressed as µmol H_2O_2 consumed/min/mg protein.

Superoxide dismutase (SOD) activity was estimated according to the method of Beauchamp and Fridovich[17]. The developed blue colour in the reaction was measured at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expressed as U/mg protein.

Glutathione peroxidase (GPx) activity was measured according to the method of Flohe and Gunzler[18]. The enzyme activity was expressed as nmol of GSH oxidized/min/mg protein.

Protein analysis in liver

Protein concentrations were measured using the method of Lowry *et al*.[19]. Briefly, 200 µl of reaction mixture ($NaCO_3$ 2%, Tartrate double of Na and K 2% and $CuSO_4$ 1%) were added to 200 µl of liver homogenate. The mixture was treated with 200 µl of Folin-phenol reagent diluted to 1/2 and then incubated in the dark for 30 min. The absorbance was measured at 500 nm. Bovine serum albumin was used as a standard.

Histopathological studies in the liver

After fixation in 10% of formalin solution, liver portions were dehydrated in an ascending graded series of ethanol, cleared in toluene and embedded in paraffin. Sections of 5-6 µm thickness were made by using a rotary microtome and stained with hematoxylin and eosin (H&E) for microscopic observations. Six slides were prepared from each liver. For degree of liver injury evaluation, slides were examined and scored for severity of changes using scores on a scale of none (‘), mild (+), moderate (++) and severe (+++).

Statistical analysis

The data were analyzed using the statistical package program Stat view 5 Software for Windows (SAS Institute, Berkley, CA). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's protected least signiûcant differ-

ence (PLSD) test as a post hoc test for comparison between groups [(STZ, STZ + CU, C + CU) vs. (C)] and [STZ + CU] vs. [STZ]. All values were expressed as means ± S.E. Differences were considered signiûcant if $p < 0.05$.

RESULTS

Hepatic biomarkers

Intraperitoneal administration of STZ to experimental rats caused liver injuries. In fact, specific biomarkers of liver damages such as aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and γ-glutamiltransferase (γ-GT) were significantly increased, respectively by 57, 71, 64 and 82% in diabetic rats when compared to controls. Administration of CU extracts countered the significant rise in the activities of the parameters cited above. Treatment with CU extracts alone did not change LDH, γ-GT, AST and ALT activities compared to controls (TABLE 1).

TABLE 1 : Serum activities of AST, ALT, LDH and γ-GT of adult rats (controls and experimental groups).

Groups	Control (C)	Diabetes (STZ)	Diabetes + CU (STZ + CU)	Control + CU (C + CU)
AST[a]	84.87 ± 7.40	197.20 ± 20.76	125.68 ± 10.20^{***++}	77.00 ± 19.82
ALT[b]	110.83 ± 18.17	382.80 ± 32.48***	251.06 ± 23.83^{***+++}	112.75 ± 7.04
LDH[c]	138.28 ± 8.26	386.74 ± 24.67***	138.62 ± 17.91^{+++}	139.63 ± 15.30
γ-GT[d]	9.12 ± 1.22	51.04 ± 1.94***	7.43 ± 0.76^{+++}	9.32 ± 1.10

Data are expressed as mean ± S.E. (n=7); Treated groups (STZ); (STZ + CU); (C + CU) vs control group (C): $^{***}p < 0.001$. (STZ + CU) group vs (STZ) group: $^{++}p < 0.01$; $^{+++}p < 0.001$; ^{a,b,c,d}: (U/l).

Liver oxidative damage and antioxidant status

Liver malondialdehyde (MDA) and protein carbonyl (PCO) levels

In the liver homogenate of diabetic rats, MDA and PCO levels increased 5-fold compared to those of control group. Administration of CU extracts to diabetic group decreased MDA and PCO contents in the liver when compared to diabetic rats. In positive controls (control group treated with CU), liver MDA and PCO contents were not significantly changed, when compared to negative controls (TABLE 2).

Liver glutathione (GSH) levels

A significant increase of GSH levels in liver (+28%) was evident in diabetic rats. Administration of CU extracts reduced GSH levels reaching control values in (STZ + CU) group when compared to diabetic group (TABLE 2).

Enzymatic antioxidant status in liver

TABLE 2 depicted the activities of enzymatic antioxidants such as SOD, CAT and GPx in the liver of the control and experimental groups. Compared to controls, SOD and GPx activities significantly decreased by 26 and 54%, respectively in diabetic rats, while CAT activity increased by 11%. Treatment of diabetic rats with CU extracts improved significantly the antioxidant enzyme activities compared to those of diabetic rats, without reaching control values. CU extract, administered alone to rats during 30 days, did not change antioxidant enzyme activities.

TABLE 2 : MDA, PCO, GSH levels and antioxidant enzymes activities (SOD, CAT, GPx) in the liver tissue of adult rats (controls and experimental groups)

Groups	Control (C)	Diabetes (STZ)	Diabetes + CU (STZ + CU)	Control + CU (C + CU)
MDA[a]	32.56 ± 1.65	162.04 ± 9.25***	88.77 ± 3.02^{***+++}	36.92 ± 0.94
PCO[b]	2.61 ± 0.01	13.05 ± 0.07***	4.89 ± 0.03^{***++}	2.71 ± 0.02
GSH[c]	82.38 ± 1.58	115.32 ± 2.59***	86.57 ± 0.90^{+++}	82.21 ± 5.99
SOD[d]	117.59 ± 9.94	86.17 ± 3.97***	91.16 ± 4.63^{***+}	118.83 ± 8.38
CAT[e]	56.63 ± 2.45	64.15 ± 7.44**	60.87 ± 7.60^{*+}	56.70 ± 6.40
GPx[f]	4.60 ± 0.26	2.11 ± 0.19***	3.55 ± 0.71^{+++}	4.40 ± 0.76

Data are expressed as mean ± S.E. (n=7); Treated groups (STZ); (STZ + CU); (C + CU) vs control group (C): $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; (STZ + CU) group vs (STZ) group: $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$; ^{a} : (nmol/g tissue); ^{b}: (nmol /mg protein); ^{c}: (mg /g tissue); ^{d} : (U/mg protein); ^{e}: (µmol H_2O_2 /min/mg protein); ^{f}: (nmol GSH/min/mg protein).

Histological studies

The histological examination of liver sections stained with Hematoxylin and Eosine showed a normal architecture of hepatocytes in control group (Figure 1, A). CU extract did not show any remarkable effects in the group treated with CU alone compared with the control group (Figure 1, B). Liver sections of STZ-induced diabetic group showed alterations on hepatocellular architecture, evidenced by small foci of hepatocytes necrosis (Figure 1, C1 and C2) and numerous dilated sinusoidal spaces (Figure 1, C3). These alterations were partially improved in the liver sections of the STZ diabetic rats co-treated with CU extract for thirty days (Figure 1, D). Grading scores were summarized in TABLE 3 to evaluate the histological changes after CU extract treatment.

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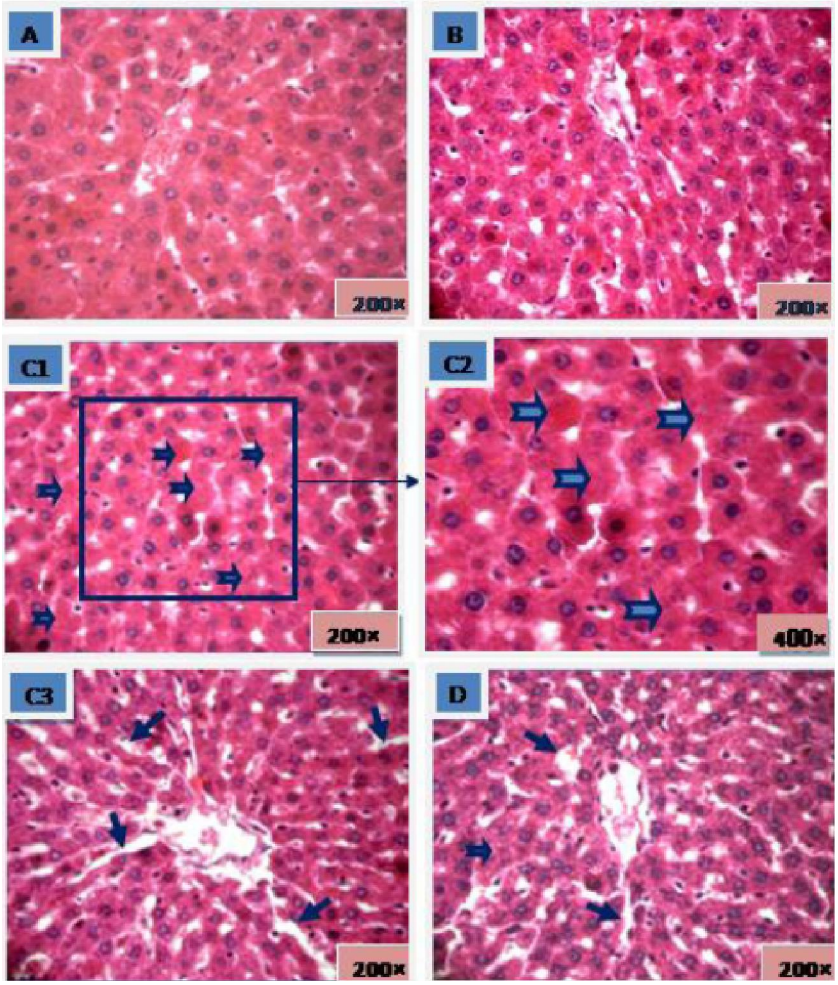

Fig. 1

Histological observation on liver sections from STZ-induced liver damage rats with or without CU extract treatment. (A) Control group. (B) CU treated control group. (C1, C2, C3) STZ diabetic group. (D) CU treated diabetic group. Optic microscopy; hematoxylin–eosin stain; magnification A, B, C, C1, C3, D: (200×) and C2: (400×).

→: sinusoidal dilatation spaces; ⇒: small foci of necrosis.

TABLE 3 : Scores of the histological changes in the liver sections.

Groups	Control (C)	Diabetes (STZ)	Diabetes + CU (STZ + CU)	Control + CU (Control + CU)
Sinusoidal dilatation	–	+++	++	–
Necrosis	–	+++	+	–

Scoring was categorized as none (“), mild (+), moderate (++) and severe (+++).

DISCUSSION

The onset and progression of diabetes resulted from the chronic accumulation of free radicals in the pancreatic β-cells[20]. Antioxidant compounds present in natural sources could scavenge free radicals, when supplemented in the diet of diabetes[21].

For the assessment of liver injury, serum concentrations of the most commonly used biochemical markers, AST, ALT and LDH were determined[22]. Injury to the hepatocytes in STZ-induced diabetic rats alters membrane permeability and affects their transport, leading to leakage of enzymes from the cells[23] . Therefore, the marked release of AST, ALT and LDH from liver cytosol into circulation and an increase of γ-GT, a membrane enzyme, found by previous study[24] indicate severe damages to hepatic tissue membranes in type 2 diabetes. Our results corroborated this study. *Centaurium umbellatum* leaf extracts, when administered to STZ diabetic rats, were able to preserve the structural integrity of the hepatocellular membrane, as can be seen from the evident reduction in the activity of these enzymes, due probably to the richness of this plant

in phenolic acids considered as antioxidant compounds, as previously reported by Hatjimanoli and Debelmas[6]. Our results were in agreement with those found by other researchers who have demonstrated that resveratrol[25] and quercetin[26] could conserve the membrane integrity of cellular organelles in the diabetic state.

During oxidative stress, auto-oxidation and the presence in excess of hydroxyl radicals lead to carbohydrate damages. The injury was well evidenced by the production of malondialdehyde (MDA) and reactive carbonyl compounds (PCO)[27]. Lipid peroxidation is frequently used as an index of tissue oxidative stress, where oxygen interacts with polyunsaturated fatty acids leading to the formation of lipid products like MDA and 4-HNE, and increase generation of free radicals, causing damage of membrane components, cell necrosis and inflammation[28]. Increased generation of free radicals can also lead to the formation of protein–protein crosslinkages, oxidation of protein backbones resulting in protein fragmentation, and modification of amino acid side chains, which including oxidation of sulphydryl moieties and formation of PCO. In the present study, we observed the enhanced formation of MDA and PCO in liver of STZ diabetic rats. *C. umbellatum* leaf extracts treatment potentially abrogated hepatic MDA and PCO concentrations by its antioxidant capacity. The normalization of these parameters might be accomplished by the bioactive constituents of *C. umbellatum*, which protected the cells from injuries as reported by Amat *et al.*[29].

Another important aspect to be discussed in our study was glutathione (GSH), which was the essential compound in maintaining cell integrity against free radicals[30]. A significant increase in hepatic GSH levels of STZ diabetic rats was obtained in our study. This could be a part of a compensatory mechanism against oxidative stress. The increase in hepatic GSH levels in diabetic rats was normalized by treatment with *C. umbellatum* leaf extracts. Similar results were reported by previous studies in diabetic rats treated by oil of *Eruca sativa* seeds[31].

In addition, to avoid oxidative stress, antioxidant enzymes function as free radical scavengers and in the repair of free radicals causing biological damage. CAT and SOD are the two major enzymes which remove ROS *in vivo*. SOD catalyzes the dismutation of superoxide anion ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2). Then it is degraded to H_2O_2 by CAT or by GPx. A decrease in the activity of these antioxidants may lead to an excess of availability of $O_2^{\cdot-}$ and H_2O_2, generating hydroxyl radicals, which result in initiation and propagation of lipid peroxidation[32]. In line with this, we observed a decrease in the activities of antioxidants enzymes like SOD and GPx in liver of diabetic rats. This may be due to the inactivation caused by the excess of free radicals and/or by non-enzymatic glycation due to the persistent hyperglycemia, which has been extensively reported to occur in diabetes[33]. On the other hand, our study demonstrated that CAT activity increased in diabetic rats. The excess of antioxidant enzymes activities has been reported by Tiedge *et al.*[34] as an adaptive response to protect cells against the toxicity of free radicals. Therefore, the occurrence of uncoordinated activities of these antioxidant enzymes in diabetes might imply that the diabetic rats were not protected against oxidative stress. *C. umbellatum* leaf extract prevented the reduction in SOD and GPx activities in liver of STZ-induced diabetic rats, indicating a possible role of the phenolic acids present in CU extract in free radical inactivation and in the antioxidant defense. These results are consistent with a reduction of oxidative stress found in other studies, where the resveratrol treatment greatly ameliorated antioxidant enzyme activities and prevented the rise of lipid peroxides in tissue and blood cells of diabetic animals[35,25].

In the present study, the histopathological examination displayed small foci of necrosis in hepatocytes of diabetic rats, as well as dilated sinusoidal spaces. This could be explained by the increased production of highly reactive intermediates of STZ as reported by Blum and Fridovich[36]. The pathological changes were partially reduced in diabetic rats treated with *C. umbellatum* leaf extract. Thus, in addition to the blood glucose-lowering effect, histopathological observations also supported the notion that CU produced a significant increase of antioxidant enzymes and partially protected the hepatic tissues of diabetic rats.

In conclusion, the findings of the present study demonstrated that *Centaurium umbellatum* treatment might provide effective protection against oxidative damage in liver of STZ-induced diabetic rats, since this plant was able to ameliorate enzymatic and non-enzymatic antioxidant defense system and to prevent the lipid peroxidation in this tissue. Moreover, it is important to point out that this is the first study to investigate the effects of CU in streptozotocin induced liver damage

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demonstrating the important role of phenolic acids as the potent antioxidant compounds. Taken together, these results might contribute to a better understanding of the hepatoprotective role of *Centaurium umbellatum* and other medicinal plants administered in the diet for human health, possibly preventing hepatic complications associated with *Diabetes mellitus*.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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