Centaurium erythraea methanol extract improves the functionality of diabetic liver and kidney by mitigating hyperglycemia-induced oxidative stress

Miloš M. Đorđević [a,*], Anja Tolić [a], Jovana Rajić [a], Mirjana Mihailović [a], Jelena Arambašić Jovanović [a], Aleksandra Uskoković [a], Nevena Grdović [a], Marija B. Đorđević [a], Danijela Mišić [b], Branislav Šiler [b], Melita Vidaković [a], Svetlana Dinić [a]

[a] *Department of Molecular Biology, Institute for Biological Research "Siniša Stanković" – National Institute of Republic of Serbia, University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia*
[b] *Department of Plant Physiology, Institute for Biological Research "Siniša Stanković" – National Institute of Republic of Serbia, University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia*

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ABSTRACT

The use of medicinal herbs can mitigate oxidative stress-induced diabetic complications and organ failure. This study investigated hepato- and reno-protective effects of methanol extract of *Centaurium erythraea* Rafn (CEE) in STZ-diabetic rats pre-treated (2 weeks) and/or post-treated (4 weeks) with CEE (orally, 100 mg/kg/day). Both CEE treatments markedly improved liver and kidney functionality in diabetic rats observed as reduced aspartate and alanine aminotransferase activities and decreased creatinine and blood urea nitrogen levels. CEE pre-treatment reduced the level of glycosylated proteins in diabetic liver more efficiently than post-treatment. Lowered levels of lipid peroxidation, DNA damage and protein glutathionylation, elevated ratio of reduced to oxidized glutathione, and mitigated disturbance of antioxidant enzyme activities reflected the antioxidant effect of CEE in diabetic liver and kidney. Although CEE pre-treatment was more effective, the obtained results indicate that both treatments protect the liver and kidney from oxidative damage by boosting the endogenous antioxidant defense system.

1. Introduction

With 537 million adult patients in 2021 and an expected rise to 784 million by 2045, diabetes represents a major health issue nowadays (International Diabetes Federation, 2021). Diabetes is characterized by hyperglycemia arising from a lack of insulin secretion or defects in insulin action. If left untreated, prolonged hyperglycemia leads to progressive tissue damage and organ failure, including both liver and kidney.

The liver plays a crucial role in maintaining glucose homeostasis by producing glucose during fasting and storing it for later use. Net glucose production in the liver results from the processes of gluconeogenesis, glycogen synthesis, glycogenolysis, and glycolysis (Petersen et al., 2017). Hepatic glucose metabolism is largely regulated by insulin (Edgerton et al., 2017), whose deficiency in diabetes conditions increases hepatic glucose production and contributes to the exacerbation of hyperglycemia. Since muscle cells and adipocytes cannot use glucose as an energy source without adequate insulin action (Tokarz et al., 2018), glucose is accumulated in insulin-insensitive tissues such as the kidneys, retina, peripheral nerves, and blood vessels, causing their

damage and development of complications.

Experimental data over the past decades recognized oxidative stress as the primary cause of long-term diabetic complications' onset and progression (King and Loeken, 2004). Oxidative stress arises when the production of reactive oxygen and nitrogen species (ROS and RNS, respectively) exceeds the capacity of the cellular antioxidant system to neutralize them. Glucose autoxidation, non-enzymatic glycosylation and subsequent formation of AGEs (advanced glycation end-products), protein kinase C activation, overactivity of hexosamine and polyol pathways, and superoxide overproduction by the mitochondrial electron transport chain altogether contribute to increased oxidative stress in hyperglycemic conditions (Giacco and Brownlee, 2010; Rains and Jain, 2011). Increased ROS and RNS levels cause oxidation of cellular proteins, lipids, carbohydrates, and DNA and ultimately lead to morphological and functional changes in tissues. Therefore, boosting the endogenous antioxidant system can protect cells from an excessive amount of free radicals.

Modern diabetes therapy relies on the use of insulin and hypoglycemic drugs, as well as on lifestyle modifications. Various drugs exert their antidiabetic effect by promoting insulin secretion, enhancing cellular glucose uptake by increasing peripheral tissue sensitivity to insulin, inhibiting hepatic gluconeogenesis, delaying gastric emptying and intestinal glucose absorption, or promoting glucose loss through urine (Zeng et al., 2020). Despite significant progress in the field of diabetes treatment, the results gained so far are way from expected and only <50% of diabetic patients achieve glycemic control (Edelman and Polonsky, 2017; Khunti et al., 2018). Moreover, the use of antidiabetic drugs carries the risk of adverse effects such as gastrointestinal intolerance, abdominal discomfort, weight gain, or fluid retention with possible cardiac arrest, while insulin therapy might induce life-threatening hypoglycemia (American Diabetes Association, 2019).

Increasing life expectancy of diabetic patients requires an integrative approach for the treatment not only of hyperglycemia but also of long-term complications, both micro- and macrovascular (Vennos et al., 2013). Insufficient efficiency and safety of current diabetic therapy, combined with the disease's emergence as a global epidemic, has prompted the development of new therapeutic strategies. To mitigate deleterious effects of diabetes and its complications, patients are increasingly turning to complementary and alternative medicine (CAM), with medicinal plants as the most commonly used CAM (Kifle, 2021). Medicinal plants are a rich source of secondary metabolites such as terpenes, phenolic compounds, and alkaloids (González Mera et al., 2019) that provide them a broad spectrum of biological activities, including antioxidative, antidiabetic, hepatoprotective, immunomodulatory, and antiinflammatory. Many of today's medications are structurally derived from these natural chemicals (Ota and Ulrih, 2017), which highlights the importance of in-depth analysis of medicinal plants and their constituents.

Centaurium erythraea Rafn (CE), from the *Gentianaceae* family, is traditionally used in many countries to treat a range of diseases, including diabetes (El Menyiy et al., 2021). Previous reports demonstrated a significant hypoglycemic effect of CE extract in diabetic rats (Đorđević et al., 2017; Sefi et al., 2011). The hepatoprotective effect of CE was demonstrated on acetaminophen-induced hepatotoxicity in rats (Mroueh et al., 2004), but none of the studies investigated the effect of CE on liver redox status. Although a diuretic effect of CE was demonstrated in adult normotensive rats (Haloui et al., 2000), there is no available data on the renoprotective effects of CE in diabetes. Therefore, the present study aimed to examine the effect of CE methanol extract on the liver and kidney of diabetic rats. We investigated CE extract effects on the functional properties as well as on protein glycosylation of diabetic liver and kidney. Since the liver and kidney are prone to oxidative damage in diabetic conditions, we evaluated the redox status parameters as well as the activities and protein levels of key antioxidant enzymes.

2. Material and methods

2.1. Plant material

The procedures for plant collection, preparation, and phytochemical analysis of CE methanol extract (CEE) used in this study have been already described by Đorđević et al. (2017). Briefly, aerial parts of plants were gathered in 2010 from the Andrijevica, Montenegro (42°44′26″N, 19°48′12″E) area. After air-drying and grinding in liquid nitrogen and overnight extraction of plant material in 96% methanol at room temperature (w:v = 1:5), the extract was sonicated for 20 min, filtrated through Whatman No. 1 filter paper, and evaporated at 30–45 °C using vacuum evaporator (Eppendorf Concentrator 5301, Germany). Thus obtained extract was subjected to metabolic profiling adopting UHPLC-qqqMS instrumentation.

2.2. Animals

Experimental procedures were conducted on albino male Wistar rats, 2.5 months old and weighing 220–250 g. The animals were maintained under climate-controlled settings, at a temperature of 22 ± 2 °C, relative humidity of 50%, and a defined 12 h day-night cycle, and had *ad libitum* access to tap water and standard food pellets. All animal procedures complied with the Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade (permit number 2–14/10).

2.3. Experimental design

Streptozotocin (STZ), freshly dissolved in sodium citrate buffer (pH 4.5), was intraperitoneally injected (40 mg/kg/i.p./five consecutive days) to rats for diabetes induction. Following 24 h after the last STZ injection, blood taken from the tail vein was used for determining glucose level with a blood glucose meter (Accu-Chek Active, India). The animals with fasting blood glucose levels higher than 20 mmol/L were considered as diabetic. CEE was dissolved in distilled water and given orally at a dosage of 100 mg/kg/day. The dose was chosen based on the common use of CE in Serbia as a herbal tea (two tablespoons of dry herbal material in 200 mL of boiling water, two to three times a day (http://www.mocbilja.rs/index. php/proizvod/caj-od-nadzemnog-dela-kicice/, assessed on August 29th, 2021)), with human-to-rat dose scaling according to Nair and Jacob (2016).

The rats were divided into five groups of seven individuals at random: C — non-diabetic control rats; CEE — non-diabetic rats treated with CEE; STZ — STZ-induced diabetic rats without treatment for four weeks; STZ/CEE — diabetic rats treated with CEE for four weeks, starting the day following last STZ injection (post-treated group); CEE/STZ/CEE — diabetic rats in whom CEE treatment started two weeks before the first dose of STZ, lasted during the induction of diabetes and continued for additional four weeks after the last dose of STZ (pre-treated group). Rats from both C and CEE groups received an equivalent volume of citrate buffer i.p. for five consecutive days. The weights of the animals were measured at the start and the end of the experiment. Additionally, CEE-treated animals were measured daily to ensure proper CEE dosage.

2.4. Tissue preparation

Four weeks after the last dose of STZ, blood collected from the tail vein of the overnight-fasted rats was clotted and centrifuged at 2000*g* for 10 min. Obtained serum was used for the determination of alanine and aspartate aminotransferase (ALT and AST, respectively) activities, blood urea nitrogen (BUN), and creatinine levels.

Following blood collection, the animals were anesthetized in the

gradual fill of CO_2 and euthanized by fast decapitation afterward liver and kidneys were isolated and measured. A portion of fresh tissue was ground in ice-cold HBSS buffer (0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 4 mM $NaHCO_3$, 140 mM NaCl, 1 mM $CaCl_2$, 5 mM KCl, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 6 mM D-glucose), containing 10% DMSO and 20 mM EDTA. The process was repeated three times with a fresh solution and the resulting cell suspension was collected and used to estimate DNA damage levels. Fresh tissue homogenate (10% in 1.15 M KCl) was prepared and used to examine the extent of lipid peroxidation in both liver and kidneys. For determination of the ratio of reduced to oxidized glutathione level (GSH/GSSG), a 20% homogenate prepared in a phosphate buffer (100 mM NaH_2PO_4, 1 mM EDTA, pH 7.5) was used. After homogenization of fresh tissue and centrifugation ($10,000g$ for 15 min at 4 °C), proteins were removed from the resulting supernatant using 5% 5-sulfosalicylic acid (SSA), followed by centrifugation ($5000g$ for 5 min at room temperature) and neutralization with triethanolamine. A 10% homogenate in sucrose buffer (0.25 M sucrose, 1 mM EDTA, 0.05 M Tris-HCl, pH 7.4) was prepared by homogenization of either fresh liver or kidney tissue, followed by sonication. After sonication, sample portion (0.5 mL) was set aside, deproteinized with 5% SSA, centrifuged ($5000g$ for 5 min), and the obtained precipitate was used for the analysis of S-glutathionylated proteins (GSSP). The remaining homogenate was centrifuged ($100,000g$ for 90 min) and the resulting supernatant was used to analyze activities of antioxidant enzymes and for immunoblot analysis. Prepared samples were stored at −80 °C until use.

2.5. Biochemical analysis

AST and ALT activities in the serum were determined by UV-kinetic method kit (ASAT (GOT) IFCC mod. to measure the production of pyruvate and ALAT (GPT) IFCC mod. to measure the production of oxaloacetate). Serum level of creatinine was measured using Creatinine (serum) Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), following the manufacturer's guidelines, while BUN level was determined using the kinetic GLDH method.

2.6. Single-cell gel electrophoresis (comet assay)

Alkaline comet assay was used to estimate DNA damage in the liver and kidneys (Singh et al., 1988). Pre-coated microscope slides were covered with a mixture of ten microliters of cell suspension and low-melting agarose, incubated in a lysis buffer (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, pH 10, 1% Triton X-100) for 2 h at 4 °C, and placed in a buffer for electrophoresis (300 mM NaOH, 1 mM EDTA, pH 13.0) for 30 min at 4 °C. Following electrophoresis, the samples were washed in a buffer for neutralization (0.4 M Tris-HCl, pH 7.4), dyed using SYBR Green I (Sigma-Aldrich, S9430), and visualized with a CCD camera-equipped fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Tail moment, quantified using TriTek CometScore™ Freeware v1.5, was used as the parameter for DNA damage.

2.7. Thiobarbituric acid reactive substance assay (TBARS)

The level of lipid peroxidation was evaluated using TBARS assay (Ohkawa et al., 1979). The reaction mixture containing 0.1 mL of liver or kidney samples, 0.7 mL water, 1.5 mL 20% acetic acid (pH 3.5), 0.2 mL 8.1% SDS, and 1.5 mL 0.8% TBA was boiled at 95 °C for 60 min and cooled to ambient temperature. After the addition of 5 mL of n-butanol/pyridine (15:1, v/v) and 1 mL of water, the mixture was stirred and centrifuged at $3000g$ for 10 min. The absorbance of the colored supernatant fraction was measured at 532 nm and the results were expressed as nmol MDA/100 mg of proteins based on the malondialdehyde (MDA) standard calibration curve.

2.8. GSH/GSSG ratio and GSSP level determination

Reduced to oxidized glutathione ratio in the liver and kidneys was determined using the Glutathione Assay Kit (Cayman Chemicals Company, Ann Arbor, MI, USA). Total GSH (reduced + oxidized) was estimated by mixing 50 μL of prepared deproteinized samples with 150 μL of the reaction mixture containing 0.1 M sodium phosphate buffer (pH 7.5), 0.3 mM 5,5′-dithio-*bis*-(2-nitrobenzoic acid), 0.4 mM NADPH, 1 mM EDTA, and 1 U of glutathione reductase I/mL. The absorbance at 412 nm was measured after incubation at room temperature for 30 min and total GSH content was estimated using a GSH calibration curve. For the GSSG determination, GSH was first removed from the samples by mixing with 10 mM 2-vinylpyridine and incubating for 1 h at room temperature. All samples and GSSG standards were analyzed as described above. GSH/GSSG ratio was calculated according to the formula: (total GSH – GSSG)/GSSG.

For GSSP determination, traces of GSH and GSSG were removed from acid-precipitated proteins by thorough washing with 1.5% trichloroacetic acid (TCA). The pellet was resuspended and incubated in a base phosphate buffer (0.1 M K_2H/KH_2PO_4, pH 7.5: 0.25 N NaOH, 9:1, v/v) for 30 min at room temperature. TCA (final concentration of 5%) was added to stop the reaction, and after centrifugation, GSH released in the supernatant was quantified enzymatically.

2.9. Determination of antioxidant enzymes' activity

Catalase (CAT) activity in the liver and kidney homogenates was measured via the rate of hydrogen peroxide degradation (Beutler, 1982). The epinephrine method described by Misra and Fridovich (1972) was used to measure total superoxide dismutase (SOD) activity. The activity of MnSOD in the samples was measured after deactivation of CuZnSOD with 8 mM KCN, while CuZnSOD activity was determined as total SOD activity – MnSOD activity. Glutathione peroxidase (GPx) activity was determined in a coupled enzyme system according to Tamura et al. (1982), while the activity of glutathione reductase (GR) was estimated from the NADPH oxidation during the reduction of GSSG (Glatzle et al., 1974). The enzyme activities were expressed as U/mg of proteins. The concentration of proteins for all assays was determined according to Lowry et al. (1951).

2.10. SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis

Liver and kidney homogenate proteins (20 μL) were subjected to SDS-PAGE and then transferred to PVDF membranes (Amersham Hybond P 0.45 PVDF, GE Healthcare Life Sciences, UK). Subsequently, the membranes were blocked with 5% non-fat dry milk (Blotto, Santa Cruz Biotechnology, USA) dissolved in a buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.6), and 0.05% Tween 20 for 1 h at room temperature and incubated overnight at 4 °C with the primary antibodies raised against CAT, GR, GPX 1 (Abcam, UK), MnSOD, CuZnSOD, GAPDH (Santa Cruz Biotechnology, USA), and β-O-linked *N*-acetylglucosamine (*O*-GlcNAc) (Sigma-Aldrich, USA). Membrane-bound primary antibodies were probed with the adequate HRP-conjugated IgG (Santa Cruz Biotechnology, USA) and visualized using the chemiluminescent technique following the manufacturer's guidelines (Western Blotting Luminol Reagent, Santa Cruz Biotechnology, USA). Immunoreactive bands were quantified using TotalLab (Phoretix, UK) electrophoresis software (v1.1).

2.11. Statistical analysis

All experimental data were analyzed with GraphPad Prism Software, ver. 5.00 (GraphPad Software, USA), and were expressed as the mean ± S.E.M. One-way ANOVA followed by Bonferroni multiple comparison test was used for comparisons among different groups. The difference

was considered to be significant with a p-value < 0.05.

3. Results

3.1. Effect of CEE on organ/body weight ratio and functional parameters of liver and kidney

The changes in selected biochemical parameters and organ/body weight ratio are presented in Table 1. Determination of the organ-to-body weight ratio, a useful and sensitive indicator of pathological changes in the internal organs, revealed increased liver/body and kidney/body weight ratios in diabetic rats by 72% and 67%, respectively when compared to the control rats. Treatment with CEE decreased liver/body weight ratio in both diabetic groups (17% in post-treated and 21% in pre-treated animals), while kidney/body weight ratio decreased only in the pre-treated group (31%). Diabetic rats displayed higher ALT and AST activities (4.75-fold and 1.77-fold, respectively) and increased serum levels of creatinine and blood urea nitrogen (BUN) (1.8-fold and 3-fold, respectively), as compared to the control group. CEE treatment did not affect any of these parameters in control rats. In contrast, post-treatment of diabetic rats with CEE decreased ALT and AST activities (2.26-fold and 1.63-fold, respectively) as well as creatinine and BUN levels (1.34-fold and 1.24-fold, respectively). In the pre-treated diabetic group, ALT and AST activities decreased 4.2-fold and 2.13-fold, respectively, while creatinine and BUN levels dropped 1.55-fold and 1.26-fold compared to diabetic rats. Also, pre-treated group had lower ALT and AST activities when compared to post-treated group (by 46.3% and 23.6%, respectively).

3.2. Effect of CEE administration on glycosylation of liver and kidney proteins in the hyperglycemic state

Posttranslational *O*-GlcNAc modification of liver and kidney proteins was examined by immunoblot analysis (Fig. 1). As expected, diabetic liver and kidney exhibited increased levels of *O*-GlcNAc modified proteins (105% and 55%, respectively). While post-treatment of diabetic rats with CEE significantly decreased the level of *O*–GlcNAc-modified proteins only in the liver (by 12%; p = 0.015), CEE pre-treatment decreased protein O–GlcNACylation in both liver and kidney of

diabetic rats (by 43% (p < 0.001) and 24% (p = 0.007), respectively). Moreover, the level of *O*–GlcNAc-modified proteins in the pre-treated group was 35.5% lower than the level observed in the post-treated group.

3.3. Effect of CEE on oxidative stress biomarkers in the liver and kidney of diabetic rats

Hyperglycemia-induced oxidative stress provokes damage to DNA, proteins, and lipids. As can be seen in Figs. 2A and 3A, the majority of liver and kidney cells of control animals did not display DNA damage, while diabetic animals had severely damaged DNA. Compared to control animals, a 24.3-fold and 21.5-fold higher tail moment was observed in diabetic liver and kidney, respectively. Administration of CEE protected DNA from oxidative damage in both diabetic groups. Compared to diabetic rats, the tail moment was notably reduced by 3.25-fold (p < 0.001) in the liver of the CEE-post-treated diabetic group and by 6.63-fold (p < 0.001) in the CEE-pre-treated group (Fig. 2A). In the kidney, CEE reduced DNA damage by 2.18-fold in post-treated and by 2.68-fold in the pre-treated group (Fig. 3A). Examination of parameters of oxidative stress in the liver and kidney of STZ-treated rats revealed a 40% and a 49.8% higher MDA level compared to normal rats, respectively (Figs. 2B and 3B). While GSSG level slightly increased in both liver and kidney (6.5% and 14.6% (p > 0.05), respectively), significantly decreased level of GSH (18.8% (p = 0.012) in liver and 21.1% (p = 0.011) in kidney) and GSH/GSSG ratio (27.3% (p < 0.001) in liver and 31.2% (p = 0.002) in kidney) were detected in diabetic animals when compared to a control group (Figs. 2C and 3C). Moreover, GSSP level was increased in both liver and kidney of diabetic animals (by 53.5% and 52.7%, respectively) (Figs. 2D and 3D). Both post- and pre-treatment of diabetic rats with CEE decreased MDA and GSSP levels and increased GSH level and GSH/GSSG ratio in both liver and kidney. Among the analyzed parameters, pre-treatment was more efficient than post-treatment in increasing GSH level in the kidney (by 21.3%).

3.4. Effect of CEE administration on antioxidant enzyme activity and protein levels in the liver and kidney of diabetic rats

Under diabetic conditions, the activities of CAT, CuZnSOD, MnSOD, GPx and GR in the liver decreased by 61%, 64%, 67%, 13%, and 22%, respectively (Fig. 4A). CEE treatment of diabetic animals potentiated the examined antioxidant enzymes' activities in the liver. Compared to the diabetic group, post-treatment with CEE boosted CAT activity by 35% and GR activity by 18%, while CuZnSOD and MnSOD activities were 57% and 44% higher, respectively. In the pre-treated group, activities of CAT, CuZnSOD, MnSOD, GPx, and GR increased by 37%, 51%, 42%, 22%, and 30%, respectively, compared to diabetic animals. In addition, GPx activity in the liver of pre-treated animals was 11.5% higher when compared to post-treated group.

In addition to changes in antioxidant enzymes' activity in the liver, changes at protein levels were also observed (Fig. 4B). Immunoblot analysis of diabetic liver homogenates revealed increased protein levels of GR by 22%, GPx by 53%, and MnSOD by 89%, decreased CuZnSOD protein level by 27%, and unchanged CAT protein level compared to control animals. Administration of CEE extract to diabetic animals decreased GPx (by 23% in the post-treated and by 19% in the pre-treated group) and MnSOD (by 37% in the post- and by 29% in the pre-treated group) protein levels. In both CEE-treated diabetic groups, a statistically insignificant decrease of GR protein level was observed (p > 0.05), while CuZnSOD protein level in the pre-treated diabetic group was slightly increased.

Changes in antioxidant enzymes' activity and protein levels were also observed in diabetic kidney (Fig. 5). When compared to the animals in the control group, activities of CAT, CuZnSOD, and MnSOD decreased by 48%, 43%, and 45%, respectively, while activities of GPx and GR increased by 65% and 45% (Fig. 5A). CEE administration to post-treated

Table 1
CEE improves liver and kidney functional parameters in diabetic rats.

	C	CEE	STZ	STZ/CEE	CEE/STZ/CEE
N	7	7	7	7	7
Liver/bw (%)	2.34 ± 0.01	2.24 ± 0.14	4.02 ± 0.15 ***	3.44 ± 0.16 **; ++	3.33 ± 0.16 +++
Kidney/bw (%)	0.66 ± 0.01	0.70 ± 0.05	1.10 ± 0.05 ***	0.96 ± 0.04 ***	0.84 ± 0.05 ++
ALT (U/L)	43.15 ± 1.83	56.67 ± 1.14	205.03 ± 14.92 ***	90.86 ± 5.59 ***; +++	48.80 ± 3.96 +++; ##
AST (U/L)	151.55 ± 4.48	170.13 ± 3.78	268.16 ± 9.68 ***	164.50 ± 9.38 +++	125.67 ± 8.58 +++; #
Creatinine (μmol/L)	26.00 ± 1.40	26.50 ± 1.70	47.00 ± 0.99 ***	35.17 ± 1.81 **; +++	30.25 ± 1.92 +++
BUN (mmol/L)	5.65 ± 0.09	5.55 ± 0.12	16.97 ± 0.16 ***	13.72 ± 0.44 ***; +++	13.42 ± 0.40 ***; +++

Liver/bw – liver to body weight ratio; Kidney/bw – kidney to body weight ratio; ALT – alanine aminotransferase; AST – aspartate aminotransferase; BUN – blood urea nitrogen. C – control animals; CEE – CEE-treated control animals; STZ – diabetic animals; STZ/CEE – post-treated diabetic animals; CEE/STZ/CEE – pre-treated diabetic animals. Values are means ± S.E.M. for N = 7 animals. * p < 0.05, ** p < 0.01, *** p < 0.001 in comparison to C; ++ p < 0.01, +++ p < 0.001 in comparison to STZ; # p < 0.05, ## p < 0.01 when compared CEE/STZ/CEE to STZ/CEE.
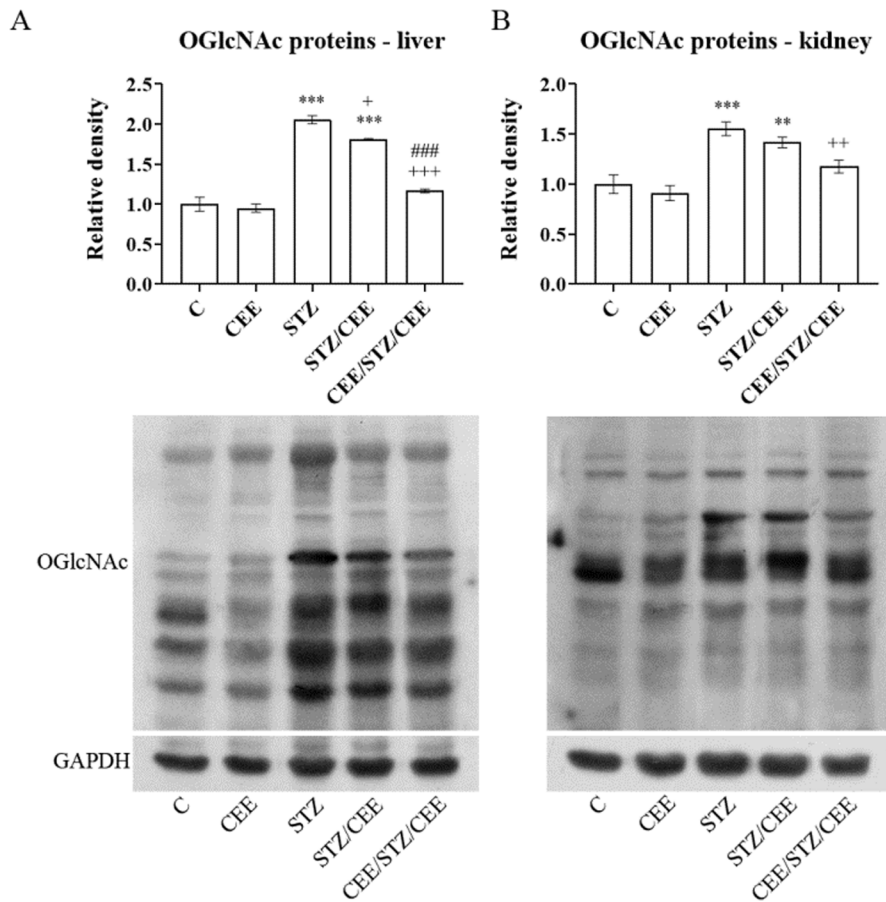
A

B

Fig. 1. CEE lowers protein glycosylation in diabetic liver (A) and kidney (B). O-GlcNAc modified proteins (whole lane) and GAPDH were quantified using TotalLab (Phoretix) electrophoresis software. Levels of *O*-GlcNAc-modified proteins are graphed in comparison to control. Presented blots are representative of three independent experiments. *O*-GlcNAc – β-*O*-linked N-acetylglucosamine; GAPDH – glyceraldehyde 3-phosphate dehydrogenase. C – control animals; CEE – CEE-treated control animals; STZ – diabetic animals; STZ/CEE – post-treated diabetic animals; CEE/STZ/CEE – pre-treated diabetic animals. Values are means ± S.E.M. for N = 7 animals. [**] $p < 0.01$, [***] $p < 0.001$ in comparison to C; [+] $p < 0.05$, [++] $p < 0.01$, [+++] $p < 0.001$ in comparison to STZ; [###] $p < 0.001$ when compared CEE/STZ/CEE to STZ/CEE.
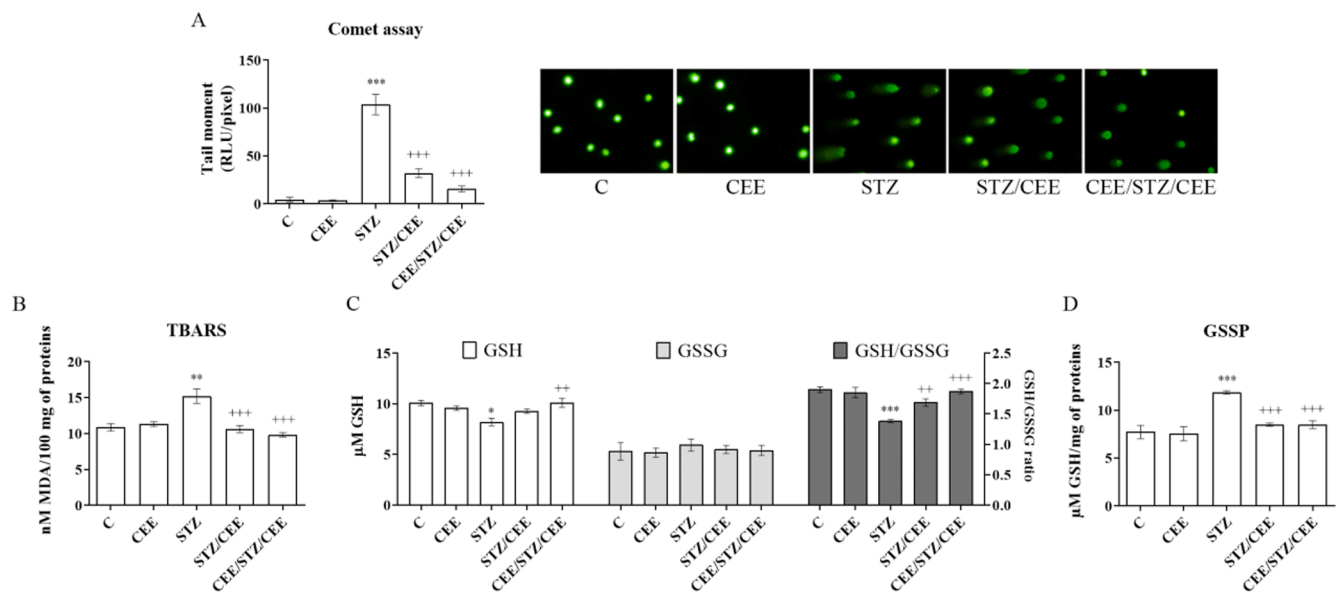

Fig. 2. CEE reduces oxidative damage in diabetic liver. (A) Assessment of DNA damage by comet assay. Presented are representative images from three independent experiments. TriTekCometScore[TM] Freeware version 1.5 was used to calculate tail moment as a measure of DNA damage. (B) Lipid peroxidation (TBARS); (C) Levels of reduced (GSH) and oxidized glutathione (GSSG) and their ratio; (D) Level of S-glutathionylated proteins (GSSP). C – control group; CEE – CEE-treated control animals; STZ – diabetic animals; STZ/CEE – post-treated diabetic animals; CEE/STZ/CEE – pre-treated diabetic animals. Values are means ± S.E.M. for N = 7 animals. [**] $p < 0.01$, [***] $p < 0.001$ in comparison to C; [++] $p < 0.01$, [+++] $p < 0.001$ in comparison to STZ.

rats stimulated CAT, CuZnSOD, and MnSOD activities by 21%, 33%, and 24%, respectively. The effect of CEE administration on CAT, CuZnSOD, and MnSOD activities was more pronounced in the pre-treated group.

Compared to diabetic animals, CAT activity in the pre-treated group increased by 30%, CuZnSOD activity by 46%, while MnSOD activity was 33% higher than the one detected in diabetic kidney. Treating diabetic
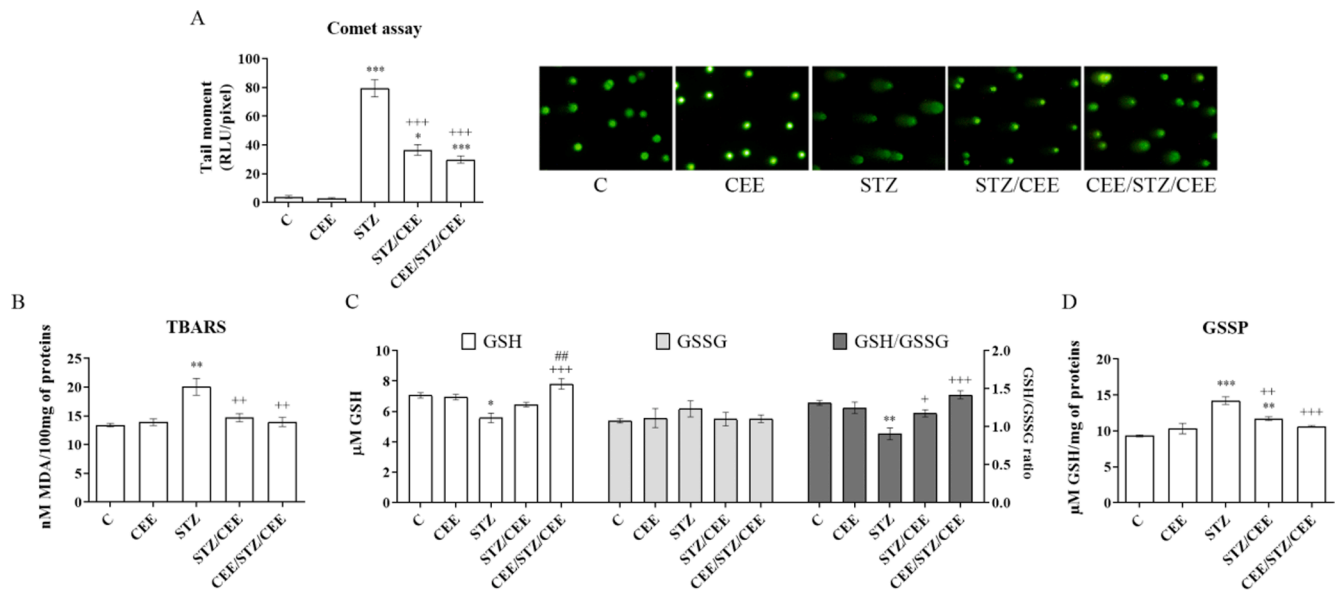
Fig. 3. CEE reduces oxidative damage in diabetic kidney. (A) Assessment of DNA damage by comet assay. Displayed are representative images from three independent experiments. TriTekCometScore™ Freeware version 1.5 was used to calculate tail moment. (B) Lipid peroxidation (TBARS); (C) Levels of reduced (GSH) and oxidized glutathione (GSSG) and their ratio; (D) Level of S-glutathionylated proteins (GSSP). C – control group; CEE – CEE-treated control animals; STZ – diabetic animals; STZ/CEE – post-treated diabetic animals; CEE/STZ/CEE – pre-treated diabetic animals. Values are means ± S.E.M. for N = 7 animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison to C; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ in comparison to STZ; ## $p < 0.01$ when compared CEE/STZ/CEE to STZ/CEE.
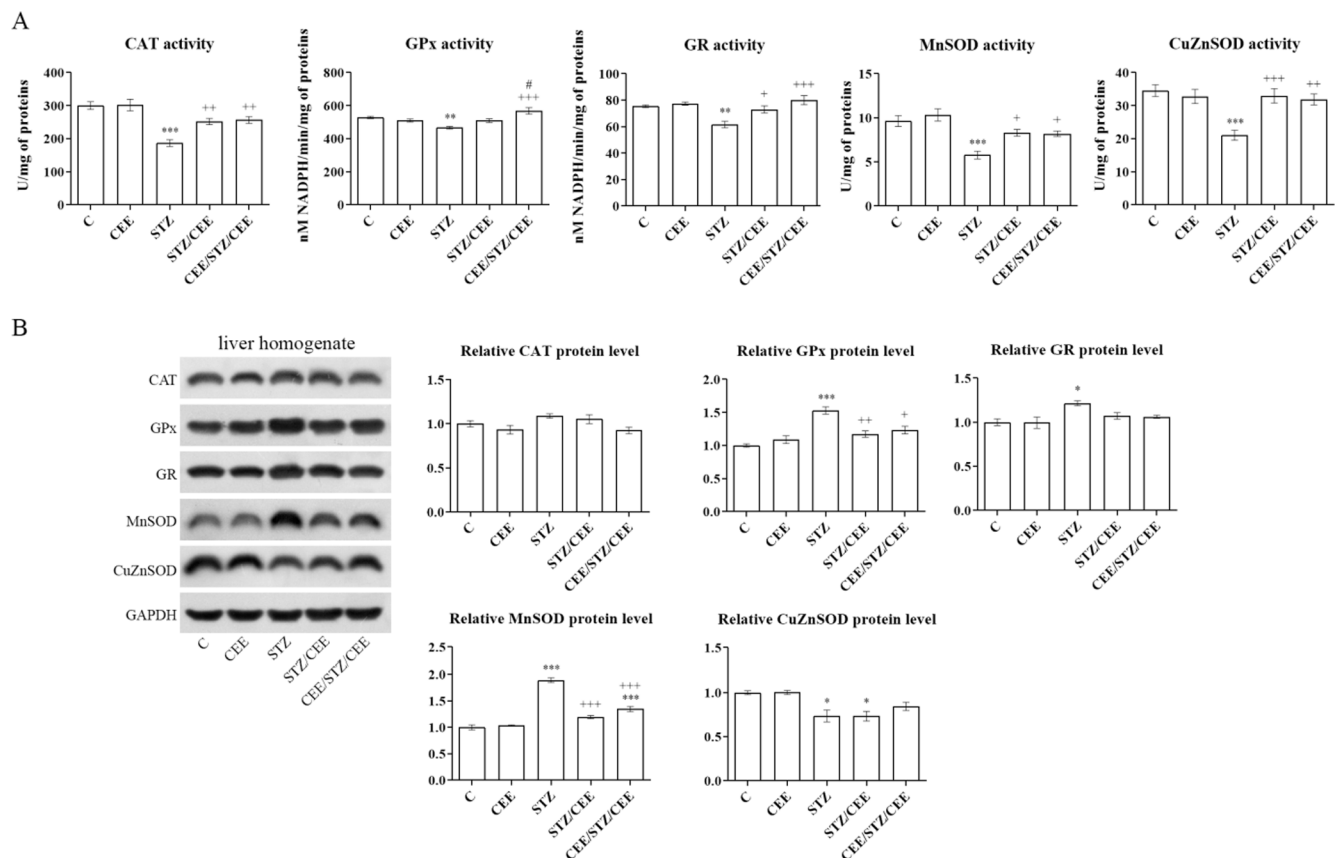

Fig. 4. CEE administration affects hepatic antioxidant enzymes activity and protein level. (A) Activity of hepatic antioxidant enzymes; (B) Representative Western blot of hepatic antioxidant enzymes. TotalLab (Phoretix) electrophoresis software was used for quantification of band intensities. Protein levels are graphed in comparison to control. CAT – catalase; CuZnSOD – copper-zinc superoxide dismutase; MnSOD – manganese superoxide dismutase; GPx – glutathione peroxidase; GR – glutathione reductase; GAPDH – glyceraldehyde 3-phosphate dehydrogenase. C – control group; CEE – CE extract-treated control animals; STZ – diabetic animals; STZ/CEE – post-treated diabetic animals; CEE/STZ/CEE – pre-treated diabetic animals. Values are means ± S.E.M. for N = 7 animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison to C; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ in comparison to STZ; # $p < 0.05$ when compared CEE/STZ/CEE to STZ/CEE.
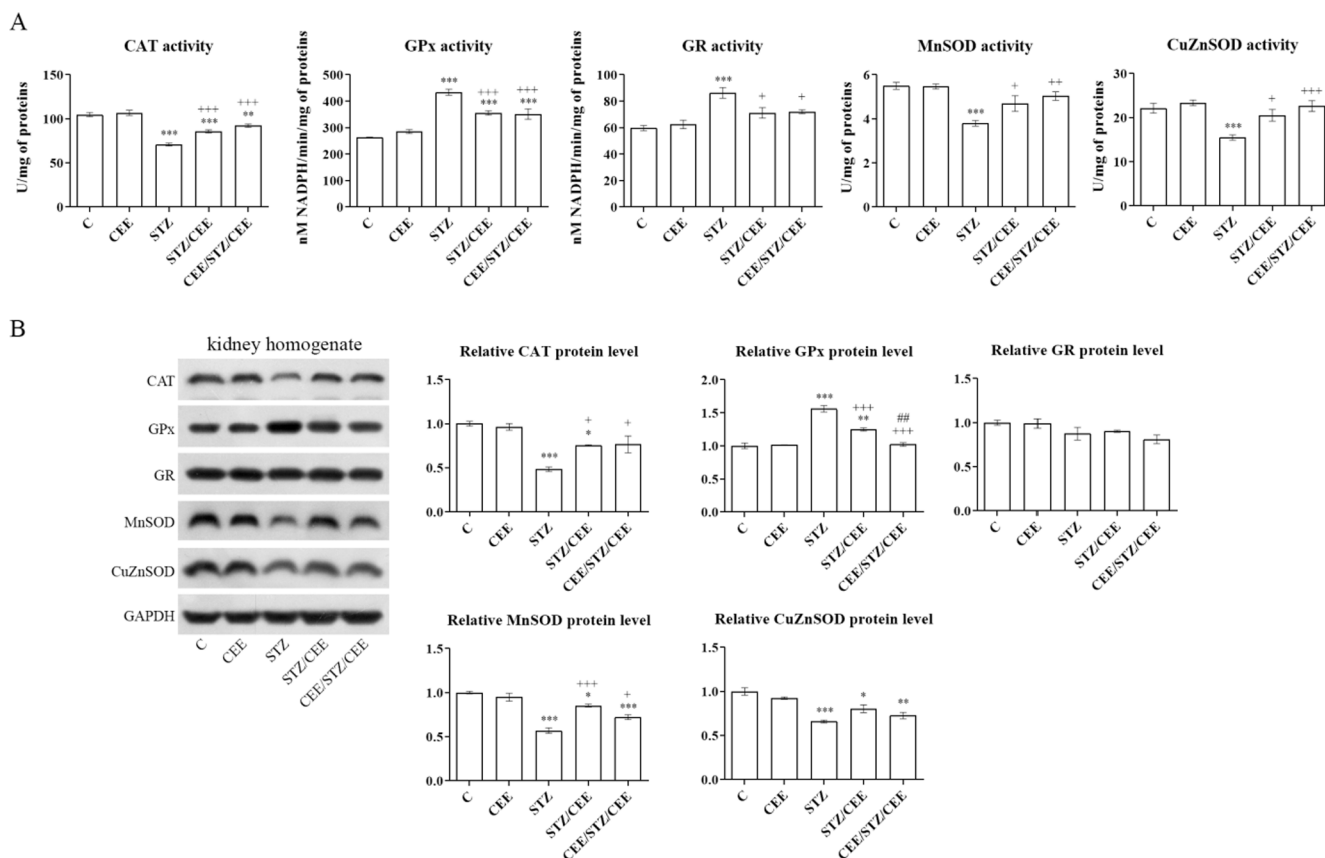
Fig. 5. CEE administration affects renal antioxidant enzymes activity and protein level. (A) Activity of renal antioxidant enzymes; (B) Representative Western blot of renal antioxidant enzymes. TotalLab (Phoretix) electrophoresis software was used for quantification of band intensities. Protein levels are graphed in comparison to control. CAT – catalase; CuZnSOD – copper-zinc superoxide dismutase; MnSOD – manganese superoxide dismutase; GPx – glutathione peroxidase; GR – glutathione reductase; GAPDH – glyceraldehyde 3-phosphate dehydrogenase. C – control group; CEE – CE extract-treated control animals; STZ – diabetic animals; STZ/CEE – post-treated diabetic animals; CEE/STZ/CEE – pre-treated diabetic animals. Values are means ± S.E.M. for N = 7 animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison to C; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ in comparison to STZ; ## $p < 0.01$ when compared CEE/STZ/CEE to STZ/CEE.

animals with CEE also affected GPx and GR activities. Compared to diabetic animals, GPx activity decreased by 22% in post-treated and by 24% in the pre-treated group, while GR activity decreased by 20% in both CEE-treated diabetic groups.

In comparison to control animals, CAT, MnSOD, and CuZnSOD protein levels in diabetic kidneys dropped by 52%, 43%, and 34%, respectively (Fig. 5B). While the level of GR protein in the kidney of diabetic animals did not alter when compared to the control group, the level of GPx protein increased by 56%. CEE treatment of diabetic animals increased CAT (by 55% in the post- and by 58% in the pre-treated group) and MnSOD (by 49% in the post- and by 27% in the pre-treated group) protein levels and decreased GPx protein level (by 25% in the post-treated group and by 53% in pre-treated animals, which was statistically different from the post-treated group (p = 0.006)). Treating control animals with CEE affected neither activities nor protein levels of antioxidant enzymes.

4. Discussion

The increasing global incidence of diabetes raises concerns regarding the onset of long-term diabetic complications, both micro- and macrovascular. Since hyperglycemia and hyperglycemia-induced oxidative stress are major causes of complications' development, dietary supplementation with natural antioxidants and hypoglycemic agents present in many plants can prevent or slow down irreversible tissue impairment. The hypoglycemic properties of *Centaurium erythraea* methanol extract (CEE) used in this study were demonstrated previously (Đorđević et al., 2017). Here presented findings provided evidence that daily treatment

with CEE protects the liver and kidney of diabetic rats from hyperglycemia-induced oxidative stress. Treatment with CEE reduced oxidative damage of DNA and lipids, increased GSH/GSSG ratio, and directed antioxidant enzymes' activities toward control values in both liver and kidney of diabetic animals. This was reflected in the improvement of functional parameters of the liver and kidney, observed as reduced ALT and AST activity, and decreased levels of BUN and creatinine in the serum.

Several enzymes and compounds are in use in clinical practice as biochemical markers for the early diagnosis of diabetes and associated complications. Serum activities of ALT and AST are used as indicators of liver damage (Soares et al., 2013) while determining serum creatinine and blood urea nitrogen levels is the most convenient method of monitoring kidney function (Dabla, 2010). Increased activities of ALT and AST enzymes, as well as creatinine and BUN levels in the serum of diabetic animals observed in this study, indicated tissue damage and impaired function of liver and kidneys. In addition, an increase in relative liver and kidney mass was observed in diabetic animals, indicating hypertrophy of the organs. Liver hypertrophy could be attributed to an abnormal synthesis and accumulation of triglyceride as a consequence of disturbed uptake, synthesis, export, and oxidation of free fatty acids (Mohamed et al., 2016). Glomerular hypertrophy occurs at an early stage of the development of pathological changes in the kidney. Hypertrophy of mesangial and proximal tubular cells is triggered by hyperglycemia and is associated with halting the cell cycle in the G1 phase due to local changes in the production of certain growth factors, like transforming growth factor-β (Malatiali et al., 2008; Wolf and Ziyadeh, 1999).

Administration of CEE decreased ALT, AST, BUN, and creatinine levels and reduced organ-to-body weight ratio, pointing to a protective effect of CEE on liver and kidney functionality. Detected stronger CEE-mediated protection of the liver in the pre-treated group implies that consuming CEE before developing diabetes may reduce the harmful impact of the disease, which is in agreement with our previous reports (Đorđević et al., 2019; Đorđević et al., 2017). Positive effect of CEE on the liver and kidney of diabetic rats could be attributed to its bioactive compounds. Targeted metabolic profiling of CEE used in this study unveiled secoiridoids (swertiamarin, gentiopicrin, sweroside, secologanin), iridoid (loganin), and polyphenols, characterized by phenolic acids (ferulic acid, ρ-coumaric acid, caffeic acid, sinapic acid), flavonoids (rutin, apigenin, quercetin, naringenin, isoquercitrin, astragalin, luteolin, kaempferol), and xanthones (desmethyleustomin, methyl-bellidifolin, eustomin, decussatin) to be abundant compounds (Đorđević et al., 2017). Swertiamarin, sweroside, and gentiopicrin, the most abundant bioactive compounds in CEE, have been shown to have hepato- and renoprotective effects (Dai et al., 2018; Han et al., 2018; Selvam et al., 2018; Sonawane et al., 2010; Wan et al., 2021). In addition, the CEE protective effect on diabetic liver and kidney could result from both hypoglycemic and antioxidant effects of accumulated polyphenolic compounds present in CEE (Đorđević et al., 2019; Đorđević et al., 2017).

Daily oral treatment with CEE prevented the development of lipid peroxidation in diabetic animals, suggesting the contribution of CEE in maintaining the membrane integrity and functionality of the liver and kidney. This is in agreement with our previous findings that CEE treatment reduces lipid peroxidation in diabetic rats' erythrocytes (Đorđević et al., 2017). Aqueous extract of CE leaves diminished MDA level in the pancreas of diabetic rats (Sefi et al., 2011), while hot water extract of CE aerial parts reduced lipid peroxidation in mouse brain homogenates *in vitro* (Gonçalves et al., 2013). In addition, treatment of alloxan-induced diabetic mice with ferulic acid, which is the constituent of CEE, reduced MDA level (Ramar et al., 2012), whilst administration of swertiamarin to D-galactosamine-treated rats decreased lipid peroxidation in the liver and kidneys (Jaishree and Badami, 2010). Other studies employing compounds contained in CEE similarly have found lowered lipid peroxidation levels in diabetic animals. Thus, treatment of diabetic animals with kaempferol and rutin, compounds belonging to flavonoids detected in CEE, reduced lipid peroxidation and increased levels of endogenous antioxidants (Al-Numair et al., 2015; Kamalakkannan and Stanely Mainzen Prince, 2006). Apigenin, another flavonoid found in CEE, reduced lipid peroxidation in the liver of alloxan-treated diabetic animals (Panda and Kar, 2010), while usage of apigenin-solid lipid nanoparticles reduced kidney lipid peroxidation in STZ-induced diabetic rats (Li et al., 2020). Besides reducing liver and kidney lipid peroxidation in diabetic animals, quercetin treatment also reduced oxidative DNA damage (Alam et al., 2014). In line with this, daily treatment of diabetic animals with CEE reduced the level of DNA damage in the liver and kidney, additionally confirming the cytoprotective effect of the extract in diabetic conditions. The DNA protective effects of quercetin and other components found in CEE have been proven in a variety of oxidative stress models. Ferulic acid and quercetin protected DNA in rat kidneys from oxidative damage caused by lead (Kelainy et al., 2019; Liu et al., 2010), while apigenin and quercetin reduced the genotoxic effect of diethylnitrosamine in the liver (Ali et al., 2014; Gupta et al., 2010). Pretreatment of Sprague-Dawley rats with caffeic acid preserved the DNA content of the liver and kidneys in methamphetamine-induced oxidative stress (Koriem et al., 2013), whilst *p*-coumaric acid ameliorated acetaminophen-induced DNA damage in the liver (Cha et al., 2018).

GSH level and GSH/GSSG ratio define the redox status of the liver and kidney and serve as a cellular health indicator. Decreased level of GSH and GSH/GSSG ratio and rise of protein glutathionylation (GSSP), recorded in this study, point to increased oxidative stress in kidney and liver tissues. These results mirror the findings of the decreased GSH level in the kidney and liver of STZ-induced diabetic rats (Sellamuthu et al.,

2013; Singh et al., 2013; Sugumar et al., 2016). CEE treatment of diabetic rats considerably reduced the presence of GSSP in the liver and kidney, which was accompanied by an elevated GSH level and GSH/GSSG ratio, suggesting that CEE protects the liver and kidneys against oxidative stress. This protective effect of CEE could arise from the improvement of the endogenous antioxidant system in diabetic animals.

The detected differences in the activity of antioxidant enzymes between diabetic liver and kidney strongly suggest an involvement of different underlying regulatory mechanisms. This is supported by conflicting data regarding the antioxidant enzymes' activity in different organs, different animal models, and models of experimental diabetes (Maritim et al., 2003). We observed a decline in CAT, CuZnSOD, MnSOD, GPx, and GR activity in the liver of diabetic animals. Decreased CuZnSOD activity could be attributed to the decreased protein level, therefore to the transcriptional regulation, while not excluding post-translational inhibition by increased O-GlcNAcylation detected in diabetic animals. On the other hand, MnSOD, GR, and GPx protein levels were significantly increased in diabetic liver, whilst CAT protein level remained unchanged. Mihailović et al. (2013a) showed that CAT, MnSOD, and CuZnSOD activity in diabetic liver declined as a result of their enhanced O-GlcNAc modification. Therefore, increased O-GlcNAcylation of CAT, MnSOD, GPx, and GR could be the cause for their detected decreased activity, despite increased or unchanged protein levels of these enzymes. Such findings indicate that transcriptional, post-transcriptional, and post-translational mechanisms could be included in the regulation of antioxidant enzyme expression/activity. This claim is backed up by the findings of Dinić et al. (2013), who suggested that the expression of CAT and CuZnSOD in diabetic liver is regulated primarily on the transcriptional level, whereas the regulation of MnSOD expression involves both post-transcriptional and post-translational mechanisms. Increased activity of GR, GPx, MnSOD, and CAT in the liver of CEE-treated diabetic animals was not mediated by an increase in protein levels, but rather by the reduced protein glycosylation detected in both CEE-treated diabetic groups. Therefore, enhanced antioxidant enzymes' activity could be driven by the hypoglycemic effect observed in the CEE treatment (Đorđević et al., 2017).

The activities of CuZnSOD, MnSOD, and CAT in the kidney of diabetic rats were decreased, while the activities of GPx and GR were increased, which corresponds to previous reports (Kedziora-Kornatowska et al., 2000; Mekinova et al., 1995; Mihailović et al., 2013b). An increased GPx activity, probably provoked by the intensified ROS production, is in line with the observed increased level of GPx protein in the diabetic kidney. Even though no increase in GR protein level was detected, the enhanced GR activity in the diabetic kidney may be driven by an increase in the level of its substrate, GSSG, produced by GPx activity. It was shown that decreased activities of both CAT and CuZnSOD in diabetic kidneys result from decreased protein and gene expression and increased O-GlcNAcylation of the enzymes (Arambašić et al., 2013; Mihailović et al., 2013a). Herein detected reduction of CAT, MnSOD, and CuZnSOD activities in the diabetic kidney could be subscribed to the observed decline in protein levels and increased protein glycosylation of these enzymes. Accordingly, the rise in activity of CAT, MnSOD, and CuZnSOD in kidneys of diabetic animals mediated by CEE could be partly attributed to the elevation of enzyme protein levels, but also the reduction of O-GlcNAcylation of proteins, especially in the pre-treated group.

5. Conclusions

Timely and effective therapy is crucial to prevent the development of diabetic complications. Although existing medications have significantly improved disease control, none of them is completely efficient in eradicating oxidative stress as one of the primary causes of diabetic complications. Therefore, the use of nutritional supplements as therapeutic adjuncts could significantly reduce the adverse effects of diabetes and its complications, and consequently the direct and indirect expenses

of treating the condition. The presented results suggest that even though both post- and pre-treatment dimished deleterious effects of diabetes on the liver and kidney, CEE was more effective in pre-treated diabetic rats. Moreover, combined CEE-mediated effects, ranging from hypoglycemic and antioxidant, to transcriptional, post-transcriptional, and post-translational regulation of antioxidant enzymes, protect the liver and kidneys of diabetic animals and improve their functionality. Multi-targeted actions of *C. erythraea* with positive effects on diabetic liver and kidney functioning open a platform for further elucidation of underlying mechanisms to improve diabetes therapy.

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Ethical statement

All animal procedures complied with the Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade (permit number 2-14/10).

CRediT authorship contribution statement

Miloš M. Đorđević: Investigation, Formal analysis, Writing – original draft. **Anja Tolić:** Investigation, Formal analysis. **Jovana Rajić:** Investigation, Formal analysis. **Mirjana Mihailović:** Investigation, Validation. **Jelena Arambašić Jovanović:** Investigation, Validation. **Aleksandra Uskoković:** Investigation, Formal analysis. **Nevena Grdović:** Investigation, Formal analysis. **Marija B. Đorđević:** Formal analysis. **Danijela Mišić:** Resources, Formal analysis. **Branislav Šiler:** Resources, Formal analysis. **Melita Vidaković:** Writing – review & editing, Supervision. **Svetlana Dinić:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Al-Numair, K. S., Chandramohan, G., Veeramani, C., & Alsaif, M. A. (2015). Ameliorative effect of kaempferol, a flavonoid, on oxidative stress in streptozotocin-induced diabetic rats. *Redox Report, 20*(5), 198–209. https://doi.org/10.1179/1351000214Y.0000000117

Alam, M. M., Meerza, D., & Naseem, I. (2014). Protective effect of quercetin on hyperglycemia, oxidative stress and DNA damage in alloxan induced type 2 diabetic mice. *Life Sciences, 109*(1), 8–14. https://doi.org/10.1016/j.lfs.2014.06.005

Ali, F., Rahul, Naz, F., Jyoti, S., & Siddique, Y. H. (2014). Protective effect of apigenin against N-nitrosodiethylamine (NDEA)-induced hepatotoxicity in albino rats. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 767*, 13–20. https://doi.org/10.1016/j.mrgentox.2014.04.006

American Diabetes Association. (2019). 9 Pharmacologic Approaches to Glycemic Treatment: Standards of Medical Care in Diabetes - 2019. *Diabetes Care, 42* (Supplement 1), S90–S102. https://doi.org/10.2337/dc19-S009

Arambašić, J., Mihailović, M., Uskoković, A., Dinić, S., Grdović, N., Marković, J., … Vidaković, M. (2013). Alpha-lipoic acid upregulates antioxidant enzyme gene expression and enzymatic activity in diabetic rat kidneys through an O-GlcNAc-dependent mechanism. *European Journal of Nutrition, 52*(5), 1461–1473. https://doi.org/10.1007/s00394-012-0452-z

Beutler, E. (1982). Catalase. In E. Beutler (Ed.), *Red cell metabolism, a manual of biochemical methods* (pp. 105–106). New York: Grune and Stratton Inc.

Cha, H., Lee, S., Lee, J. H., & Park, J.-W. (2018). Protective effects of p-coumaric acid against acetaminophen-induced hepatotoxicity in mice. *Food and Chemical Toxicology, 121*, 131–139. https://doi.org/10.1016/j.fct.2018.08.060

Dabla, P. K. (2010). Renal function in diabetic nephropathy. *World Journal of Diabetes, 1* (2), 48–56. https://doi.org/10.4239/wjd.v1.i2.48

Dai, K., Yi, X.-J., Huang, X.-J., Muhammad, A., Li, M., Li, J., … Gao, Y. (2018). Hepatoprotective activity of iridoids, seco-iridoids and analog glycosides from Gentianaceae on HepG2 cells via CYP3A4 induction and mitochondrial pathway. *Food and Function, 9*(5), 2673–2683. https://doi.org/10.1039/C8FO00168E

Dinić, S., Arambašić, J., Mihailović, M., Uskoković, A., Grdović, N., Marković, J., … Vidaković, M. (2013). Decreased O-GlcNAcylation of the key proteins in kinase and redox signalling pathways is a novel mechanism of the beneficial effect of alpha-lipoic acid in diabetic liver. *British Journal of Nutrition, 110*(3), 401–412. https://doi.org/10.1017/S0007114512005429

Đorđević, M., Grdović, N., Mihailović, M., Arambašić Jovanović, J., Uskoković, A., Rajić, J., … Dinić, S. (2019). *Centaurium erythraea* extract improves survival and functionality of pancreatic beta-cells in diabetes through multiple routes of action. *Journal of Ethnopharmacology, 242*, Article 112043. https://doi.org/10.1016/j.jep.2019.112043

Đorđević, M., Mihailović, M., Arambašić Jovanović, J., Grdović, N., Uskoković, A., Tolić, A., … Dinić, S. (2017). *Centaurium erythraea* methanol extract protects red blood cells from oxidative damage in streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology, 202*, 172–183. https://doi.org/10.1016/j.jep.2017.03.016

Edelman, S. V., & Polonsky, W. H. (2017). Type 2 Diabetes in the Real World: The Elusive Nature of Glycemic Control. *Diabetes Care, 40*(11), 1425. https://doi.org/10.2337/dc16-1974

Edgerton, D. S., Kraft, G., Smith, M., Farmer, B., Williams, P. E., Coate, K. C., … Cherrington, A. D. (2017). Insulin's direct hepatic effect explains the inhibition of glucose production caused by insulin secretion. *JCI Insight, 2*(6), e91863. https://doi.org/10.1172/jci.insight.91863

El Menyiy, N., Guaouguaou, F.-E., El Baaboua, A., El Omari, N., Taha, D., Salhi, N., … Bouyahya, A. (2021). Phytochemical properties, biological activities and medicinal use of *Centaurium erythraea* Rafn. *Journal of Ethnopharmacology, 276*, Article 114171. https://doi.org/10.1016/j.jep.2021.114171

Giacco, F., & Brownlee, M. (2010). Oxidative stress and diabetic complications. *Circulation research, 107*(9), 1058–1070. https://doi.org/10.1161/CIRCRESAHA.110.223545

Glatzle, D., Vuilleumier, J. P., Weber, F., & Decker, K. (1974). Glutathione reductase test with whole blood, a convenient procedure for the assessment of the riboflavin status in humans. *Experientia, 30*(6), 665–667. https://doi.org/10.1007/BF01921531

Gonçalves, S., Gomes, D., Costa, P., & Romano, A. (2013). The phenolic content and antioxidant activity of infusions from Mediterranean medicinal plants. *Industrial Crops and Products, 43*, 465–471. https://doi.org/10.1016/j.indcrop.2012.07.066

González Mera, I. F., González Falconí, D. E., & Morera Córdova, V. (2019). Secondary metabolites in plants: Main classes, phytochemical analysis and pharmacological activities. *Bionatura, 4*, 1000–1009. https://doi.org/10.21931/rb/2019.04.04.11.

Gupta, C., Vikram, A., Tripathi, D. N., Ramarao, P., & Jena, G. B. (2010). Antioxidant and antimutagenic effect of quercetin against DEN induced hepatotoxicity in rat. *Phytotherapy Research, 24*(1), 119–128. https://doi.org/10.1002/ptr.2883

Haloui, M., Louedec, L., Michel, J.-B., & Lyoussi, B. (2000). Experimental diuretic effects of *Rosmarinus officinalis* and *Centaurium erythraea*. *Journal of Ethnopharmacology, 71* (3), 465–472. https://doi.org/10.1016/S0378-8741(00)00184-7

Han, H., Xu, L., Xiong, K., Zhang, T., & Wang, Z. (2018). Exploration of Hepatoprotective Effect of Gentiopicroside on Alpha-Naphthylisothiocyanate-Induced Cholestatic Liver Injury in Rats by Comprehensive Proteomic and Metabolomic Signatures. *Cellular Physiology and Biochemistry, 49*(4), 1304–1319. https://doi.org/10.1159/000493409

International Diabetes Federation (2021). *IDF Diabetes Atlas* (10th ed.).

Jaishree, V., & Badami, S. (2010). Antioxidant and hepatoprotective effect of swertiamarin from *Enicostemma axillare* against D-galactosamine induced acute liver damage in rats. *Journal of Ethnopharmacology, 130*(1), 103–106. https://doi.org/10.1016/j.jep.2010.04.019

Kamalakkannan, N., & Stanely Mainzen Prince, P. (2006). Rutin improves the antioxidant status in streptozotocin-induced diabetic rat tissues. *Molecular and Cellular Biochemistry, 293*(1–2), 211–219. https://doi.org/10.1007/s11010-006-9244-1

Kedziora-Kornatowska, K. Z., Luciak, M., & Paszkowski, J. (2000). Lipid peroxidation and activities of antioxidant enzymes in the diabetic kidney: Effect of treatment with angiotensin convertase inhibitors. *IUBMB Life, 49*(4), 303–307. https://doi.org/10.1080/15216540050033177

Kelainy, E. G., Ibrahim Laila, I. M., & Ibrahim, S. R. (2019). The effect of ferulic acid against lead-induced oxidative stress and DNA damage in kidney and testes of rats. *Environmental Science and Pollution Research, 26*(31), 31675–31684. https://doi.org/10.1007/s11356-019-06099-6

Khunti, K., Ceriello, A., Cos, X., & De Block, C. (2018). Achievement of guideline targets for blood pressure, lipid, and glycaemic control in type 2 diabetes: A meta-analysis. *Diabetes Research and Clinical Practice, 137*, 137–148. https://doi.org/10.1016/j.diabres.2017.12.004

Kifle, Z. D. (2021). Prevalence and correlates of complementary and alternative medicine use among diabetic patients in a resource-limited setting. *Metabolism Open, 10*, 100095. https://doi.org/10.1016/j.metop.2021.100095

King, G. L., & Loeken, M. R. (2004). Hyperglycemia-induced oxidative stress in diabetic complications. *Histochemistry and Cell Biology, 122*(4), 333–338. https://doi.org/10.1007/s00418-004-0678-9

Koriem, K. M. M., Abdelhamid, A. Z., & Younes, H. F. (2013). Caffeic acid protects tissue antioxidants and DNA content in methamphetamine induced tissue toxicity in Sprague Dawley rats. *Toxicology Mechanisms and Methods, 23*(2), 134–143. https://doi.org/10.3109/15376516.2012.730561

Li, P., Bukhari, S. N. A., Khan, T., Chitti, R., Bevoor, D. B., Hiremath, A. R., … Gubbiyappa, K. S. (2020). Apigenin-Loaded Solid Lipid Nanoparticle Attenuates Diabetic Nephropathy Induced by Streptozotocin Nicotinamide Through Nrf2/HO-

1/NF-kB Signalling Pathway. *International journal of nanomedicine, 15*, 9115–9124. https://doi.org/10.2147/IJN.S256494

Liu, C.-M., Ma, J.-Q., & Sun, Y.-Z. (2010). Quercetin protects the rat kidney against oxidative stress-mediated DNA damage and apoptosis induced by lead. *Environmental Toxicology and Pharmacology, 30*(3), 264–271. https://doi.org/10.1016/j.etap.2010.07.002

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*(1), 265–275.

Malatiali, S., Francis, I., & Barac-Nieto, M. (2008). Phlorizin Prevents Glomerular Hyperfiltration but not Hypertrophy in Diabetic Rats. *Experimental Diabetes Research, 2008*, Article 305403. https://doi.org/10.1155/2008/305403

Maritim, A. C., Sanders, R. A., & Watkins, J. B., 3rd (2003). Diabetes, oxidative stress, and antioxidants: A review. *Journal of Biochemical and Molecular Toxicology, 17*(1), 24–38. https://doi.org/10.1002/jbt.10058

Mekinova, D., Chorvathova, V., Volkovova, K., Staruchova, M., Grancicova, E., Klvanova, J., & Ondreicka, R. (1995). Effect of intake of exogenous vitamins C, E and beta-carotene on the antioxidative status in kidneys of rats with streptozotocin-induced diabetes. *Nahrung, 39*(4), 257–261. https://doi.org/10.1002/food.19950390402

Mihailović, M., Arambašić, J., Uskoković, A., Dinić, S., Grdović, N., Marković, J., … Vidaković, M. (2013a). β-Glucan administration to diabetic rats alleviates oxidative stress by lowering hyperglycaemia, decreasing non-enzymatic glycation and protein O-GlcNAcylation. *Journal of Functional Foods, 5*(3), 1226–1234. https://doi.org/10.1016/j.jff.2013.04.005

Mihailović, M., Arambašić, J., Uskoković, A., Dinić, S., Grdović, N., Marković, J., … Vidaković, M. (2013b). β-Glucan administration to diabetic rats reestablishes redox balance and stimulates cellular pro-survival mechanisms. *Journal of Functional Foods, 5*(1), 267–278. https://doi.org/10.1016/j.jff.2012.10.016

Misra, H. P., & Fridovich, I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological Chemistry, 247*(10), 3170–3175.

Mohamed, J., Nazratun Nafizah, A. H., Zariyantey, A. H., & Budin, S. B. (2016). Mechanisms of Diabetes-Induced Liver Damage: The role of oxidative stress and inflammation. *Sultan Qaboos University Medical Journal, 16*(2), e132–e141. https://doi.org/10.18295/squmj.2016.16.02.002.

Mroueh, M., Saab, Y., & Rizkallah, R. (2004). Hepatoprotective activity of *Centaurium erythraea* on acetaminophen-induced hepatotoxicity in rats. *Phytotherapy Research, 18*(5), 431–433. https://doi.org/10.1002/ptr.1498

Nair, A. B., & Jacob, S. (2016). A simple practice guide for dose conversion between animals and human. *Journal of Basic and Clinical Pharmacy, 7*(2), 27–31. https://doi.org/10.4103/0976-0105.177703

Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical biochemistry, 95*(2), 351–358. https://doi.org/10.1016/0003-2697(79)90738-3

Ota, A., & Ulrih, N. P. (2017). An Overview of Herbal Products and Secondary Metabolites Used for Management of Type Two Diabetes. *Frontiers in Pharmacology, 8*, 436. https://doi.org/10.3389/fphar.2017.00436

Panda, S., & Kar, A. (2010). Apigenin (4',5,7-trihydroxyflavone) regulates hyperglycaemia, thyroid dysfunction and lipid peroxidation in alloxan-induced diabetic mice. *Journal of Pharmacy and Pharmacology, 59*(11), 1543–1548. https://doi.org/10.1211/jpp.59.11.0012

Petersen, M. C., Vatner, D. F., & Shulman, G. I. (2017). Regulation of hepatic glucose metabolism in health and disease. *Nature Reviews Endocrinology, 13*(10), 572–587. https://doi.org/10.1038/nrendo.2017.80

Rains, J. L., & Jain, S. K. (2011). Oxidative stress, insulin signaling, and diabetes. *Free Radical Biology and Medicine, 50*(5), 567–575. https://doi.org/10.1016/j.freeradbiomed.2010.12.006

Ramar, M., Manikandan, B., Raman, T., Priyadarsini, A., Palanisamy, S., Velayudam, M., … Vaseeharan, B. (2012). Protective effect of ferulic acid and resveratrol against alloxan-induced diabetes in mice. *European Journal of Pharmacology, 690*(1–3), 226–235. https://doi.org/10.1016/j.ejphar.2012.05.019

Sefi, M., Fetoui, H., Lachkar, N., Tahraoui, A., Lyoussi, B., Boudawara, T., & Zeghal, N. (2011). *Centaurium erythrea* (Gentianaceae) leaf extract alleviates streptozotocin-induced oxidative stress and beta-cell damage in rat pancreas. *Journal of Ethnopharmacology, 135*(2), 243–250. https://doi.org/10.1016/j.jep.2011.02.029

Sellamuthu, P. S., Arulselvan, P., Kamalraj, S., Fakurazi, S., & Kandasamy, M. (2013). Protective Nature of Mangiferin on Oxidative Stress and Antioxidant Status in Tissues of Streptozotocin-Induced Diabetic Rats. *ISRN Pharmacology, 2013*, 10. https://doi.org/10.1155/2013/750109

Selvam, R., Muruganantham, K., & Subramanian, S. (2018). Biochemical evaluation of antidiabetic properties of swertiamarin, a secoiridoid glycoside of *Enicostemma littorale* leaves, studied in high-fat diet-fed low-dose streptozotocin-induced type 2 diabetic rats. *Asian Journal of Pharmacology and Clinical Research, 11*(10), 486–492. https://doi.org/10.22159/ajpcr.2018.v11i10.28152

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research, 175*(1), 184–191. https://doi.org/10.1016/0014-4827(88)90265-0

Singh, R., Bhardwaj, P., & Sharma, P. (2013). Antioxidant and toxicological evaluation of *Cassia sopherain* streptozotocin-induced diabetic Wistar rats. *Pharmacognosy Research, 5*(4), 225–232. https://doi.org/10.4103/0974-8490.118767

Soares, A. A., De Sá-Nakanishi, B. A., Bracht, A., Da Costa, M. S., Koehnlein, A. E., De Souza, G. C., & Peralta, M. R. (2013). *Hepatoprotective Effects of Mushrooms, 18*. https://doi.org/10.3390/molecules18077609

Sonawane, R. D., Vishwakarma, S. L., Lakshmi, S., Rajani, M., Padh, H., & Goyal, R. K. (2010). Amelioration of STZ-induced type 1 diabetic nephropathy by aqueous extract of *Enicostemma littorale* Blume and swertiamarin in rats. *Molecular and Cellular Biochemistry, 340*(1), 1–6. https://doi.org/10.1007/s11010-010-0393-x

Sugumar, M., Doss, D. V. A., & Maddisetty, P. N. P. (2016). Hepato-renal protective effects of hydroethanolic extract of *Senna alata* on enzymatic and nonenzymatic antioxidant systems in streptozotocin induced diabetic rats. *Integrative Medicine Research, 5*(4), 276–283. https://doi.org/10.1016/j.imr.2016.06.005

Tamura, M., Oshino, N., & Chance, B. (1982). Some characteristics of hydrogen- and alkylhydroperoxides metabolizing systems in cardiac tissue. *Journal of Biochemistry, 92*(4), 1019–1031. https://doi.org/10.1093/oxfordjournals.jbchem.a134017

Tokarz, V. L., MacDonald, P. E., & Klip, A. (2018). The cell biology of systemic insulin function. *Journal of Cell Biology, 217*(7), 2273–2289. https://doi.org/10.1083/jcb.201802095

Vennos, C., Schwabl, H., & Bommeli, C. (2013). Phytotherapeutics in Treating Diabetes. In R. R. Watson, & V. R. Preedy (Eds.), *Bioactive Food as Dietary Interventions for Diabetes* (pp. 139–144). San Diego: Academic Press. https://doi.org/10.1016/B978-0-12-397153-1.00013-5.

Wan, Z., Li, H., Wu, X., Zhao, H., Wang, R., Li, M., … Li, X. (2021). Hepatoprotective effect of gentiopicroside in combination with leflunomide and/or methotrexate in arthritic rats. *Life Sciences, 265*, Article 118689. https://doi.org/10.1016/j.lfs.2020.118689

Wolf, G., & Ziyadeh, F. N. (1999). Molecular mechanisms of diabetic renal hypertrophy. *Kidney International, 56*(2), 393–405. https://doi.org/10.1046/j.1523-1755.1999.00590.x

Zeng, Z., Huang, S.-Y., & Sun, T. (2020). Pharmacogenomic Studies of Current Antidiabetic Agents and Potential New Drug Targets for Precision Medicine of Diabetes. *Diabetes Therapy, 11*(11), 2521–2538. https://doi.org/10.1007/s13300-020-00922-x