Journal of Ethnopharmacology 242 (2019) 112043

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Centaurium erythraea extract improves survival and functionality of pancreatic beta-cells in diabetes through multiple routes of action

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ARTICLE INFO

Keywords:
Centaurium erythraea
Diabetes mellitus
Beta-cells
Oxidative stress
Pro-survival
Antioxidant

ABSTRACT

Ethnopharmacological relevance: *Centaurium erythraea* Rafn (CE) is used as a traditional medicinal plant in Serbia to treat different ailments due to its antidiabetic, antipyretic, antiflatulent and detoxification effects.
Aim of the study: Elucidation of the mechanisms that underlie the antioxidant and pro-survival effects of the CE extract (CEE) in beta-cells and pancreatic islets from streptozotocin (STZ)-treated diabetic rats.
Material and methods: Diabetes was induced in rats by multiple applications of low doses of STZ (40 mg/kg intraperitoneally (i.p.), for five consecutive days). CEE (100 mg/kg) was administered orally, in the pre-treated group for two weeks before diabetes induction, during the treatments with STZ and for four weeks after diabetes onset, and in the post-treatment group for four weeks after diabetes induction. The impact of CEE on diabetic islets was estimated by histological and immunohistochemical examination of the pancreas. Molecular mechanisms of the effects of CEE were also analyzed in insulinoma Rin-5F cells treated with STZ (12 mM) and CEE (0.25 mg/mL). Oxidative stress was evaluated by assessing the levels of DNA damage, lipid peroxidation, protein S-glutathionylation and enzymatic activities and expression of CAT, MnSOD, CuZnSOD, GPx and GR in beta-cells. The presence and activities of the redox-sensitive and islet-enriched regulatory proteins were also analyzed.
Results: Treatment with CEE ameliorated the insulin level and glycemic control in STZ-induced diabetic rats by improving the structural and functional properties of pancreatic islets through multiple routes of action. The disturbance of islet morphology and islet cell contents in diabetes was reduced by the CEE treatment and was associated with a protective effect of CEE on the levels of insulin, GLUT-2 and p-Akt in diabetic islets. The antioxidant effect of CEE on STZ-treated beta-cells was displayed as reduced DNA damage, lipid peroxidation, protein S-glutathionylation and alleviation of STZ-induced disruption in MnSOD, CuZnSOD and CAT enzyme activities. The oxidative stress-induced disturbance of the transcriptional regulation of CAT, MnSOD, CuZnSOD, GPx and GR enzymes in beta-cells was improved after the CEE treatment, and was observed as readjustment of the presence and activities of redox-sensitive NFκB-p65, FOXO3A, Sp1 and Nrf-2 transcription factors. The observed CEE-mediated induction of proliferative and pro-survival pathways and insulin expression/secretion after STZ-induced oxidative stress in beta-cells could be partially attributed to a fine-tuned modulation of the

Abbreviations: CEE, *Centaurium erythraea* Rafn extract; STZ, streptozotocin; GSH, glutathione; GSSG, oxidized glutathione; GSSP, S-glutathionylated proteins; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; •OH, hydroxyl radical; $•O_2^-$, superoxide anion; HOCl, hypochlorous acid; H_2O_2, hydrogen peroxide; NO, nitric oxide; GLUT2, glucose transporter 2; GSIS, glucose-stimulated insulin secretion

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https://doi.org/10.1016/j.jep.2019.112043
Received 17 April 2019; Received in revised form 24 June 2019; Accepted 24 June 2019
Available online 25 June 2019

activities of pro-survival Akt, ERK and p38 kinases and islet-enriched Pdx-1 and MafA regulatory factors.
Conclusions: The results of this study provide evidence that CEE improves the structural and functional properties of pancreatic beta-cells by correcting the endogenous antioxidant regulatory mechanisms and by promoting proliferative and pro-survival pathways in beta-cells.

1. Introduction

Diabetes mellitus is a complex metabolic disease accompanied by hyperglycaemia resulting from a deficiency in insulin secretion and/or action, which leads to multiple organ failure and long-term diabetic complications (American Diabetes Association, 2014). Type 1 diabetes is caused by the autoimmune destruction of islet beta-cells by reactive T cells, and it is assumed that about 70–90% of the beta-cell mass is lost at the time of diagnosis (reviewed in Cernea and Dobreanu, 2013). In type 2 diabetes, high insulin demand due to peripheral insulin resistance induces compensatory beta-cell expansion and hyperinsulinemia, gradually leading to a glucotoxic loss of beta-cell mass (reviewed in Remedi and Emfinger, 2016). Recent data indicate that chronic inflammatory responses associated with obesity and metabolic stressors result in autoimmune destruction of islet beta-cells in type 2 diabetes as well (Brooks-Worrell and Palmer, 2012). Various underlying factors and mechanisms are involved in the decline in beta-cell mass and function, and elucidation of these processes is of crucial importance for the development of new therapeutic interventions that could improve beta-cell function (Wajchenberg, 2007). It has been shown that many of these processes are mediated by oxidative stress.

Autoimmune destruction of islet beta-cells occurs as the result of insulitis – the activation of infiltrated immune-cells and increased local cytokine production in inflammation of the islets of Langerhans (Brooks-Worrell and Palmer, 2012; Donath et al., 2008). Such an environment leads to inhibition of insulin secretion, induction of DNA damage and beta-cell death mediated primarily by NO and related free radicals and oxidant species (Delaney et al., 1993; Eizirik et al., 1996; Fehsel et al., 1993; Virág and Szabó, 2002). NO is produced in islets directly from the infiltrating macrophages (Kleemann et al., 1993) and indirectly from the induction of inducible NO synthase (iNOS). The prolonged exposure to hyperglycemia in diabetes triggers structural and functional damage of beta-cells (glucotoxicity) through several mechanisms, further impairing their functioning. By increasing the metabolic flux into the mitochondria and by promoting different mechanisms such as glucose autooxidation and non-enzymatic glycation, hyperglycemia stimulates increased production of reactive oxygen species (ROS) resulting in chronic oxidative stress (Poitout and Robertson, 2008; Robertson et al., 2003). Oxidative stress induces protein oxidation, lipid peroxidation, DNA damage (Acharya and Ghaskadbi, 2010) and is implicated in the gradual decline of insulin gene expression and reduced activity of the key regulators of insulin and other beta-cell-specific genes (Kim and Yoon, 2011; Poitout et al., 1996). Hyperglycemia-induced endoplasmic reticulum (ER) stress amplifies ROS production that in turn further increases the ER stress that impedes insulin production and causes beta-cell death (Kaufman et al., 2010; Leibowitz et al., 2010). Clinical studies have corroborated the presence of chronic oxidative stress in diabetic patients, manifested as increased levels of serum markers, reflecting oxidative tissue damage and a positive correlation between parameters of oxidative stress and impairment of glucose-stimulated insulin secretion (GSIS) in islets (Del Guerra et al., 2005; Sakuraba et al., 2002; Shin et al., 2001).

Pancreatic beta-cells are distinguished by a lower antioxidant capacity than most other tissues (Lenzen et al., 1996; Tiedge et al., 1997). The levels of $^{\bullet}O_2^-$ eliminating superoxide dismutase (SOD) isoenzymes in beta-cells is about 50% lower, while the levels of H_2O_2-inactivating enzymes, catalase (CAT) and glutathione peroxidase (GPx), are only about 1% of their respective levels in the liver. Such antioxidant properties of islets enable ROS-mediated GSIS, however, amplified and persistent oxidative stress can lead to impaired GSIS and beta-cell damage and destruction (Gerber and Rutter, 2017). Considering that ROS production is indispensable and at the same time potentially detrimental to normal beta-cell functioning, a suitable modulation of the antioxidant capacity of beta-cells under chronic oxidative stress (as in diabetes) could exert beneficial effects. Thus, studies using experimental models of diabetes revealed diabetes amelioration after treatment with plant extracts possessing antioxidant activity (reviewed in Dinić et al., 2013).

Centaurium erythraea Rafn (CE), known by the common name centaury (fam. *Gentianaceae*), is widely used for food and beverage bittering and is a popular component of many modern gastric herbal preparations and dietary supplements (Botion et al., 2005; Šiler et al., 2014). CE is used as a folk medicinal plant in Serbia to treat different ailments due to its antidiabetic, antipyretic, antiflatulent and detoxification effects (Jarić et al., 2015; Zlatković et al., 2014). The extract prepared from different parts of CE improved hyperglycemia and the lipid status in the serum of diabetic animals (Đorđević et al., 2017; Sefi et al., 2011; Stefkov et al., 2014). Sefi et al. (2011) showed that the extract of CE leaves alleviates oxidative stress and reduces degenerative injury of the pancreas in diabetic rats. The methanol extract of the aerial parts of CE protected red blood cells from hyperglycemia-triggered oxidative damage in STZ-induced diabetic rats by scavenging free radicals and by reducing protein glycation/glycosylation (Đorđević et al., 2017). In the present work we examined the molecular mechanisms that underlie the antioxidant and potentially protective effects of the CE extract on pancreatic beta-cells in STZ-induced oxidative stress in the pancreas of STZ-induced diabetic rats and STZ-treated insulinoma beta-cells. We assessed the parameters of the redox status of beta-cells and analyzed the presence and activities of factors involved in the transcriptional regulation of antioxidant enzymes, as well as of mediators of the pathways involved in beta-cell survival and insulin expression/secretion.

2. Materials and methods

2.1. Plant material

Plant material was collected at the locality Andrijevica (Montenegro; 42° 44′ 26″ N, 19° 48′ 12″ E) in 2010. *Centaurium erythraea* Rafn (CE) was authenticated in the field by the authors and was deposited in the Department of Plant Physiology, Institute for Biological Research "Siniša Stanković" (IBISS), University of Belgrade, Serbia. The plant name was verified with www.theplantlist.org, accessed on March 6th, 2019.

2.2. Preparation of the methanol extract

The aerial parts of the plants were air-dried and stored in paper bags until use. The dried material was ground into a fine powder using liquid nitrogen and extracted with 96% methanol (w:v = 1:5) overnight at room temperature. The methanol extract was sonicated for 20 min, filtered through Whatman No. 1 filter paper and evaporated in a vacuum evaporator (Eppendorf Concentrator 5301, Germany) at 30–45 °C until dry. The extraction yielded 14.09 ± 2.60% (w/w) of dry extract. Dry extracts were kept at −20 °C until use. Phytochemical characterization of *C. erythraea* methanol extract (CEE) that was used in the present study was previously described by Đorđević et al. (2017). UHPLC-qqqMS metabolic profiling revealed that predominant

compounds were secoiridoids (sweroside, gentiopicrin, swertiamarin, loganin, secologanin) and polyphenols, represented by: (1) phenolic acids i.e. hydroxycinnamic acids (caffeic acid, p-coumaric, ferulic and sinapic acid); (2) flavonoids from the group of flavones (luteolin and apigenin), flavonols (quercetin, rutin, isoquercitrin, kaempferol, astragalin) and flavanones (naringenin); (3) xanthones (decussatin, eustomin, desmethyleustomin and methylbellidifolin) (Đorđević et al., 2017). The UHPLC/(±)HESI − MS total ion scanning chromatogram (TIC) of CEE is provided as a Supplementary figure (Fig. S1). The analysis was performed using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Bremen, Germany) configured with a triple-quadrupole mass spectrometer (TSQ Quantum Access Max, Thermo Fisher Scientific, Basel, Switzerland).

2.3. Animals

Experiments were performed on 2.5-month-old adult male albino Wistar rats weighing 220–250 g. The rats were provided with standard food pellets and tap water *ad libitum* and were kept under controlled environmental conditions (12 h light/dark cycle, 22 ± 2 °C, 50% relative humidity). All animal procedures were in compliance with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes, approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade.

2.4. Experimental design

Diabetes was induced in rats by multiple i.p. injections of a low dose of STZ (40 mg/kg) for five consecutive days (O'Brien et al., 1996). STZ was dissolved before use in sodium citrate buffer (0.1 M, pH 4.5). Glucose was measured with a blood glucose meter (Accu-Chech Active) using blood samples obtained from the tail vein. Rats were considered diabetic when the fasting blood glucose exceeded 20 mmol/L, 24 h after the last STZ injection. The dried extract was dissolved in distilled water (10 mg/mL) and applied to the rats orally at a dose of 100 mg/kg. The dose was chosen based on the traditional use of CE as a herbal tea in Serbia (i.e. two tablespoons of ground herbal substance in 200 mL of boiling water, two to three times daily (http://www.mocbilja.rs/index.php/proizvod/caj-od-nadzemnog-dela-kicice/) (accessed on March 6th, 2019)), with allometric dose scaling as described by Nair and Jacob (2016). Determination of initial glucose and insulin levels served for the selection of rats subjected to further experimental procedure (Table S1 in Supplementary material). The animals were randomly divided into five groups of seven animals each as follows: Group I (C) – the non-diabetic control that received an equivalent volume of citrate buffer i.p. for 5 consecutive days; Group II (CEE) – CEE-treated non-diabetic rats that received an equivalent volume of citrate buffer i.p. for 5 consecutive days; Group III (D) – diabetic rats that received STZ (40 mg/kg/day, 5 days i.p.) and were left untreated throughout the 4-week period; Group IV (D/CEE) – diabetic rats that received the CEE for 4 weeks, starting from 24 h after the last STZ injection (post-treated group); Group V (CEE/D/CEE) – diabetic rats that received the CEE for 2 weeks before, the 5-day treatment with STZ, and for 4 weeks after the last STZ injection (pre-treated group).

2.5. Biochemical analyses

Serum was used for the determination of both blood glucose and insulin. After four weeks of diabetes, the rats fasted overnight and 2 mL of blood was collected from the tail vein. Following collection site cleaning with 70% ethanol, blood was collected from the lateral tail vein using a 21–23 gauge needle. Blood flow was stopped by applying pressure with a sterile gauze to achieve hemostasis. Serum was collected after blood clotting and centrifugation at 2000 × g for 10 min. Blood glucose was measured with a commercial kit (Gluco-quant Glucose/HK, Boehringer, Mannheim, Germany), based on the hexokinase/G6P-DH enzymatic method. Serum insulin was determined using an enzyme-linked immunosorbent assay (ELISA) kit (EMD Milipore, St. Charles, Missouri, USA).

2.6. Histological and immunohistochemical examination of the pancreas

For histological and immunohistological examinations, the pancreata were removed and fixed in 10% buffered formalin, embedded in paraffin wax and sectioned at 5 µm thickness. For histological analysis, tissue sections were stained with hematoxylin and eosin (H&E) and observed under a DM RB Photomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a Leica DFC 320 CCD camera at 40 × magnification. For immunohistochemical analysis, deparaffinized sections were passed through xylene and rehydrated in sequentially graduated ethyl alcohol. Slides were incubated in 0.3% hydrogen peroxide/methanol for 20 min to reduce nonspecific background staining due to endogenous peroxidase. For antigen retrieval, the slides were treated with 0.01 M sodium citrate buffer (98 °C). The cooled sections were washed in phosphate buffered saline (PBS) and permeabilized for 10 min in 0.3% Triton X-100 (Amersham Biosciences, Little Chalfont, UK) prior to application of the blocking serum (0.05% Tween 20, 3% bovine serum albumin) for 60 min. The primary antibody was applied overnight at +4 °C. Polyclonal antibodies raised against insulin (H-86), glucagon (C-18), somatostatin (H-106) and phosphorylated protein kinase B (pAkt) (Ser 473) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1:100, while Glut-2 (H-67) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:50 in PBS with 0.2% Tween 20. After washing in PBS, sections were incubated with secondary antibody conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), at a dilution of 1:100 for 1 h at room temperature. The sections were then contrasted with hematoxylin, dehydrated in a series of ethanol concentrations and cleared in xylol. After mounting of DPX plates, sections were visualized on a DM RB Photomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) at 40 × magnification.

2.7. Cell culture and treatment

Pancreatic islet tumor Rin-5F (ATCC-CRL-2058) cells were cultivated in RPMI 1640 medium (Biological Industries, Beit HaEmek, Israel) supplemented with fetal bovine serum (FBS) (10%) (Bio West, Nuaillé, France), L-glutamine (2 mM) (Biological Industries, Beit HaEmek, Israel) and antibiotics penicillin (100 U/mL) and streptomycin (100 µg/mL) (Biological Industries, Beit HaEmek, Israel), at 37 °C under 5% CO_2 in a humidified atmosphere (95%). The medium was exchanged every 72 h. All experiments on Rin-5F cells were performed with cells between passages 5 and 35; as indicated in the literature, the use of early passages (from 5 to 35) of the Rin-5F cell line provided stable insulin expression (reviewed in Skelin et al., 2010). Rin-5F cells were seeded in sterile plates and after reaching confluency of 60–70%, the cells were treated with an IC_{50} dose of STZ dissolved in citrate buffer (0.1 M, pH 4.5), or with CEE dissolved in RPMI medium and used for cell treatment at a concentration of 0.25 mg/mL for 24 h (unless otherwise indicated). The cells were processed immediately after incubation with STZ and/or CEE.

2.8. MTT viability test

Rin-5F cell viability was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, Cat. No. M5655-1G) assay. Cell viability was estimated based on the quantification of mitochondrial activity by measuring the formation of dark-purple formazan formed by reduction of the tetrazolium ring of MTT. Rin-5F cells grown in 96-well plates were treated with 12 mM STZ in combination with CEE, and after 24 h, 200 µL of MTT (0.5 mg/mL RPMI medium)

was added to each well. After incubation for 2 h in the dark, the insoluble formazan products formed in living cells were dissolved in dimethyl sulfoxide (DMSO). Formazan product formation was quantified by measuring the absorbance at 570 nm. Cell viability was expressed as the percentage after comparison to the control cells that were assumed to be 100%.

2.9. Comet assay

DNA damage was estimated using the alkaline Comet assay. Each experimental group of Rin-5F cells was mixed with low-melting agarose and placed onto a microscope slide. Cells were lysed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% Triton X-100) for 2 h at 4 °C. The slides were incubated in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13.0) for 30 min at 4 °C and subjected to electrophoresis in order to separate the damaged DNA fragments. After electrophoresis, the slides were placed in neutralization buffer (0.4 M Tris–HCl, pH 7.4) and stained with Sybr Green I (Sigma-Aldrich, S9430). Tail moment was used as an index of induced DNA damage, considering both the migration of the genetic material and the relative amount of DNA in the tail. Quantification of images was performed by measuring the displacement of the genetic material between the nucleus ('comet head') and the resulting 'comet tail' using TriTekCometScore Freeware version 1.5.

2.10. Lipid peroxidation assay

The level of lipid peroxidation was estimated by measuring the concentration of the reactive by-product malondialdehyde (MDA) in the thiobarbituric acid-reactive substance (TBARS) assay as described by Ohkawa et al. (1979). Briefly, an aliquot (0.1 mL) of the cell homogenates was mixed with 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of 0.8% thiobarbituric acid (TBA) and 0.7 mL of water. The mixture was heated at 95 °C for 60 min, supplemented with 1 mL of water and 5 mL of n-butanol-pyridine (15:1, v/v), mixed and centrifuged at 3000 × g for 10 min. The red pigment in the supernatants was evaluated by absorbance at 532 nm. A calibration curve was prepared using a MDA standard. The concentration of MDA was expressed as nM MDA/mg proteins. Protein concentrations were determined according to Lowry et al. (1951).

2.11. Determination of reduced glutathione (GSH), glutathione disulfide (GSSG) and protein S-glutathionylation (GSSP)

Measurement of GSH, GSSG and GSSP was performed as described previously (Grdović et al., 2012). Briefly, Rin-5F cells were resuspended in 2.5% sulfosalicylic acid and homogenized using a Potter–Elvehjem Teflon-glass homogenizer. The homogenate was centrifuged at 8000 × g for 5 min at 4 °C, and an aliquot of the supernatant was analyzed for the content of GSH, while the acid-precipitated proteins were used for measurement of glutathionylated proteins (GSSP). For the estimation of GSH, samples and the reaction mixture (0.1 M sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.3 mM-5,5′-dithiobis-(2-nitrobenzoic acid), 0.4 mM NADPH and 1 U/mL glutathione reductase I (GR) were incubated for 30 min at room temperature. Absorption was measured at 412 nm and the GSH content was evaluated using a calibration curve. GSSG was quantified after derivatization of GSH with 10 mM 2-vinylpyridine and assayed as described above using a calibration curve. For GSSP determination, acid-precipitated proteins were resuspended and washed twice with 1.5% trichloracetic acid (TCA). After washing, the proteins were resuspended in 0.5 mL of basic solution (9:1, v/v, 0.1 mM phosphate buffer (pH 7·4) and 0.25 mM NaOH), and stirred for 30 min at room temperature. Proteins were precipitated with 60% TCA and GSH was determined in the supernatant as described above.

2.12. Determination of antioxidant enzyme activities

The cells were resuspended in sucrose buffer (0.25 M sucrose, 1 mM EDTA and 0.05 M Tris-HCl, pH 7.4), sonicated at 20 kHz/30s on ice, and centrifuged at 14000 × g/4 °C for 1 min. Aliquots of the obtained supernatants were used for determination of protein concentrations and for measurement of enzyme activities. CAT activity was determined according to Beutler (1982) by the rate of H_2O_2 decomposition and expressed as U/mg proteins. Total SOD activity was measured according to the epinephrine method (Misra and Fridovich, 1972) and expressed as U/mg proteins. MnSOD activity was performed after preincubation with 8 mM KCN. CuZnSOD activity was calculated from the difference between total SOD and MnSOD activities. The activity of GPx was determined following oxidation of NADPH as a substrate with *tert*-butyl hydroperoxide (Tamura et al., 1982) and expressed in nmol NADPH/min/mg protein. The activity of GR was measured as described by Glatzle et al. (1974) and expressed as nmol NADPH/min/mg proteins.

2.13. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

Cell lysates and nuclear fractions were prepared using the ProteoJET Mammalian Cell Lysis Reagent (Fermentas, Lithuania) and NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, lL, USA), respectively, according to the manufacturer's instructions. Cells (1×10^6) were transferred to a 1.5 mL microcentrifuge tube and pelleted by centrifugation at 500 × *g* for 3 min. The supernatant was carefully removed leaving the cell pellet as dry as possible. After addition of 100 µL of ice-cold Cytoplasmic Extraction Reagent I (CER I) buffer, the cells were vortexed for 15 s and then incubated for 10 min on ice. After incubation, 5.5 µL of CER II buffer was added to the tubes, the samples were vortexed for 5 s, incubated for 1 min on ice and centrifuged at 16 000 x *g*, 5 min at 4 °C. Insoluble (pellet) fraction which contains nuclei was suspended in the ice-cold Nuclear Extraction Reagent (NER) buffer (50 µL), the samples were vortexed for 15 s and then incubated for 40 min on ice with vortexing for 15 s every 10 min. At the end of the incubation, the samples were centrifuged at 16 000 x *g* for 10 min at 4 °C. Supernatants containing nuclear fractions were transferred to new tubes and stored at −80 °C until use. The nuclear fraction and homogenate proteins (20 µg) were separated by (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes (Amersham Hybond P 0.45 PVDF, GE Healthcare Life Sciences, UK), blocked for 1 h at room temperature with 5% non-fat dry milk (Blotto, non-fat dry milk, Santa Cruz Biotechnology, USA) in blotto base buffer (0.2% Tween 20, 20 mM Tris-HCl pH 7.6, 150 mM NaCl), and examined by immunoblot analysis using the following antibodies: anti-MnSOD (FL-222), anti-CuZnSOD (C-17), anti-Akt 1/2/3 (H-136), anti-pAkt 1/2/3 (Ser 473), anti-ERK1/2 (K-23), anti-pERK (E − 4), anti-p38 (C-20), anti-p-p38 (Tyr 182), anti-Nrf2 (C-20), anti-NFκB-p65 (C-20), anti-pNFκB-p65 (Ser 311), anti-Sp1 (E − 3), anti-FOXO3a (H-144), anti-Pdx1 (H-140), anti-MafA (F-6), anti-lamin B (M-20) and anti-GAPDH (FL-335) (all from Santa Cruz Biotechnology, USA), anti-catalase, anti-GR and anti-GPX 1 (Abcam, USA). The blots were then probed with appropriate HRP-conjugated IgG (all from Santa Cruz Biotechnology, USA). Staining was performed by the chemiluminescent technique according to the manufacturer's instructions (Western Blotting Luminol Reagent, Santa Cruz Biotechnology, USA). Quantification of immunoreactive bands was performed using TotalLab (Phoretix, USA) electrophoresis software (v1.1).

2.14. RNA isolation and real-time quantitative PCR analysis (RT-qPCR)

Total RNA was isolated from Rin-5F cells using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, USA) following the

manufacturer's instructions. Cells seeded in 6-well plates were treated with STZ (IC_{50}) and/or CEE for 24 h. For complementary DNA synthesis, total RNA (1 μg) was treated with DNAse I and reverse transcribed with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) using oligo(dT) primers. The levels of mRNA were quantified by RT-qPCR using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fischer Scientific, USA) and QuantStudio 3 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). The program for RT-qPCR included an initial denaturation step (95 °C/10 min), followed by 40 cycles of a two-step PCR program at 95 °C for 15 s and at 60 °C for 1 min. Negative controls without the template were used in all RT-qPCR reactions. The expression levels of the target genes were related to the averaged expression level of rat GAPDH gene. RT-qPCR reactions were carried out in triplicate. The fragments were amplified using the following primers (Invitrogen, USA): for the rat CAT gene: Fw 5′-GCGAATGGAGAGGCAGTGTAC-3′ and Rev 5′- GAGTGACGTTGTCT TCATTAGCACTG -3' (652 bp); for the rat MnSOD gene: Fw 5′-CAGAT CATGCAGCTGCACCA-3′ and Rev 5′-AGTCCAGGCTGAAGAGCA-3' (133 bp); for the CuZnSOD gene: Fw 5′-GCAGAAGGCAAGCGGTGAAC-3′ and Rev 5′-CGGCCAATGATGGAATGCTC-3' (282 bp); for the GPx gene: Fw 5′-AGTTCGGACATCAGGAGAATGG-3′ and Rev 5′-TAAAGAGCGGGTG AGCCTTC-3' (141 bp); for the GR gene: Fw 5′-CACTTCCCGGTAGGAA ACCC-3′ and Rev 5′-GATCGCAACTGGGGTGAGAA-3' (227 bp); for the insulin 1 (Ins1) gene: Fw 5′-ATGGCCCTGTGGATGCGCTT-3′ and Rev 5′-ACAATGCCACGCTTCTGCCG-3' (275 bp); for the GAPDH gene: Fw 5′-CAAGGTCATCCATGACAACTTTG-3′ and Rev 5′-GTCCACCACCCTGT TGCTGTAG-3' (496 bp).

2.15. Statistical analysis

All data were analyzed using GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA). Statistical differences between groups were analyzed using one-way Analysis of Variance (one-way ANOVA), followed by Bonferroni's Multiple Comparison Test. The difference was considered statistically significant at $p < 0.05$. The data were expressed as the mean ± S.E.M. (standard error of mean).

3. Results

3.1. CEE improves structural and functional properties of pancreatic islets in diabetic rats

As can be seen in Table 1, STZ-treated rats displayed typical signs of diabetes: elevation of blood glucose concentration (4-fold) and reduction of serum insulin level (3-fold) in comparison to control animals. CEE treatment of diabetic animals significantly improved glycemic control and insulin production especially in the pre-treated group. This beneficial effect of CEE administration was associated with preservation of the histological features of pancreatic islets in diabetic rats observed after examination of H&E-stained pancreatic sections (Fig. 1A). Control islets of Langerhans displayed typical oval or elongated shapes surrounded by a thin connective sheath and separated from the exocrine pancreatic tissue. Besides a disturbed shape of islets, the number and size of islets and the number of present cells were significantly lower in diabetic rats compared to control animals. In the pre- and post-treated animals, the morphology of pancreatic islets was more similar to that of the control, which points to the protective effect of CEE. This observation was consonant with the immunohistochemical staining with anti-insulin, anti-glucagon and anti-somatostatin antibodies, reflecting the number and distribution of beta, alpha and delta-cells (respectively) along the islets. In contrast to the control islets which were characterized by insulin-stained beta-cells that were uniformly distributed throughout the entire central portion of the islet, the islets of diabetic rats displayed a disturbed distribution of a significantly reduced number of insulin-positive cells (Fig. 1B). The treatment with CEE increased the number of insulin-positive cells that occupied the central region of the diabetic islets, notably in the pre-treated group. As can be seen on Fig. 1C, glucagon-stained alpha-cells were organized in clusters scattered at the periphery of the control islets, and arranged centrally in the islets prepared from diabetic rats. Application of CEE led to a mostly peripheral localization of glucagon-staining cells, particularly in post-treated rats. As expected, in the controls most of the somatostatin-positive delta-cells were in the form of clusters dispersed peripherally, with only a few cells placed in the intermediate position (Fig. 1D). The central localization of somatostatin-positive cells observed in diabetic islets was improved after the treatment with CEE as delta-cells were detected at the periphery of the islets.

The glucose transporter GLUT-2, which is responsible for glucose entry into rodent beta-cells, functions as part of the glucose sensing mechanism for the stimulation of insulin secretion (Hou et al., 2009). In pancreatic sections prepared from control and CEE-treated control rats, strong positive GLUT-2 staining with a uniform distribution within the islets was observed (Fig. 1E). As expected, the pattern of GLUT-2 distribution in the islets coincided with the insulin-staining of beta-cells. Contrary to diabetic islets where GLUT-2 was not detected, CEE treatment of diabetic rats resulted in GLUT-2 positive staining in the central portion of the islets, in particular in the pre-treated group. Akt plays an important role in the regulation of pancreatic beta-cell growth and survival. Immunohistochemical analysis of the control pancreas revealed the presence of activated Akt (p-Akt) uniformly distributed throughout the islets, and a similar pattern of p-Akt was observed in CEE-treated controls. While diabetic islets did not display p-Akt-positive staining, treatment of diabetic animals with CEE was accompanied by the preservation of p-Akt presence in islets comparable to the control.

The positive effects of the CEE treatment on the structure and functionality of the endocrine pancreas in diabetic rats stimulated further examination of the potential impact of CEE on the redox-related molecular pathways that were triggered by STZ in pancreatic beta-cells.

3.2. CEE positively affects the survival and functionality of insulinoma (Rin-5F) beta-cells

The effect of CEE on beta-cell survival after STZ treatment was assessed by the cytotoxicity test. Insulin-secreting pancreatic (Rin-5F) beta-cells were treated with increasing concentrations of CEE which were previously analyzed for their *in vitro* antioxidant activity of the extract (Đorđević et al., 2017). According to the cell viability assay, the

Table 1
Centaurium erythraea extract improves fasting blood glucose and serum insulin level in diabetic rats.

	C	CEE	D	D/CEE	CEE/D/CEE
N	7	7	7	7	7
Glucose (mmol/L)	5.5 ± 0.09	5.75 ± 0.28	22.5 ± 1.04***	15.1 ± 2.15***; ++	11.27 ± 1.28*; +++
Insulin (ng/mL)	0.57 ± 0.04	0.59 ± 0.02	0.19 ± 0.02***	0.33 ± 0.01***; ++	0.45 ± 0.03*; +++

C – non-diabetic control; CEE – CE extract-treated non-diabetic group; D – diabetic group; D/CEE – post-treated diabetic group; CEE/D/CEE – pre-treated diabetic group. Values are means ± S.E.M. for the indicated number of animals for each group (N). *$p < 0.05$, ***$p < 0.001$ as compared to C; ++ $p < 0.01$, +++ $p < 0.001$ as compared to D.

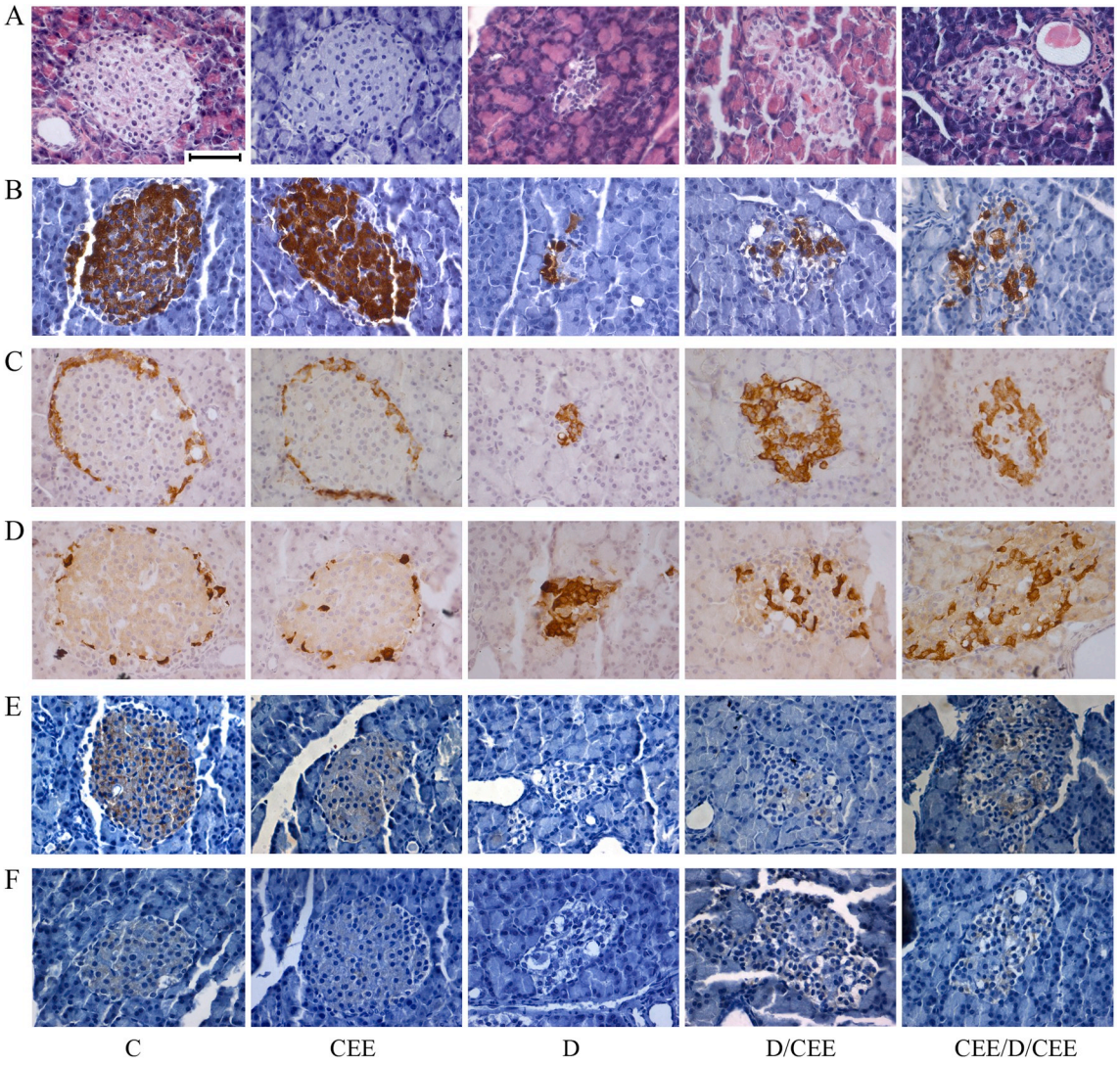

Fig. 1. *Centaurium erythraea* extract improves the structural and functional properties of pancreatic islets. (A) H&E staining of pancreatic sections (magnification 40x). Scale bar, 50 µm. Immunohistochemical localization of insulin (B), glucagon (C), somatostatin (D), GLUT-2 (E) and p-Akt (F) in pancreatic islets (magnification 40x). Scale bar, 50 µm. C – non-diabetic control; CEE – CE extract-treated non-diabetic group; D – diabetic group; D/CEE – post-treated diabetic group; CEE/D/CEE – pre-treated diabetic group.

concentrations of extract ranging from 0.05 to 0.25 mg/mL did not exhibit any cytotoxic effect on Rin-5F cells (Fig. 2A). Treatment of Rin-5F cells with increasing concentrations of STZ for 24 h revealed that the concentration of 12 mM STZ corresponded to IC_{50} that was used in all subsequent experiments (Fig. 2B). When Rin-5F cells were treated in parallel with IC_{50} of STZ and with a non-cytotoxic concentration of CEE, the most significant increase (for 12%) of Rin-5F cell viability was observed when 0.25 mg/mL CEE was applied (Fig. 2C). This dose of CEE was used in all further examinations after taking into account its effect on cell survival and *in vitro* antioxidant properties (Đorđević et al., 2017). To estimate the functionality of viable beta-cells, we further examined insulin gene expression and insulin secretion in Rin-5F cells (Fig. 2D and E). The treatment with STZ reduced the level of Ins1 mRNA to 63% in comparison to control cells, whereas the STZ/CEE co-treatment improved Ins1 gene expression significantly (to 77% of the control). Concurrently, the treatment with STZ reduced insulin secretion to 76% of the level measured in the control, while CEE application improved insulin secretion to 90% of the control level. Treatment of control cells with CEE had no effect on insulin expression and secretion.

3.3. CEE alleviates STZ-mediated oxidative stress in rat pancreatic beta-cells

Given that STZ promotes pancreatic beta-cell death and dysfunction by DNA alkylation and subsequent induction of oxidative stress (Lenzen, 2008), we examined whether CEE provided DNA protection and affected the antioxidant defense system in STZ-treated beta-cells. As can be seen on Fig. 3, STZ-treated Rin-5F cells displayed a 9-fold higher tail moment in comparison with the control. The co-treatment of Rin-5F cells with STZ and CEE significantly protected DNA from damage. The antioxidant effect of the CEE in STZ-treated beta-cells was evaluated by measuring the levels of lipid peroxidation (MDA level), the reduced glutathione/oxidized glutathione (GSH/GSSG) ratio, protein S-glutathionylation (GSSP) and the activity of antioxidant enzymes (Table 2). In STZ-treated cells, the levels of MDA and GSSP were significantly elevated, by 77% and 125%, respectively. The GSH/GSSG ratio was lowered by 25% when compared to the control. CEE improved the measured oxidative stress parameters, as the levels of MDA and GSSP were reduced significantly with respect to the STZ treatment (by 27% and 40%, respectively), while the GSH/GSSG ratio was slightly increased (by 8%). Treatment of control cells with the CEE did not

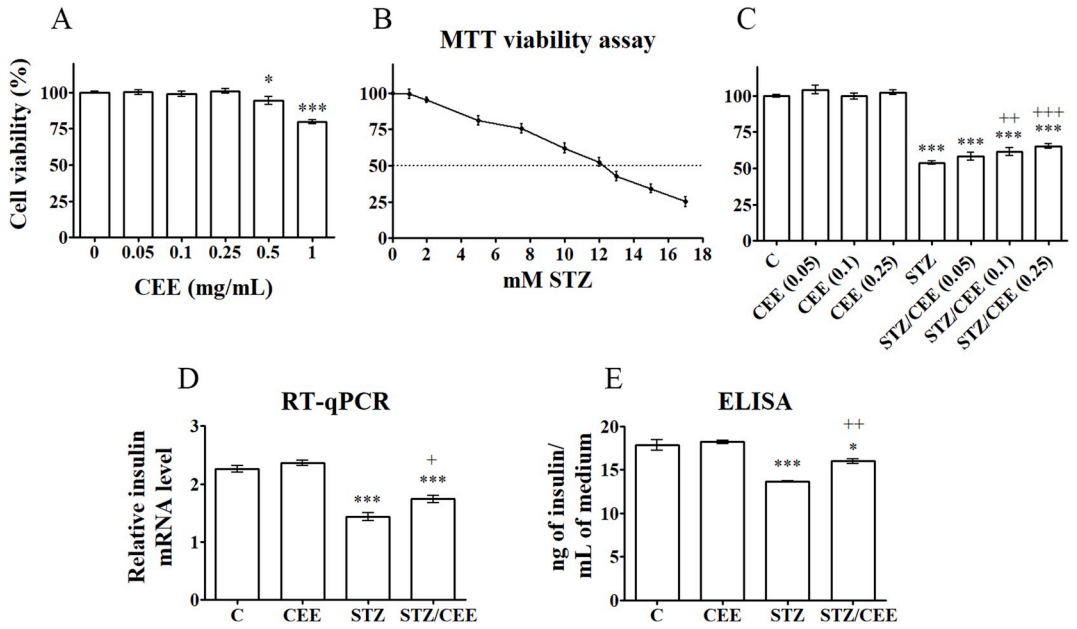

Fig. 2. *Centaurium erythraea* extract improves the viability and functionality of Rin-5F insulinoma beta-cells. (A) Viability assay performed on Rin-5F cells after treatment with increasing concentrations of *Centaurium erythraea* extract (CEE). (B) Viability assay after treatment of Rin-5F cells with increasing concentrations of streptozotocin (STZ). (C) Viability assay after co-treatment of Rin-5F cells with 12 mM STZ (IC_{50}) and CEE (non-toxic concentrations). (D) Relative expression of insulin mRNA against GAPDH in Rin-5F cells after co-treatment with 12 mM STZ and CEE (0.25 mg/mL). (E) Released amount of insulin after co-treatment of Rin-5F cells with STZ and CEE. The values are means ± S.E.M. from at least three separate experiments. *$p < 0.05$, ***$p < 0.001$ as compared to C; +$p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ as compared STZ/CEE to STZ.

affect the levels of MDA, GSSP nor the GSH/GSSG ratio.

Further estimation of the beneficial contribution of CEE to an antioxidant milieu in STZ-treated beta-cells included the analysis of the activities of antioxidant enzymes in cell homogenates (Table 2). The activities of CAT, MnSOD and CuZnSOD were significantly increased (by 53%, 97% and 55%, respectively) after the STZ treatment as compared to the control. Co-treatment with STZ and CEE significantly lowered MnSOD and CuZnSOD activities with respect to the STZ treatment (by 22% and 11%, respectively), whereas CAT activity was reduced to the control level. GPx and GR activities did not change significantly, even though a slight increase in GR activity was detected after the STZ treatment. Treatment of control cells with CEE did not alter the enzyme activities. The detected changes in the activities of antioxidant enzymes point to the potential of CEE to correct the disturbed redox balance in STZ-treated beta-cells.

3.4. The effect of CEE on gene expression and protein levels of antioxidant enzymes in beta-cells

The observed changes in antioxidant enzyme activities could have been the result of different mechanisms that become activated in STZ-mediated oxidative stress and after CEE treatment. Considering that transcriptional regulation plays an important role in protein expression and activity, the following experiments were aimed at investigating gene transcription of enzymes by measuring the relative changes in their mRNA levels and comparing them with the respective relative changes of the protein levels (Fig. 4). CAT mRNA was induced in STZ-treated cells by 62%, while in STZ/CEE-treated cells induction was weaker as CAT mRNA was increased by 32% as compared to the control (Fig. 4A). GPx mRNA was significantly induced after the STZ treatment (2.7-fold), whereas the co-treatment with STZ and CEE caused a smaller increase (2.1-fold) in GPx mRNA than in control cells. GR mRNA was induced by 30% in comparison to the control in both STZ- and STZ/CEE-treated cells. The level of MnSOD mRNA was increased by 52% after the STZ treatment, while the co-treatment with STZ and CEE resulted in lower MnSOD induction (26%) in comparison to control cells. CuZnSOD mRNA was induced after the STZ treatment, increasing by 15%, and remaining at the control level after the co-treatment with STZ and CEE. Treatment of control cells with CEE did not affect the mRNA levels of the analyzed enzymes.

The changes observed at the transcriptional level were associated with changes at the protein level of the examined antioxidant enzymes (Fig. 4B). Western blot analysis of cell homogenates showed that the relative level of CAT protein in STZ-treated cells was 30% higher than in the control, while after the co-treatment with STZ and CEE, the presence of CAT was increased by 14% above the control. STZ and STZ/CEE treatments did not affect the relative amount of GPx protein, whereas the relative level of GR protein was significantly (3-fold) higher after the STZ and STZ/CEE treatments in comparison to the control. The relative amounts of MnSOD and CuZnSOD were increased after the treatment with STZ by 21% and 53%, respectively, in comparison to the control, and remained unchanged after the co-treatment with STZ and CEE. Treatment of control cells with CEE had no effect on the relative levels of proteins.

The detected alterations in antioxidant enzyme expression pointed to changes at the level of the upstream regulators of this process. Considering that oxidative stress affects the stability, distribution and activities of different proteins, we assumed that transcription factors would be among the primary targets of oxidative stress, with the changed expression of their target genes contributing to beta-cell malfunctioning (Grdović et al., 2012; Guo et al., 2013).

3.5. The effect of CEE on the activities of transcription factors involved in the transcriptional regulation of antioxidant enzymes in beta-cells

To further elucidate the effects of CEE on the regulatory mechanisms activated by STZ-mediated oxidative stress, we examined the presence of NFκB-p65, FOXO3A, Sp1 and Nrf-2 factors that are involved in the transcriptional regulation of the analyzed antioxidant enzymes. For this analysis, cell nuclear fractions were prepared from Rin-5F cells subjected to STZ treatment and STZ/CEE co-treatment for 24 h (Fig. 5). Upon translocation to the nucleus, redox-sensitive transcription factor

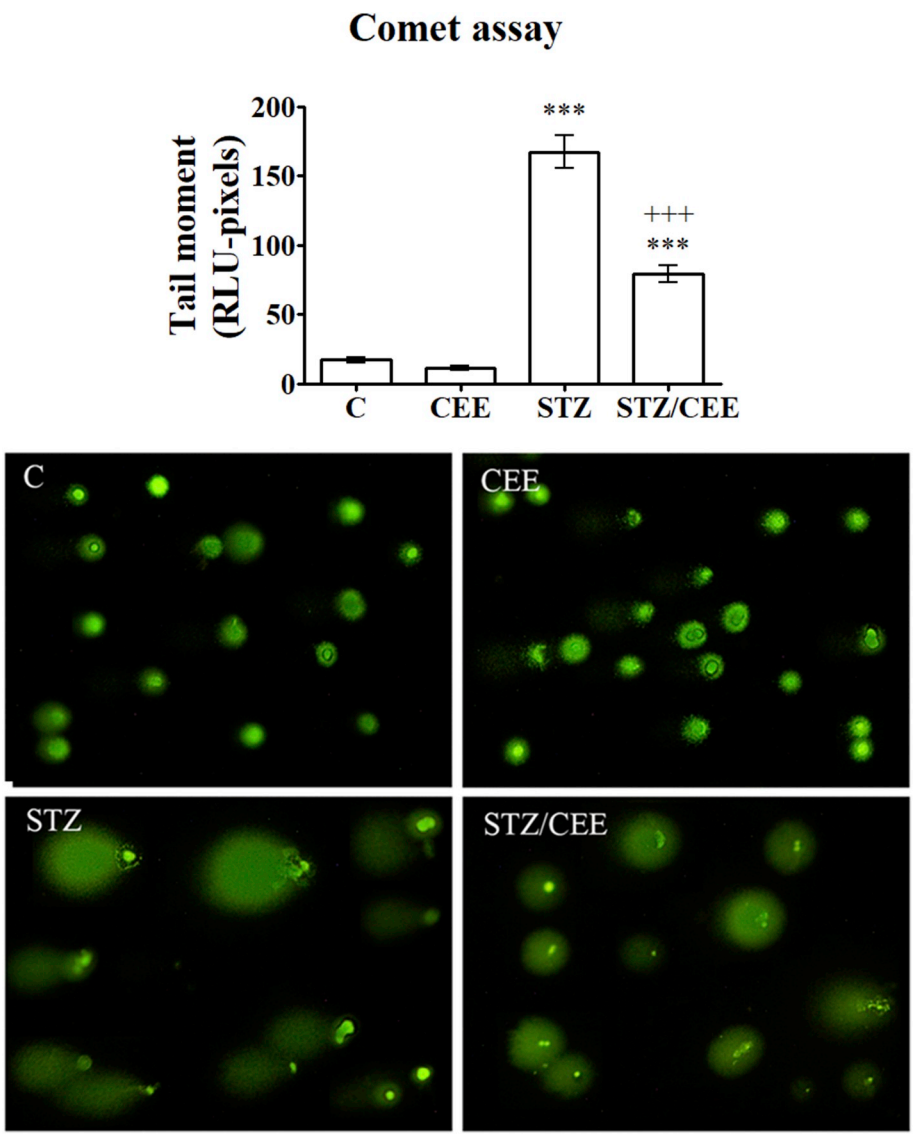

Fig. 3. *Centaurium erythraea* extract reduced DNA damage in STZ-treated Rin-5F beta-cells. DNA damage was assessed by the alkaline comet assay, using tail moment as the parameter of damage. Representative images of comets from three independent experiments are shown. Presence of DNA in the comet tail (tail moment) estimated by using TriTekCometScore™ Freeware version 1.5 and graphically presented as mean ± SEM. C – control cells; CEE – CE extract (0.25 mg/mL)-treated cells; STZ – cells treated with 12 mM streptozotocin (STZ); STZ/CEE – cells co-treated with STZ (12 mM) and CE extract (0.25 mg/mL). ***$p < 0.001$ as compared to C; $^{+++}p < 0.001$ as compared STZ/CEE to STZ.

NFκB-p65 is phosphorylated to its transcriptionally active form. Analysis of the Ser311 phosphorylation site which is essential for NFκB-p65 transcriptional activity and function (Duran et al., 2003) revealed NFκB-p65 activation after the STZ treatment. Namely, the relative level of the phosphorylated form of NFκB-p65 (p-NFκB-p65) in STZ-treated cells was 38% above the control level. In beta-cells co-treated with STZ/CEE, the activity of NFκB-p65 was reduced as the level of p-NFκB-p65 was 14% higher than in the control. The relative nuclear levels of

Table 2
General markers of oxidative stress in Rin-5F beta-cells.

	C	CEE	STZ	STZ/CEE
TBARS [a]	41.13 ± 2.06	42.54 ± 2.13	72.68 ± 3.63 ***	52.78 ± 2.64 ++
GSH/GSSG	23.95 ± 1.2	22.98 ± 1.15	17.91 ± 0.9 *	19.4 ± 0.97
GSSP [b]	65.31 ± 3.27	68.36 ± 3.42	146.95 ± 7.35 ***	88.35 ± 4.42 +++
CAT activity [c]	4.83 ± 0.2	5.16 ± 0.42	7.41 ± 0.37 ***	5.06 ± 0.5 ++
GPx activity [c]	2.92 ± 0.18	2.86 ± 0.18	3.06 ± 0.3	2.98 ± 0.25
GR activity [c]	42.19 ± 1.85	43.69 ± 3.1	51.52 ± 3.77	42.28 ± 4.75
MnSOD activity [c]	1.03 ± 0.04	1.04 ± 0.05	2.03 ± 0.08 ***	1.59 ± 0.08 **; ++
CuZnSOD activity [c]	3.44 ± 0.14	3.62 ± 0.11	5.34 ± 0.08 ***	4.77 ± 0.08 ***; +

Lipid peroxidation (TBARS), ratio of reduced and oxidized glutathione (GSH/GSSG), level of S-glutathionylated proteins (GSSP), enzyme activities of catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), manganese and copper-zinc superoxide dismutase (MnSOD and CuZnSOD). The values are means ± S.E.M. from three experiments performed in triplicate. C – control cells; CEE – CE extract (0.25 mg/mL)-treated cells; STZ – cells treated with 12 mM streptozotocin (STZ); STZ/CEE – cells co-treated with STZ (12 mM) and CE extract (0.25 mg/mL). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared to C; +$p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ as compared STZ/CEE to STZ.

[a] nM MDA/100 mg of proteins.
[b] μM GSH/mg of proteins.
[c] U of activity/mg of proteins.

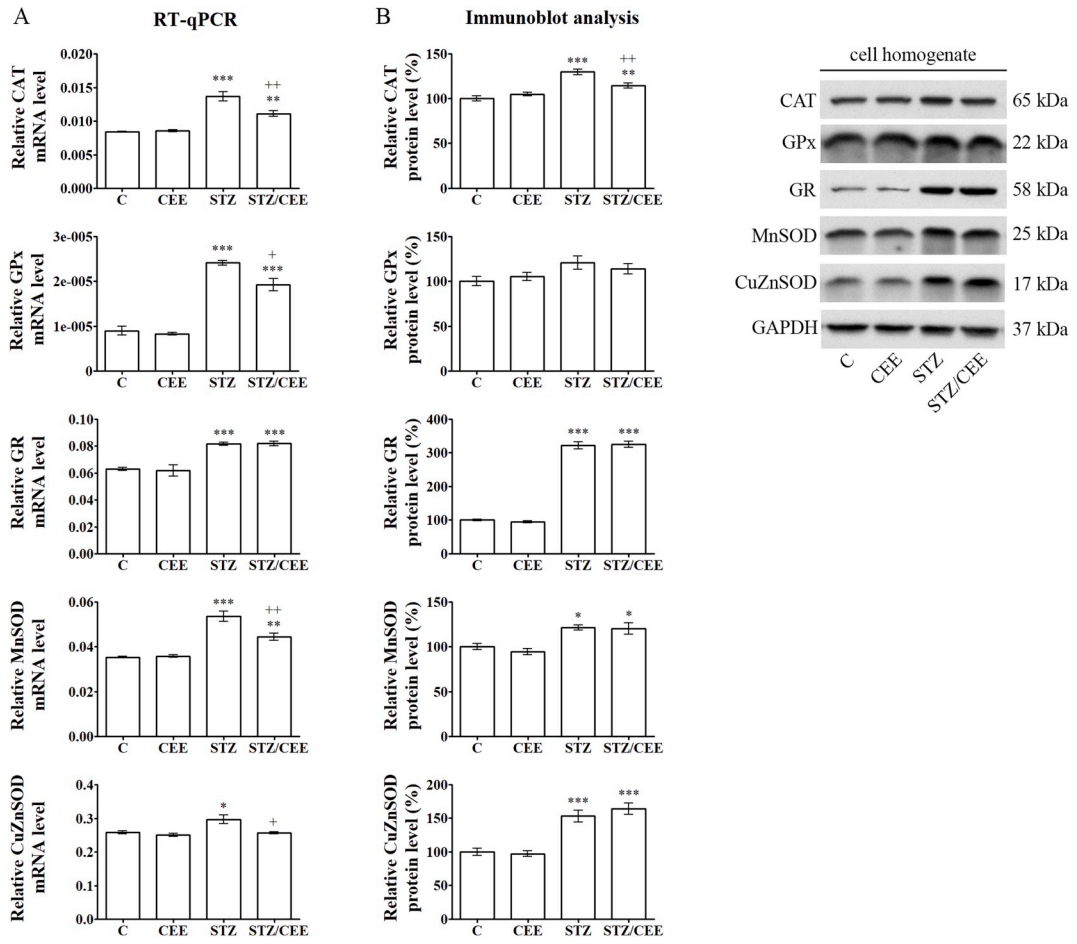

Fig. 4. The effect of *Centaurium erythraea* extract on antioxidant enzyme expression in Rin-5F cells. (A) Quantitative RT-PCR analysis of CAT, GPx, GR, MnSOD and CuZnSOD gene expression. RNA isolation and complementary DNA (cDNA) preparation from Rin-5F cells was performed after treatment with 12 mM streptozotocin (STZ) for 24 h with/without CE extract (0.25 mg/mL). Gene expression of antioxidant enzymes was corrected for the amount of GAPDH mRNA. The values plotted are the means ± S.E.M. from three experiments performed in triplicate. (B) Immunoblot analysis of CAT, GPx, GR, MnSOD and CuZnSOD in Rin-5F cell homogenates prepared after treatment with 12 mM streptozotocin (STZ) for 24 h with/without CE extract (0.25 mg/mL). Representative blots from three independent experiments are shown. Blots were quantified using TotalLab (Phoretix) electrophoresis software. The changes in antioxidant enzyme protein levels relative to the control and corrected for the expression of GAPDH are presented on the graphs. The values plotted are the means ± S.E.M. from three independent experiments. CAT – catalase; GPx – glutathione peroxidase; GR – glutathione reductase; MnSOD – manganese superoxide dismutase; CuZnSOD – copper-zinc superoxide dismutase; GAPDH - glyceraldehyde 3-phosphate dehydrogenase. C – control cells; CEE – CE extract (0.25 mg/mL)-treated cells; STZ – cells treated with 12 mM streptozotocin (STZ); STZ/CEE – cells co-treated with STZ (12 mM) and CE extract (0.25 mg/mL). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared to C; $^{+}p < 0.05$, $^{++}p < 0.01$ as compared STZ/CEE to STZ.

FOXO3A, a transcription factor involved in cellular antioxidant defense, was increased by a similar amount (32% and 41%, respectively) in both STZ and STZ/CEE treatments. The nuclear presence of the ubiquitously expressed factor Sp1, which is involved in redox-controlled transcriptional regulation, was reduced in STZ-treated beta-cells by 24% in comparison to control cells. However, in STZ/CEE-treated cells, Sp1 was at the control level. The level of Nrf-2, the central regulator of constitutive and inducible antioxidant response element (ARE)-associated gene expression, was increased 7-fold in the nuclear fraction isolated from STZ-treated cells, whereas after co-treatment with STZ and CEE, the relative level of Nrf-2 was reduced, remaining 4-fold above the control. Treatment of control cells with CEE did not affect the presence of the examined transcription factors. The obtained results suggest that the STZ-induced oxidative stress in beta-cells was associated with changes in the presence and activities of redox-sensitive regulatory transcription factors, and that the treatment with CEE exhibited a propensity to rebalance their abundance and consequently their actions.

3.6. The effect of CEE on pro-survival pathway mediators and regulators of insulin expression and secretion in STZ-treated beta-cells

To assess the potential impact of CEE on the oxidative stress-induced alterations in signaling pathways involved in apoptosis, survival and insulin expression in beta-cells, we examined the changes in activities of protein kinase B (Akt), extracellular signal-regulated kinase (ERK) and p38 kinases, and the relative changes in nuclear levels of pancreatic duodenal homeobox (Pdx1) and musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) transcription factors that are responsible for the regulation of beta-cell proliferation and insulin expression and secretion (Guo et al., 2013). Taking into account that changes in the activities of kinases and transcription factors involved in pro-survival and insulin signaling pathways occurred at earlier time points within the analyzed 24 h period, and in order to closely monitor the potential changes, Rin-5F cells were exposed to STZ and the combination of STZ and CEE for 4 h, 6 h, 8 h, 12 h and 24 h. Western blot analysis of cell lysates revealed dynamic changes in Akt, ERK and p38 kinase activities, estimated by analysing the alterations in the relative levels of their phosphorylated forms (Fig. 6). The highest levels of p-Akt

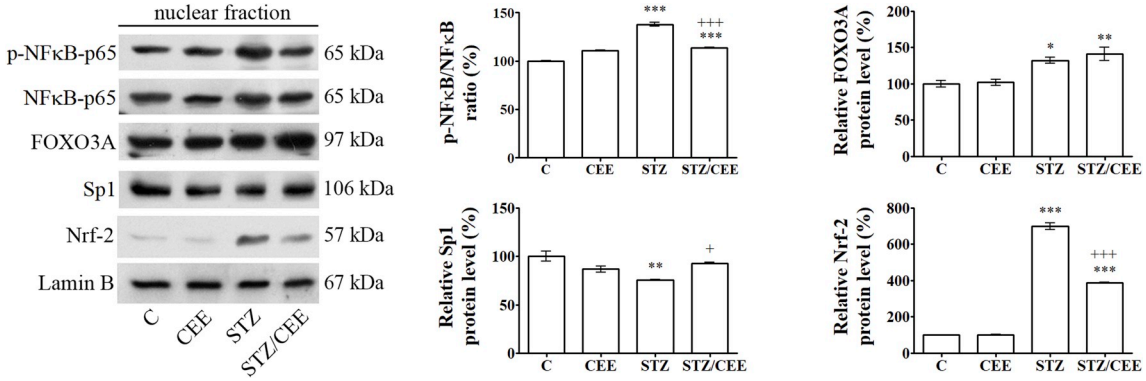

Fig. 5. The effect of *Centaurium erythraea* extract on the activity of transcriptional regulators of the antioxidant enzymes in beta-cells. Immunoblot analysis of the nuclear presence of NFκB-p65 (phosphorylated (p-) and total amount), FOXO3A, Sp1 and Nrf-2 was performed on nuclear fractions prepared from Rin-5F cells after 24 h of treatment with 12 mM streptozotocin (STZ) with/without CEE (0.25 mg/mL). Representative blots from three independent experiments are shown. Blots were quantified using TotalLab (Phoretix) electrophoresis software. The ratio of p-NFκB-p65/NFκB-p65 and nuclear levels of FOXO3A, Sp1 and Nrf-2 corrected for the expression of Lamin B are presented relative to the control. The values plotted are the means ± S.E.M. from three independent experiments. C – control cells; CEE – CE extract (0.25 mg/mL)-treated cells; STZ – cells treated with 12 mM streptozotocin (STZ); STZ/CEE – cells co-treated with STZ (12 mM) and CE extract (0.25 mg/mL). *p < 0.05, **p < 0.01, ***p < 0.001 as compared to C; +p < 0.05, $^{+++}$ p < 0.001 as compared STZ/CEE to STZ.

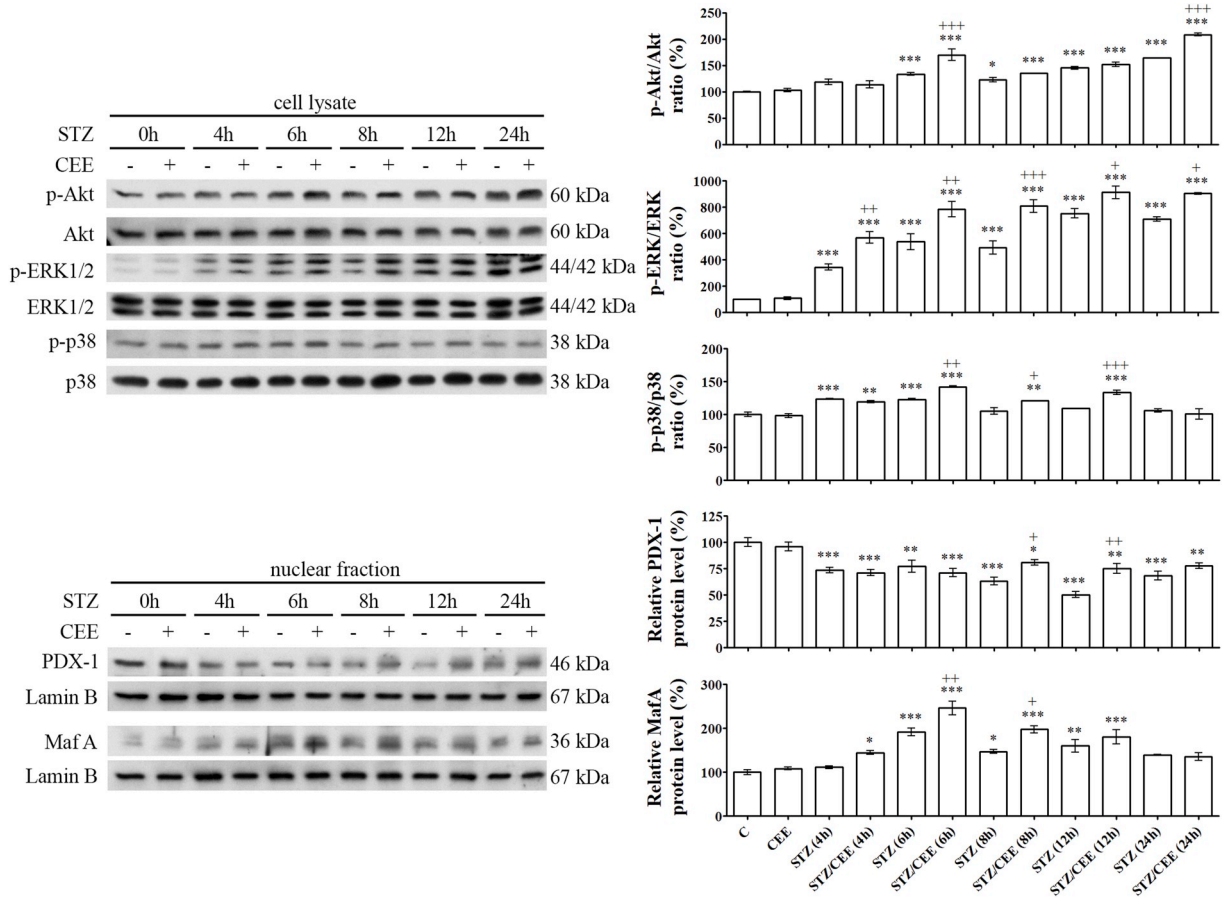

Fig. 6. The effect of *Centaurium erythraea* extract on the mediators of pro-survival pathways and insulin expression in STZ-treated beta-cells. Immunoblot analysis of cell lysates on active (p-) and inactive form of Akt, ERK1/2 and p38 kinases (left) and of nuclear fractions on PDX-1 and MafA (right) was performed after treatment of Rin-5F cells with 12 mM streptozotocin (STZ) for indicated time (0 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h) with/without CEE (0.25 mg/mL). Representative blots from three independent experiments are shown. Blots were quantified using TotalLab (Phoretix) electrophoresis software. The ratio of p-Akt/Akt, p-ERK/ERK and p-p38/p38 and nuclear levels of PDX-1 and MafA corrected for the expression of Lamin B are presented relative to the control. The values plotted are means ± S.E.M. from three independent experiments. C – control cells (STZ 0 h); CEE – CE extract (0.25 mg/mL)-treated cells (STZ 0 h); STZ (Xh) – cells treated with 12 mM streptozotocin (STZ) for indicated time; STZ/CEE (Xh) – cells co-treated with STZ (12 mM) and CE extract (0.25 mg/mL) for indicated time. *p < 0.05, **p < 0.01, ***p < 0.001 as compared to C; +p < 0.05, $^{++}$ p < 0.01, $^{+++}$ p < 0.001 as compared corresponding STZ/CEE (Xh) to STZ (Xh).

were detected 24 h after the treatments with STZ and STZ/CEE (and were 64% and 101% higher than in the control, respectively). In comparison to control cells, p-ERK1/2 displayed a substantial increase after all treatments and was consistently higher following the co-treatment with STZ and CEE than after the treatment with STZ alone. The peak in p-ERK (7-and 9-fold increases relative to the control following STZ and STZ/CEE applications, respectively) was observed at the 12 h time point. The level of p-p38 displayed a different pattern of change, as maximal activity was detected 4 h and 6 h after the STZ treatment (a 23% increase with respect to the control), whereas after the co-treatment with STZ and CEE, maximal p38 phosphorylation was observed at 6 h (a 42% increase in comparison to the control). At 24 h post STZ and STZ/CEE treatments, p-p38 was at the control level, however, p-p38 was significantly higher in STZ/CEE-than in STZ-treated cells at 6 h, 8 h and 12 h post-application. The treatment of control cells with CEE had no influence on the levels of the phosphorylated forms of the analyzed kinases.

Pdx1 and MafA act in the nucleus by providing transcriptional regulation which is directed at beta-cell functioning and survival, while their cytoplasmic sequestration serves to attenuate their nuclear action (reviewed in Fujimoto and Polonsky, 2009; Harmon et al., 2009). The nuclear presence of Pdx1 was persistently reduced in STZ- and STZ/CEE-treated cells in comparison to the control, with similar levels of Pdx1 observed at 4 h, 6 h and 24 h after both treatments (Fig. 6). Significantly higher Pdx1 levels were measured at 8 h and 12 h in STZ/CEE-than in STZ-treated cells which exhibited a greater reduction in Pdx1 levels (by 63% and 50% of the control, respectively) as compared to cells co-treated with STZ and CEE (by 19% and 25% of the control, respectively). In contrast to Pdx1, MafA was induced by the treatments with STZ and STZ/CEE, and at 24 h after both treatments MafA was at the control level. Maximal nuclear presence of MafA was detected at 6 h when it was 92% and 146% above the control level in STZ- and STZ/CEE treatments, respectively. The treatment with CEE did not affect the levels of the analyzed kinases and transcription factors, however, in STZ-induced oxidative stress, the treatment with CEE significantly affected the presence and activity of the analyzed mediators of the mechanisms involved in cell survival and insulin expression and secretion.

4. Discussion

Recovery and maintenance of a functional beta-cell mass is an ongoing challenge in the treatment of diabetes (Remedi and Emfinger, 2016). Due to its deleterious actions, oxidative stress is one of the potential therapeutic targets for preventing or slowing down beta-cell loss and dysfunction. An increasing amount of data indicates that oxidative stress can be mitigated by antioxidants contained in plant extract preparations either by free radical scavenging or by modulation of antioxidant enzyme activities (reviewed in Dinić et al., 2013). The results presented herein show that treatment with CEE improved insulin production and glycemic control in STZ-induced diabetic rats by improving the structural and functional properties of pancreatic islets. Analysis of the molecular mechanisms that underlie this protective effect provides evidence for the CEE-mediated alleviation of STZ-induced DNA damage, lipid peroxidation, protein S-glutathionylation and activities of MnSOD, CuZnSOD and CAT enzymes in STZ-treated beta-cells that are exposed to a very high level of oxidative stress. The oxidative stress-induced disturbance of the transcriptional regulation of CAT, MnSOD, CuZnSOD, GPx and GR was improved after the treatment with CEE by a readjustment of the presence and the activities of redox-sensitive transcription factors, NFκB-p65, FOXO3A, Sp1 and Nrf-2. Besides the described mechanisms, the beneficial effect of CEE on beta-cell survival and insulin expression and secretion could be partially attributed to the fine-tuned modulation of the activities of Akt, ERK and p38 kinases and of Pdx-1 and MafA regulatory factors.

The lowering of blood glucose concentration in diabetic rats by CEE is in agreement with previous reports, suggesting that the *Centaurium erythraea* extract stimulates peripheral glucose utilization, reduces glucose absorption from the gastrointestinal tract, enhances glycogen synthesis and reduces gluconeogenesis in the liver (Đorđević et al., 2017; Sefi et al., 2011; Stefkov et al., 2014). Constituents of CEE such as secoiridoids and xanthones, due to their bitter taste, could contribute to normalization of glucose concentrations by stimulating the excretion of hormones and enzymes in the gastrointestinal tract and by increasing the secretion of insulin from stimulated remnant pancreatic beta cells (Stefkov et al., 2014). Additionally, the reduction of hyperglycemia could have been caused by improved serum insulin concentration after CEE treatment of diabetic rats as a result of increased protection of pancreatic islets. This finding is in agreement with the previously reported protective effect of CE leaf extract on the number and the size of pancreatic islets in STZ-induced diabetic rats (Sefi et al., 2011). The histological and immunohistochemical examination of diabetic pancreas described in the present study revealed that the insulin-staining cell area was reduced, while glucagon- and somatostatin-positive cells predominated along the central region of the islets. The disturbance in islet morphology and islet cell contents in diabetes was reduced by the CEE treatment, thus contributing to the preservation of beta-cell mass and consequently to the increased production of insulin. This observation is further supported by the displayed protective effect of CEE on GLUT-2 and p-Akt levels in diabetic islets. Loss of GLUT-2 expression in pancreatic beta-cells is associated with impaired GSIS and hyperglycemia (Hou et al., 2009). Since the phosphatidylinositide 3-kinase (PI3K)/Akt signaling pathway plays an important role in the regulation of beta-cell mass and insulin secretion, impaired functioning of this pathway consequently leads to impaired insulin release and beta-cell dysfunction (Leibiger et al., 2010). Our findings also suggest a positive correlation between Akt activity and GLUT-2 expression in pancreatic islets, which is in agreement with recent data pointing to a link between Akt activation and GLUT-2 synthesis in diabetic liver (Rathinam and Pari, 2016). The pronounced positive effect of CEE administration on the regulation of blood glucose concentration and serum insulin and on insulin- and GLUT-2-positive staining of islets in pre-treated diabetic rats suggests that CEE consumption before diabetes onset could lessen the immediate negative effects of diabetes, which was previously discussed in the context of the protective effects of CEE against the oxidative challenge in red blood cells of diabetic rats (Đorđević et al., 2017). The observed protective effect of CEE on beta cells is in agreement with the finding that the administration of swertiamarin, which is the predominant compound in CEE, prevented and protected beta cells from STZ-induced damage (Dhanavathy, 2015). The displayed antihyperglycemic, antihyperlipidemic, cytoprotective and immune activities of swertiamarin in the same study pointed to its potential for treating diabetes and other diabetes-related complications. Quercetin, the other constituent of CEE, demonstrated protective effects on the pancreas in mice under inflamatory conditions by acting as an antiinflammatory and antioxidant agent (Carvalho et al., 2010). Several animal models revealed that consumption of flavonoids reduce blood glucose concentrations and that these substances are capable of improving, stabilizing and sustaining insulin secretion by human islets for long periods (rewieved in Cid-Ortega and Monroy-Rivera, 2018). Flavonoids from the group of flavones, such as luteolin and apigenin, which are constituents of CEE, could be also responsible for its beneficial effect on the regulation of glucose and insulin levels in diabetic rats.

CEE displayed the ability to increase the percentage of viable beta-cells in relation to STZ-induced cell death and to alleviate the STZ-induced reduction in insulin expression and secretion. Increased viability and functionality of beta-cells caused by CEE, was accompanied by a reduction of STZ-induced oxidative damage of beta-cells, estimated by the general biomarkers of oxidative stress i.e. DNA damage, lipid peroxidation and protein S-glutathionylation. This finding is in correlation with the previously reported ameliorative effect of the common centaury extract on oxidative damage of DNA, lipids and proteins in the

pancreas and red blood cells in STZ-induced diabetic rats (Đorđević et al., 2017; Sefi et al., 2011). The antioxidant activity of CEE could be due to the presence of luteolin, apigenin and quercetin, which were shown to protect DNA from H_2O_2-induced damage (Romanova et al., 2001). In addition, CEE constituents, such as swertiamarin, kaempferol, quercetin, rutin and ferulic acid, reduced lipid peroxidation in STZ-induced diabetic rats (Al-Numair et al., 2015; Elbe et al., 2015; Jaishree and Badami, 2010; Roy et al., 2013; Wang et al., 2015).

The antioxidant effect of CEE in STZ-treated beta-cells was also reflected in the activities of antioxidant enzymes; CEE application significantly lowered the levels of STZ-induced increases in CAT, MnSOD and CuZnSOD activities, suggesting the potential of CEE to lower oxidative stress via a mechanism other than further induction of antioxidant enzyme activities. The ability of CEE to alleviate the disturbance in redox balance in STZ-treated beta-cells could be explained by the potential of CEE to inhibit free radicals, thus directly reducing the need for enzyme activity. We have previously reported high H_2O_2 and NO scavenging activities of CEE (Đorđević et al., 2017) that could be attributed to its constituents luteolin, apigenin, quercetin and sinapic acid (Romanova et al., 2001; Nićiforović and Abramovič, 2014). An indirect effect of CEE on antioxidant enzyme activities could have been accomplished through its impact on the molecular events involved in transcriptional regulation of antioxidant enzymes. In line with the assumption that transcription factors are among the primary targets of oxidative stress, with effects on the expression of their target genes (Guo et al., 2013), our results indicate that STZ treatment of beta-cells was associated with changes in the presence and activities of redox-sensitive transcription factors involved in the transcriptional regulation of antioxidant enzymes. STZ treatment of beta-cells was accompanied by the increased nuclear presence of NFκB-p65 and Nrf-2 factors which are activated upon translocation to the nucleus (Karin and Ben-Neriah, 2000; van den Berg et al., 2001; Itoh et al., 2003). Activation of NFκB-p65 in STZ-treated beta-cells is probably stimulated by ROS such as H_2O_2 and/or its derivatives (Sen and Packer, 1996). A high ROS level promotes the stabilization of Nrf-2, leading to its nuclear translocation and transcriptional activation of several antioxidant genes through ARE regulatory elements (Jaiswal, 2004). Induction of NFκB and Nrf-2 was attenuated in the presence of CEE probably through its ROS-scavenging activity. Considering that NFκB regulates the expression of SOD genes (Hoffmann et al., 2006; Xu et al., 1999), while Nrf-2 controls basal and/or inducible expression of a number of endogenous antioxidants and phase 2 enzymes including CAT, SOD, GR and Gpx (Dinić et al., 2016; Kim and Vaziri, 2010; Zhu et al., 2005), the detected increases in CAT, GPx, GR and MnSOD mRNAs in STZ-treated beta-cells could have resulted from NFκB and Nrf-2 activation. By reducing the activity of NFκB and Nrf-2, the treatment of STZ-treated beta-cells with CEE reduced CAT, GPx and SOD gene expression. Considering that CEE treatment did not affect FOXO3A activity, it is likely that FOXO3A equally contributed to the transcriptional induction of its target CAT and MnSOD genes (Kops et al., 2002; Tan et al., 2008) in STZ and STZ/CEE conditions. The nuclear level of Sp1 was reduced in STZ-treated beta-cells but remained at the control level after the treatment with CEE. Sp1 is essential for the constitutive and inducible expression of CuZn/MnSOD (Minc et al., 1999; Xu et al., 2002), and it plays an essential role in the positive regulation of CAT expression (reviewed in Glorieux et al., 2015). We suggest that Sp1 exerted a greater influence on SOD and CAT transcriptional activities after the CEE treatment. CAT mRNA and protein levels were significantly reduced following the treatment with CEE, while increased gene expression of GR was accompanied by its increased protein expression in STZ- and STZ/CEE-treated cells, suggesting that CAT and GR were regulated at the transcriptional level. The detected slight but not significant differences in SOD and GPx protein levels after STZ and STZ/CEE treatments point to the involvement of additional post-translational mechanisms in the regulation of the levels of these enzymes and activities.

The CEE-mediated reduction in STZ-induced beta-cell death could in part be the result of a significant induction in the activities of Akt, ERK and p38 kinases, known to be involved in the regulation of cell growth and survival as well as insulin expression, secretion and signaling. Experiments with transgenic mice constitutively expressing Akt linked to an insulin gene promoter revealed that an increase in pancreatic beta-cell mass was at least in part due to increased cell proliferation (Bernal-Mizrachi et al., 2001; Cho et al., 2001; Tuttle et al., 2001). In addition, constitutive expression of Akt has also been associated with increased resistance of transgenic mice to STZ-induced diabetes. Subsequent investigations of (Wijesekara et al.,2010) revealed that phosphorylation/activation of both Akt and ERK1/2 increased the viability of islet and beta-cells after adiponectin treatment, by decreasing apoptosis accompanied by increased insulin gene expression and GSIS. The decrease in the phosphorylation level of Akt Ser^{473} was shown to be accompanied by attenuation of insulin signaling (Bozulic and Hemmings, 2009). In the present study, the level of p-Akt was increased after CEE application to STZ-treated beta-cells, and correlated with preservation of p-Akt and insulin presence in the islets of diabetic rats after administration of CEE, revealing the potential of CEE to improve insulin secretion and signaling in preserved beta-cells. It has been reported that multiple growth factors and hormones induce the proliferation of rodent beta-cells through the activation of ERK1/2 (reviewed in Stewart et al., 2015). Thus, hormones such as prolactin, insulin and triiodothyronine can achieve full stimulation of replication of rodent beta-cell only upon activation of the ERK1/2 and PI3K/Akt/mTOR signaling pathways (Amaral et al., 2004; Beith et al., 2008; Kim et al., 2014). However, only sustained activation of ERK promotes beta-cell proliferation since transient activation of ERK was not sufficient to induce beta-cell proliferation (Lingohr et al., 2002). ERK1/2 also exerts a positive effect on beta-cell survival through an anti-apoptotic effect under conditions of lipotoxicity (Panse et al., 2015). The results from the same study revealed that ERK1/2 activity was not crucial for GSIS, but the authors did not exclude the possibility that ERK1/2 exert indirect effects on insulin secretion. This observation is supported by studies showing that ERK1/2 mediates glucose-dependent regulation of insulin gene transcription and thus supports insulin production (Khoo et al., 2003; Lawrence et al., 2008). Based on the described findings, our results suggest that increased viability and functionality of STZ/CEE-treated beta-cells was partially the result of substantial activation of ERK1/2 by CEE. The activity of p38 appears to be cell-type specific and depends on the type of activating stressors (Hou et al., 2008). For example, inhibition of the p38 pathway suppresses production of pro-inflammatory cytokines in human islet graft and improves its functioning (Matsuda et al., 2005), which could be explained by the suppression of p38 in resident macrophages producing cytokines and in endothelial cells expressing cyclooxygenase-2 and inducible nitric oxide synthase. Investigations have also suggested that p38 is activated in oxidative stress and plays a role in cell survival and apoptosis (Mendelson et al., 1996). Hou et al. (2008) reported that induced phosphorylation of p38 displayed a suppressive effect on ROS-induced beta-cell death. Accordingly, CEE-stimulated p38 phosphorylation in our system could contribute to improved beta-cell survival. Our results strongly suggest that CEE, by stimulating the activity of Akt, ERK1-2 and p38 kinases, induces pro-survival and proliferation processes in beta-cells in oxidative stress, consequently leading to improved insulin expression/secretion/signaling and preservation of the beta-cell mass.

CEE improved the serum insulin level in diabetic rats and induced insulin gene expression and secretion in STZ-treated Rin-5F cells. This effect could be, in part, connected with CEE-mediated transient induction of MafA and compensation for the decline in Pdx1 nuclear level after STZ-induced oxidative stress. Pdx1 and MafA are islet-enriched transcription factors that play key roles in the regulation of insulin gene expression (Zhao et al., 2005). MafA is involved in the regulation of not only insulin expression but also of insulin secretion (Wang et al., 2007). The significance of Pdx1 for beta-cell functioning is supported by the findings that partial deficiency of Pdx1 increases destruction and

dysfunction of beta-cells and leads to diabetes in rodents and humans. In turn, hyperglycemia causes dysfunction of beta-cells via reduced expression of Pdx1 (Robertson, 2004). It remains to be clarified how Pdx1 regulates beta-cell survival and function, but investigations of Johnson et al. (2006) revealed that Pdx1 is a signaling target of insulin and that insulin protects islets from apoptosis through Pdx1. The potential role of Pdx1 in non-apoptotic beta-cell death should also be considered in future studies and attempts at developing novel agents aimed at preventing and treating diabetes (reviewed in Fujimoto and Polonsky, 2009). Our results are in correlation with studies showing that the nuclear level of Pdx1 was significantly reduced in HIT-T15 beta-cells cultured under conditions of chronic exposure to glucotoxicity-mediated oxidative stress, and that treatment with the antioxidant N-acetylcysteine (NAC) prevented loss of Pdx1 and its binding to the insulin promoter which at least partially prevented a decrease in insulin expression (Tanaka et al., 1999). Examination of the sensitivity of crucial islet-enriched transcription factors to oxidative stress revealed that H_2O_2 treatment of βTC-3 cells dramatically reduced the ability of nuclear Pdx1 to bind target gene promoters, while the activity of other key transcriptional regulators of beta-cells was unaffected (Guo et al., 2013). The same study revealed that the nuclear level of MafA was reduced after H_2O_2 treatment of βTC-3 cells, as well as upon development of hyperglycemia in diabetes model db/db mice (Guo et al., 2013). In HIT-T15 beta-cells cultured in glucotoxic conditions, the nuclear level of MafA was significantly reduced while antioxidant treatment with NAC preserved the level of MafA and its activity on the insulin gene promoter (Harmon et al., 2005). However, STZ treatment of Rin-5F cells provoked a transient induction of nuclear MafA which was further increased by the treatment with CEE. This discrepancy in the MafA level between our and the previous research could be ascribed to different mechanisms that are induced by different pro-oxidants and/or to the cell-specific response since different beta-cell lines were used by the authors. Timing may also be a factor, so that MafA protein stability could have been affected later by oxidative stress, while our analysis was performed in early cell passages. In agreement with this result is the observed reduction in MafA in HIT-T15 beta-cells under glucotoxic conditions that was detected in the later passage (p123-128) as compared to the early passage (p71-75) (Harmon et al., 2005), which indicates that the final effect of oxidative stress on the reduction of MafA level was probably time-dependent. Our study shows that the beneficial effect of CEE treatment on Pdx1 and MafA nuclear levels influenced the improvement in insulin gene expression and survival of beta-cells in oxidative stress; this is in accordance with previous investigations using treatments with antioxidants. Consequently, improved insulin expression and secretion could have contributed to beta-cell survival via Pdx1 activity.

Beta-cell death in islets exposed to chronic hyperglycemia is attributed to increased inflammatory cytokine production, increased oxidative stress and other factors. Recent data indicate that apoptotic cell death is not the only contributor to the reduction of beta-cell mass in diabetes and that it could also involve the loss of mature beta-cell identity (Butler et al., 2007; Cinti et al., 2016; Guo et al., 2013; Jonas et al., 1999; Rahier et al., 2008). Dedifferentiation to progenitor cells and transdifferentiation to glucagon-producing alpha-cells are important mechanisms of beta-cell failure in diabetes (reviewed in Remedi and Emfinger, 2016). Pdx1 and MafA are transcription factors that are essential for the development and maintenance of the mature beta-cell identity. Loss of Pdx1 and MafA from pancreatic islets results in loss of the beta-cells phenotype and reduction of insulin, leading to dedifferentiation of beta-cells and upregulation of 'beta-cell disallowed genes' (Ahlgren et al., 1998; Cinti et al., 2016; Guo et al., 2013; Nishimura et al., 2015). Diabetes progression is associated with failure of insulin signaling to suppress glucagon secretion and action, indicating that disrupting glucagon signaling can correct hyperglycemia in the absence of insulin secretion. It is believed that early and intensive alleviation of glucotoxicity during progression of beta-cell dysfunction in diabetes could preserve endogenous beta-cells (reviewed in Remedi and Emfinger, 2016). Our results strongly suggest that CEE or its components possess the ability to be used to ameliorate such devastating glucotoxic effects.

5. Conclusions

Deficiency of functional beta-cells in both prevalent types of diabetes requires discovery of novel therapeutic approaches to induce beta-cell expansion. Increasing knowledge of factors and signaling pathways involved in beta-cell survival and proliferation is of crucial importance in the identification of targets for such a therapeutic intervention. This is tightly associated with attempts to develop novel agents that could be used to target critical regulatory processes. The presented results suggest that CEE improved the structural and functional properties of pancreatic islets by influencing endogenous antioxidant mechanisms and by promoting proliferative and pro-survival pathways in beta-cells. This encourages efforts aimed at the elucidation of the mechanisms that underlie the antioxidant and pro-survival properties of CEE and its constituent compounds/metabolites. This opens new avenues for a potential therapeutic approach in diabetes treatment involving the expansion of beta-cell mass and improving its functioning.

Author contributions

S.D. and M.V. planned and designed the experiments. M.Đ., M.M., J.A.J. and A.U. performed *in vivo* experiments. M.Đ., N.G. and S.D. performed *in vitro* experiments. J. R., M. S. and A.T. contributed to data acquisition. D. M. and B. Š. prepared plant extract. S.D. and M.Đ. analyzed the experimental data. M.Đ., S.D. and G.P. wrote the manuscript. All authors have read and approved the final manuscript.Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grant No. 173020.

Conflicts of interest

The authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2019.112043.

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