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Trans-3,5-dicaffeoylquinic acid from *Geigeria alata* Benth. & Hook.f. ex Oliv. & Hiern with beneficial effects on experimental diabetes in animal model of essential hypertension

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ABSTRACT

Geigeria alata Benth. & Hook.f. ex Oliv. & Hiern (Asteraceae) is used in Sudanese folk medicine for treatment of diabetes. The study aimed to estimate the acute oral toxicity of *trans*-3,5-dicaffeoylquinic acid (3,5-diCQA) from *G. alata* roots and to assess its antihypeglycemic, antioxidant and antihypertensive effects on chemically-induced diabetic spontaneously hypertensive rats (SHRs). The structure of 3,5-diCQA was established by NMR and HRMS spectra. Type 2 diabetes was induced by intraperitoneal injection of streptozotocin. 3,5-diCQA was slightly toxic with LD_{50} = 2154 mg/kg. At 5 mg/kg 3,5-diCQA reduced significantly ($p < 0.05$) the blood glucose levels by 42%, decreased the blood pressure by 22% and ameliorated the oxidative stress biomarkers reduced glutathione, malondialdehyde, and serum biochemical parameters. The beneficial effect on antioxidant enzymes was evidenced by the elevated glutathione peroxidase, glutathione reductase, and glutathione S-transferase activitiy in the livers of diabetic animals. 3,5-diCQA prevents the histopathological changes related to diabetes and hypertension. 3,5-diCQA was more potent α-glucosidase inhibitor (IC_{50} 27.24 μg/mL) than acarbose (IC_{50} 99.77 μg/mL). The antihyperglycemic action of the compound was attributed to the α-glucosidase inhibition. The beneficial effects of 3,5-diCQA on streptozotocin-induced diabetic hypertensive rats support the traditional use of *G.alata* for the management of diabetes.

1. Introduction

Sudan is renowned for its rich flora and ethnomedicinal tradition (El-Ghazali et al., 1997; El-Kamali, 2009; Khalid et al., 2012) Sudanese medicinal plants have been reported to possess a wide range of biological activity, including among others, antidiabetic properties (Alamin et al., 2015). In the recent years, there are several reports on the hypoglycemic effect of *Geigeria alata* (Hafizur et al., 2012). *Geigeria alata* Benth. & Hook.f. ex Oliv. & Hiern (Asteraceae) is an annual herb distributed in north and south Africa (Khalid et al., 2012). The species is commonly known as 'Gud-gad' and the whole plant is used as a spice (El-Kamali, 2009). *G. alata* roots and leaves are used in Sudanese folk medicine for the treatment of diabetes, epilepsy, pneumonia, and rheumatism (El-Ghazali et al., 1997). In a recent study, Elbashir et al. (2018) investigated the antioxidant, α-glucosidase and lipase inhibitory activity of eighteen Sudanese medicinal plants (70% ethanol and water extracts). The study results show that *G. alata* (aerial parts) is pointed out as one of the plants that exhibited an appreciable range of activity

Abbreviations: 3,5-diCQA, dicaffeoylquinic acid; DTNB, 2,2-dinitro-5,5-dithiodibenzoic acid; EDTA, ethylenediaminetetraacetic acid; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; MDA, malondialdehyde; NADPH, beta-nicotinamide adenine dinucleotide 2-phosphate reduced tetrasodium salt; SHR, spontaneously hypertensive rats; STZ, streptozotocin; TBARS, thiobarbituric acid reactive substances

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and could be regarded as a natural antidiabetic agent with strong antioxidant and anti-hyperlipidemic effects (Elbashir et al., 2018) An *in vitro* study demonstrated the antioxidant and α-glucosidase inhibitory activity of *G. alata* methanol-aqueous root extract (Hafizur et al., 2012). Further, the antidiabetic activity was evidenced by the enhanced insulin secretion, modulation of β-cell function and improvement of antioxidant status in streptozotocin (STZ)-induced diabetic rats.

As a part of our ongoing investigation of Sudanese medicinal plants, we reported the isolation of acylquinic acids from *G. alata* roots and their antioxidant capacity (Zheleva-Dimitrova et al., 2017). A variety of acylquinic acids were identified in *G. alata* roots by liquid chromatography – high resolution mass spectrometry (LC-HRMS). Their total content reached up to 6.22%. 3,5-dicaffeoylquinic acid (3,5-diCQA) was the most abundant acid, being present at 25.96 ± 2.08 mg/g dry weight. The compound exerted a free radical scavenging potential and reducing power, assessed by *in vitro* methods (Zheleva-Dimitrova et al., 2017). Further, the antioxidant and hypoglycemic effects of 3,5-diCQA on STZ-induced diabetic normotensive Wistar rats was established (Vitcheva et al., 2018).

Despite the accumulating evidence that caffeoylquinic acids have antihyperglycemic potential in *in vitro* (Anaya-Eugenio et al., 2014; Matsui et al., 2004) and *in vivo* models (Pari et al., 2010), much of the research focus has been centred on the benefits of crude plant extracts from *Artemisia* spp. (Anaya-Eugenio et al., 2014; Boudjelal et al., 2015; Nurul Islam et al., 2013), *Cichorium inthybus* (Ferrare et al., 2018), *Gynura divaricata* (Yin et al., 2018). There is no published data reporting *in vivo* antihyperglycemic and antihypertensive activity of 3,5-diCQA on experimental diabetes in an animal model of essential hypertension. The effects of 3,5-diCQA on oxidative stress biomarkers and antioxidant enzymes have not been thoroughly investigated under the conditions of coexisting diabetes and hypertension.

The connection of experimentally induced diabetes and hypertension has been investigated in spontaneously hypertensive rats (SHRs) (Alves-Wagner et al., 2014). Chemically induced diabetes produces more profound effects in SHRs than it does in normotensive rats (Simeonova et al., 2016). The use of antioxidants belonging to the caffeoylquinic acids as supplementation to conventional antidiabetic and antihypertensive treatment could be beneficial in reducing the cardiovascular complications.

Recently, Clifford et al. (2017) reviewed the bioavailability and metabolism of acylquinic acids in humans (Clifford et al., 2017). Dicaffeoylquinic acids (diCQA) are more rapidly absorbed than monoacylquinic acids. The metabolic pathway of the caffeoylquinic acids has been proposed (Stalmach et al., 2009). Among the metabolites, the powerful antioxidants caffeic acid, dihydrocaffeic acid, dihydroferulic acids, caffeic acid-4′-sulfate, isoferulic acid, and ferulic acid were detected.

Based on all this data, it is reasonable to investigate *in vivo* the antidiabetic, antioxidant and antihypertensive potential of *trans*-3,5-dicaffeoylquinic acid, a major caffeoylquinic acid isolated from *G. alata,* using a model of streptozotocin-induced type 2 diabetes in SHRs. The antihyperglycemic effect of 3,5-diCQA was compared with the effect of the positive control acarbose.

2. Materials and methods

2.1. Plant material, extraction and isolation of 3,5-diCQA

Geigeria alata roots were collected in July 2011 from El-obeid (Latitude: 13° 09′ 7.20″ N; Longitude: 30° 13′ 34.80″ E), west Kordofan (Sudan). Botanical identification was performed by Dr. Wail El Sadig, and a voucher specimen № 41935/HNC was deposited in the herbarium of Botany Department, Faculty of Sciences, University of Khartoum, Sudan. *G. alata* was air-dried at room temperature and the powdered roots (300 g) were extracted with aqueous methanol (80%, v/v) (1:10, w/v) by ultrasound assisted extraction (2 × 15 min). The crude extract aliquots (1 g) were purified by solid-phase extraction on VacElut 10 manifold (Varian, CA, USA) using Strata cartridges C18-E, 10 g/60 mL (Phenomenex, USA), conditioned subsequently with 10 mL methanol and 10 mL water (Figs. S1A and B). After the washing step with water, the elution step was accomplished consequently with 30%, 70% and 100% methanol. Eluates obtained with 30% methanol were subjected to repeated low pressure liquid chromatography on a RP C18 column (310 mm × 25 mm, 40–63 μm, Merck, Germany) using binary elution system methanol/water and step-gradient as described earlier to yield 0.950 g 3,5-dicaffeoylquinic acid (3,5-diCQA) (96% HPLC purity) (Zheleva-Dimitrova et al., 2017).

2.2. Identification of 3,5-diCQA

The identity of 3,5-diCQA was established by ^{1}H, ^{13}C NMR, together with homonuclear (^{1}H–^{1}H) COSY, and heteronuclear (^{1}H–^{13}C) HSQC NMR (Table S1). The ^{1}H NMR together with 2D NMR (COSY and HSQC) data revealed the presence of 17 protons. The most upfield resonances of the ^{1}H NMR spectrum at δH 2.10–2.35 ppm corresponded to the two quinic methylenes, integrating for a total of 4 protons. The COSY spectrum showed 2 methylene protons at H-2 and H-6 in the quinic moiety (m, 2.10–2.35 ppm, 4H) coupled to the H-3 and H-5 (m, 5.36–5.44 ppm, 2H), respectively. In the cynnamoyl moieties, the H-8' (d, 6.26 ppm, 1H) and H-8" (d, 6.35 ppm, 1H) signals were shown as coupled to the H-7′ and H-7″ protons (dd, 7.59 ppm, 2H). The coupling constant value (J = 15.9 Hz) at the cinnamoyl double bonds indicated a *trans*-configuration. The ^{13}C NMR data and one-bond ^{1}H–^{13}C correlations in the HSQC experiment revealed a total of 25 carbon atoms. Therefore, the compound was suggested to be *trans*-3,5-dicaffeoyl quinic acid. The NMR spectra were consistent with reported literature data (Wald et al., 1989) (Vermerris and Nicholson, 2008).

The structure was confirmed by ultra high-performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS). In negative ion mode the deprotonated molecule $[M-H]^-$ was observed at m/z 515.1213 (Fig. S1C). The MS/MS spectrum of 3,5-diCQA gave the fragment ions as follows: m/z 191.0552 (100% relative abundance) $[M-H-2\times162]^-$, 179.0340 (70.83%) $[M-H-(162+174)]^-$, 135.0438 (12.35%) $[M-H-(162+174+CO_2)]^-$, 353.0879 (14.03%) $[M-H-162]^-$, 173.0445 (4.69%) $[M-H-(2\times162+H_2O)]^-$, 161.0232 (3.91%) [caffeoyl-H]$^-$, 155.0339 (1.56%) $[M-H-(2\times162+2H_2O)]^-$, 335.0767 (0.48%) $[M-H-(162+H_2O)]^-$ (Zheleva-Dimitrova et al., 2017).

2.3. Animals

Experiments were performed in male SHRs, inbred strain Okamoto-Aoki (initial body weight 200–250 g), obtained from Charles River Laboratories (Sulzfeld, Germany). The SHR strain was created during the 1960s by Okamoto and co-workers, who started breeding Wistar-Kyoto rats with high blood pressure. Hypertension develops around 5–6 weeks of age, and reaches systolic blood pressures between 180 and 220 mmHg in the adult animals (Okamoto and Aoki, 1963). The SHR strain has been widely used as a model of human essential hypertension and metabolic syndrome as well as a number of other pathophysiological phenotypes including cardiac hypertrophy, insulin resistance and defects in lipid metabolism (Diaz et al., 2016). The animals were housed in Plexiglas cages (2 per cage) at 20 ± 2 °C and 12/12 h light/dark cycle. During the procedure, rats were given *ad libitum* access to food and water. All studies were carried out in accordance with the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) and proved by the Bulgarian Agency of Food Safety (permission № 185).

2.4. Chemicals

Streptozotocin, acarbose, bovine serum albumin (fraction V),

beta–Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), and cumene hydroperoxide were purchased from Sigma Chemical Co. (Taufkirchen, Germany). 2,2-Dinitro-5,5 dithiodibenzoic acid (DTNB) and methanol were obtained from Merck (Darmstadt, Germany). α-glucosidase was provided by Sigma (Aldrich, USA). All reagents were of analytical grade.

2.5. In vitro evaluation of α-glucosidase inhibitory activity

3,5-diCQA aqueous solution (200.0, 100.0, 50.0, 25.0, 12.5 μg/mL) (50 μL) was mixed with α-glucosidase solution (from *Saccharomyces cerevisiae* type I, 19.6 units/mg solid, 28 units/mg protein, Sigma) (50 μL) in phosphate buffer (0.1 M, pH 6.8) and PNPG (4-N-trophenyl-α-D-glucopyranoside (50 μL, 0.005 M) in 96-well microplate and incubated for 15 min at 37 °C (Zengin et al., 2014). A blank solution was prepared without enzyme, while a negative control – without 3,5-diCQA. The reaction was stopped with the addition of sodium carbonate (50 μL, 0.2 M). The absorbance of the reaction solutions was measured at 400 nm on Microplate Reader EZ Read 800. Acarbose (in the same range as 3,5-diCQA) was used as a positive control. The percentage inhibition of α-glucosidase activity was calculated via the following equation:

$$I\ (\%) = (A_{neg.\ control} - A_{blank}) - (A_{sample} - A_{sample\ blank}) \times 100/(A_{neg.\ control} - A_{blank})$$

The concentration of the tested samples giving 50% inhibition of the enzyme activity (IC_{50}) was estimated from the plots of the concentration *vs* the inhibitory activity, all samples were assayed in triplicate.

2.6. Blood pressure and body weight measurement

Blood pressure and body weight (b.w.) measurements were performed as previously described (Simeonova et al., 2016). The blood pressure was measured in conscious animals using an automated tail-cuff device (CODA non-invasive blood pressure system, Kent Scientific Corporation, USA). SHRs with highest blood pressure values were taken for the *in vivo* experiment. The body weight is measured by a simple laboratory scale.

2.7. Induction of diabetes

Type 2 diabetes was induced by the method of Pari et al. (2010). Rats were injected intraperitoneally (i.p.) with nicotinamide (NA) (110 mg/kg b.w.) and after 15 min followed by i.p. injection of STZ (45 mg/kg b.w.), dissolved in 0,1 M citrate buffer, pH 4.4. Rats with blood glucose levels of 9 mmol/L or higher were considered to be diabetic and were included in the experiment.

2.8. Design of the experiment

The design of the experiment is depicted on Table 1. Twenty male SHRs were randomly divided into five groups of four animals (n = 4) as follow:

Group 1: control SHR animals, orally treated with the physiological saline at 5 mL/kg b.w. per day, for 28 days.
Group 2: SHRs, orally exposed to 3,5-diCQA alone, dissolved by sonication in a physiological saline buffer (5 mg/kg/day, for 28 days) (Pari et al., 2010).
Group 3: SHRs, treated orally with the physiological saline, on the 7th day rats were challenged with 45 mg/kg, i.p. STZ dissolved in citrate buffer 0.1M, pH 4.4, 15 min after the i.p. administration of NA (110 mg/kg bw).
Group 4: SHRs were treated orally with a saline vehicle (5 mL/kg b.w./day, for 7 days). On the 7th day, the animals were challenged with NA-STZ (110/45 mg/kg bw, i.p.) and subsequently treated orally with acarbose (5 mg/kg b.w./day) for 3 weeks (Kurt, 2012).
Group 5: SHRs treated with 3,5-diCQA (5 mg/kg, oral-gavage) for 7 days. On day 7th the animals were challenged with NA-STZ (110/45 mg/kg bw, i.p.) and subsequently treated with 3,5-diCQA for another 3 weeks.

On the 28th day, the animals were placed individually for 24 h in metabolic cages "Ugo Basile - Italy" for assessment of urine parameters. The next day the animals from all groups were sacrificed, blood was collected and biochemical parameters in serum were measured. Afterwards, the livers were taken to assess the oxidative stress biomarkers – MDA, GSH, and the activity of the antioxidant enzymes GR, GPx and GST. For all following experiments, the excised livers were perfused with cold saline solution (0.9% NaCl), blotted dry, weighed, and homogenized with appropriate buffers. Small pieces from livers, kidneys and pancreatic tissues from all rats were preserved in 10% formalin for histopathological assessment.

2.9. Blood biochemical assays and oxidative stress markers

AST, ALT, urea, creatinine, cholesterol and triglycerides were assessed using commercially available standard diagnostic kits (Mindray, Shenzhen, China) with an automatic biochemistry analyzer (BS-120, Mindray, Shenzhen, China).

Oxidative damage was determined by measuring the quantity of thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde (MDA) equivalents as described by Polizio and Peña (2005). Reduced glutathione (GSH) was assessed by measuring the non-protein sulfhydryls after precipitation of proteins with trichloracetic acid (TCA), using the method, described by Bump (Bump et al., 1983). The antioxidant enzymes activities were measured in the supernatant of 10% homogenates, prepared in 0.05M phosphate buffer (pH 7.4). Glutathione peroxidase (GPx) was measured by NADPH oxidation, using a coupled reaction system consisting of GSH, glutathione reductase (GR), and cumene hydroperoxide (Tappel, 1978). Glutathione reductase activity (GR) was measured spectrophotometrically at 340 nm according to the method of Pinto (Pinto et al., 1984) by following NADPH oxidation. GST was measured by the method of Habig (Habig et al., 1974) using CDNB as substrate.

Table 1
Experimental design.

Groups	Week 1	7th day	Week 2	Week 3	Week 4
Control	NaCl 0.9% 5 mL/kg p.o.	NaCl 0.9% 5 mL/kg p.o.	NaCl 0.9% 5 mL/kg p.o.	NaCl 0.9% 5 mL/kg p.o.	NaCl 0.9% 5 mL/kg p.o.
DCQA	DCQA 5mg/kg/day, p.o.	DCQA 5mg/kg/day, p.o.	DCQA 5mg/kg/day, p.o.	DCQA5mg/kg/day, p.o.	DCQA 5mg/kg/day, p.o.
DM	NaCl 0.9% 5 mL/kg p.o.	NA 110 mg/kg i.p.+ STZ 45 mg/kg i.p	NaCl 0.9% 5 mL/kg p.o.	NaCl 0.9% 5 mL/kg p.o.	NaCl 0.9% 5 mL/kg p.o.
DM + Acb	NaCl 0.9% 5 mL/kg p.o.	NA 110 mg/kg i.p.+ STZ 45 mg/kg i.p	Acarbose po 5 mg/kg/day	Acarbose po 5 mg/kg/day	Acarbose po 5 mg/kg/day
DM + DCQA	DCQA 5mg/kg/day, p.o.	NA 110 mg/kg i.p.+ STZ 45 mg/kg i.p	DCQA p.o. 5 mg/kg/day	DCQA p.o. 5 mg/kg/day	DCQA p.o. 5 mg/kg/day

2.10. Urine analysis

The urine volume, collected for 24 h, protein and glucose presence in the urine, pH, and ketones were assessed using urine reagent strips for urinalysis "Condor-Teco Medical Technology - China".

2.11. Acute oral toxicity of 3,5-diCQA in SHR

The acute oral toxicity test of 3,5-diCQA was performed using the method of Lorke (1983). 3,5-diCQA was dissolved in physiological saline (0.9% NaCl) by sonification and administered orally (p.o.) at different doses to 18 male SHR. Firstly, three groups of SHRs, 3 animals per group, were treated with 10, 100 and 1000 mg/kg 3,5-diCQA (first phase). The survived animals were observed for symptoms of toxicity and death up to 24 h. Secondly, higher doses (1600, 2900 and 5000 mg/kg) of 3,5-diCQA were administered orally to another 9 animals in the same manner, and they were observed until the next day (second phase).

The LD_{50} was calculated using the following equation:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

where D_0 is the highest dose that gave no mortality and D_{100} is the lowest dose that produced mortality.

2.12. Histopathological examination

Histopathological examination was performed using the method of Bancroft and Gamble (2002) (Bancroft and Gamble, 2002).The sections were observed under high power microscope and photomicrographs were taken using "Olympus" CX31 and Camera "Olympus x Optical zoom" with objective "PlanaC" 4/0.10 (Karl Zeiss, Germany).

2.13. Statistical analysis

Statistical analysis was performed by MEDCALC program. Results are expressed as mean ± SEM on four rats in each group. Experimental groups were compared by the Kruskal–Wallis variance analysis test, and a post-hoc analysis using Mann–Whitney *U* test was performed. Statistically significant were considered values $p \leq 0.05$.

3. Results

3.1. In vitro α-glucosidase inhibitory activity and in vivo changes in blood glucose level

In the α-glucosidase inhibitory assay, 3,5-diCQA exerted higher activity when compared to the well-known α-glucosidase inhibitor acarbose. IC_{50} of 3,5-diCQA was 27.24 μg/mL, while for acarbose 99.77 μg/mL was measured.

At the end of the experiment, glucose concentration was significantly elevated in the STZ-induced diabetic SHRs (by 103%, $p < 0.05$), compared to their matched controls (Fig. 1A). In the diabetic animals, the treatment with 3,5-diCQA or acarbose resulted in significant ($p < 0.05$) reduction in the blood glucose levels by 44% and 42% ($p < 0.05$) respectively, compared to diabetic SHR at 29th day.

3.2. Changes in systolic blood pressure (SBP)

The SBP of the SHRs treated with STZ was gradually increased throughout the experiment and at the end of the experiment it was higher by 14% ($p < 0.05$) compared to the control animals. The treatment of both non-diabetic and diabetic SHRs with 3,5-diCQA decreased the blood pressure in a statistically significant manner ($p < 0.05$) by 22% and 17%, respectively at 29th day. (Fig. 1B). The acarbose also slightly decreased the SBP with 10% in comparison with STZ-treated rats.

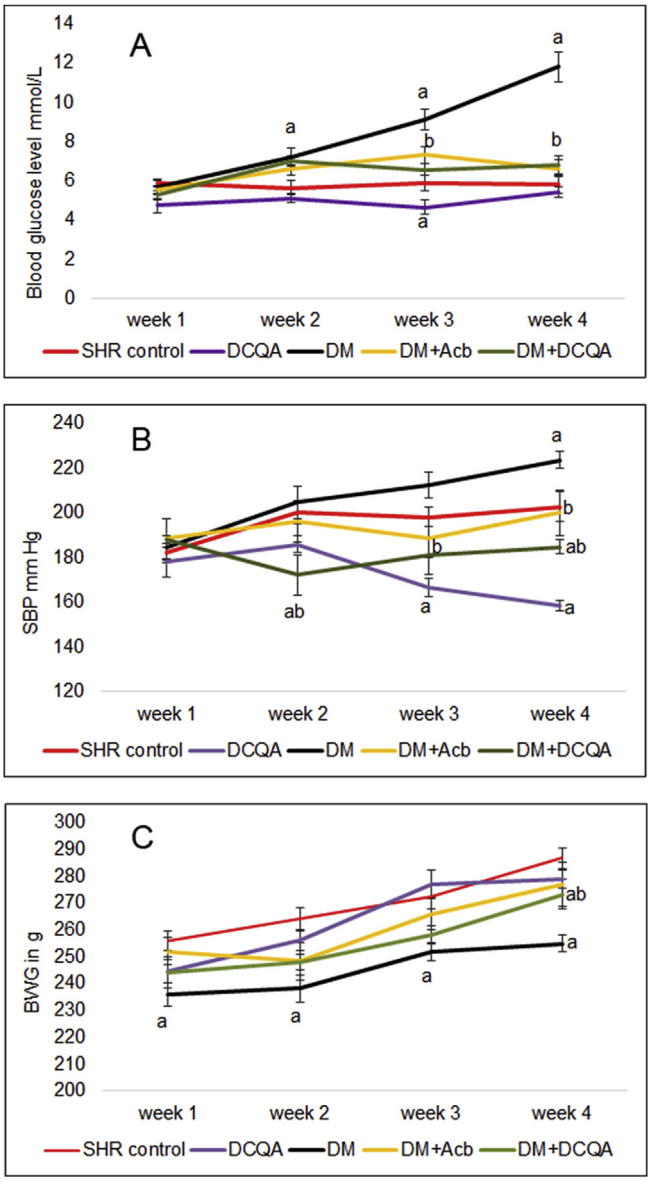

Fig. 1. Weekly dynamic changes in systolic blood glucose levels (A), systolic blood pressure (B) and body weight gain (C). Treatment: as described in the experimental design section. Data are expressed as mean ± SEM of four rats ($n = 4$). For comparison between groups, Mann—Whitney *U* test was performed. [a]$p < 0.05$ vs SHR control group; [b]$p < 0.05$ vs diabetic SHR group.

3.3. Changes in body weight gain (BWG)

When compared to the control animals, the final body weight gain of the diabetic SHRs was significantly lower by 33% ($p < 0.05$), while the animals treated with 3,5-diCQA alone, showed an increase in the final body weight gain by 17% (Fig. 1C). The diabetic rats, treated with 3,5-diCQA or acarbose, showed a significant increase in the final body weight gain. The changes were more pronounced in the 3,5-diCQA treated diabetic group where the body weight gain at 29th day was 50% higher, when compared to the respective control group. Acarbose also increased the final body weight gain in diabetic rats, but the elevation was less than in the 3,5-diCQA treated group.

Table 2
Changes in blood biochemical parameters.

Parameters	Controls	3,5-diCQA	DM	DM + acarbose	DM + 3,5-diCQA
Cholesterol mmol/L	1.59 ± 0.24	1.61 ± 0.26	3.51 ± 0.8[a]	1.87 ± 0.61[b]	1.89 ± 0,34[b]
Triglicerides mmol/L	0.57 ± 0.13	0.64 ± 0.22	1.24 ± 0.2[a]	0.62 ± 0.11[b]	0.55 ± 0.16[b]
ASAT U/L	92.2 ± 8.0	82 ± 9.21	267 ± 16.2[a]	293.2 ± 14.8[a]	203 ± 19[abc]
ALAT U/L	36.6 ± 4.2	42.2 ± 4.7	68.8 ± 8.2[a]	72.3 ± 5.8[a]	62.2 ± 7.2[a]
Urea mmol/L	6.94 ± 2.24	9.45 ± 0.26	14.7 ± 1.5[a]	12.4 ± 3.2[a]	13.8 ± 1.8[a]
Creatinine μmol/L	53.7 ± 2.6	52.2 ± 0.36	66.6 ± 3.9[a]	50.6 ± 2.8[b]	51.9 ± 1.09[b]
Total protein g/L	74.4 ± 2.1	72.6 ± 1.45	61.3 ± 1.0[a]	66.8 ± 2.1[ab]	66 ± 1.06[a]

Treatment: as described in the experimental design section.
Data are expressed as mean ± SEM of four rats ($n = 4$). For comparison between groups, Mann—Whitney U test was performed.
[a]$p < 0.05$ vs SHR control group; [b]$p < 0.05$ vs diabetic SHR group; [c]$p < 0.05$ vs diabetic SHR + acarbose.

3.4. Serum biochemical parameters

STZ-induced diabetes provoked significant changes in all investigated parameters (Table 2). Cholesterol and triglycerides raised by 120% and 118% respectively ($p < 0.05$). AST and ALT activity increased by 190% and 88% respectively ($p < 0.05$). Urea and creatinine levels were augmented in diabetic SHRs by 112% and 24%, respectively ($p < 0.05$) and total protein was decreased by 18%. 3,5-diCQA treatment of diabetic rats ameliorated most of the above-mentioned serum biochemical parameters. Cholesterol and triglycerides concentrations declined by 48% and 56%, respectively. AST activity and creatinine levels diminished in a statistically significant manner by approximately 22%, compared to the diabetic SHRs. Similar changes were observed in diabetic rats treated with the α-glucosidase inhibitor. Acarbose reduced significantly the cholesterol, triglycerides and creatinine concentrations by 47%, 50%, and 24% respectively, but it did not influence the higher ALT and AST activity measured in diabetic SHRs.

3.5. Urine analysis

Diabetes led to the excretion of extremely large amounts of ketone bodies (1100%), proteinuria, glycosuria, and polyuria (Table 3). It should be noted that administration of 3,5-diCQA was also accompanied by significant increase of the urine volume (up to 41%) when compared to control rats and up to 24%, when compared to diabetic group. The experimental diabetes led to significant acidification of the urine.

3.6. Oxidative stress markers in the liver

STZ-induced diabetes resulted in liver oxidative stress, discerned by markedly increased MDA formation by 170% ($p < 0.05$) and GSH depletion by 45% ($p < 0.05$) (Table 4). However, the beneficial effect of 3,5-diCQA treatment in diabetic animals was evidenced by significant increase of GSH levels by 84% ($p < 0.05$), while MDA production was decreased by 56% ($p < 0.05$) compared to the diabetic SHRs. The similar changes were observed in acarbose-treated group, were the MDA concentrations diminished by 42% ($p < 0.05$) and GSH increased by 80% ($p < 0.05$) in comparison with the diabetic animals. The treatment of non-diabetic animals with 3,5-diCQA alone did not change the investigated parameters.

3.7. Changes in antioxidant enzymes

The activity of antioxidant enzymes in the diabetic SHRs were significantly ($p < 0.05$) reduced, compared to their non-diabetic controls. GPx activity was decreased by 46% ($p < 0.05$). GR was reduced by 31% ($p < 0.05$), and GST activity declined by 22% ($p < 0.05$) (Table 4). 3,5-diCQA, administered alone, did not affect the enzymes activity. However, 3,5-diCQA treatment of diabetic rats, exerted an antioxidant potential, discerned by normalizing the antioxidant enzymes activity to the control levels. GPx, GR and GST activities were increased ($p < 0.05$) by 43%, 22% and 17%, respectively. Acarbose, after three weeks of administration, increased GPx activity by 28%, compared to STZ group, but GR and GST were not affected by the α-glucosidase inhibitor.

3.8. Acute oral toxicity of 3,5-diCQA

The results show that all animals survived the first phase of the experiment without apparent symptoms of toxicity (Table 5). According to Hodge and Sterner scale (Derelanko and Hollinger, 2002) the investigated compound could be classified as slightly toxic when administered orally to SHRs ($LD_{50} = 2154$ mg/kg).

3.9. Histopathological examination

Histopathological alterations were not observed in the livers from the control, 3,5-diCQA treated SHRs, and 3,5-diCQA treated diabetic rats (Fig. 2A, B, and 2E). Livers from diabetic animals revealed changes manifested by sinusoidal dilatation and congestion with portal inflammation, and small droplets focal steatosis (toxic type, due to STZ and hypertension) (Fig. 2C). Livers from acarbose treated diabetic rats had less pronounced small focal hepatosteatosis (Fig. 2D).

Due to the hypertension, some tubules alterations in the control kidneys were visible (Fig. 2A). 3,5-diCQA protected the glomeruli and tubules by the hypertension influence (Fig. 2B). The kidney sections from diabetic rats showed dilated tubules with cloudy swelling, diabetic

Table 3
Changes in urine parameters.

Parameters	Controls	3,5-diCQA	DM	DM + acarbose	DM + 3,5-diCQA
Ketone mmol/L	1.0 ± 0.58	0.75 ± 0.5	12.0 ± 4.62[a]	2.75 ± 1.44[b]	2.13 ± 1.25[b]
Protein g/L	0.00 ± 0.00	0.00 ± 0.00	15.0 ± 5.77[a]	2.50 ± 1.00[b]	2.0 ± 1.15[b]
Glucose mmol/L	0.00 ± 0.00	0.00 ± 0.00	26.25 ± 7.5[a]	10.0 ± 5.77[b]	7.5 ± 5.0[b]
pH	7 ± 0.41	7.13 ± 0.25	4.63 ± 0.25[a]	6.38 ± 0.63[b]	6.75 ± 0.29[b]
Volume ml/24h	32.0 ± 4.32	45.0 ± 5.29[a]	68.50 ± 8.70[a]	59.0 ± 4.16[b]	85.0 ± 4.16[b]

Treatment: as described in the experimental design section.
Data are expressed as mean ± SEM of four rats ($n = 4$). For comparison between groups, Mann—Whitney U test was performed.
[a]$p < 0.05$ vs SHR control group; [b]$p < 0.05$ vs diabetic SHR group.

Table 4
Effects of 3, 5-diCQA on the markers of oxidative stress and the activity of antioxidant enzymes in the liver.

Parameter	Controls	3,5-diCQA	DM	DM + acarbose	DM + 3,5-diCQA
MDA[a]	3.78 ± 0.17	3.5 ± 0.36	10.2 ± 0.6[a]	5.91 ± 0.42[ab]	4.5 ± 0.46[abc]
GSH[a]	5.60 ± 0.52	5.27 ± 0.43	3.07 ± 0.3[a]	5.54 ± 0.31[b]	5.66 ± 0.33[b]
GPx[b]	0.267 ± 0.056	0.267 ± 0.02	0.143 ± 0.014[a]	0.183 ± 0.019[b]	0.205 ± 0.054[b]
GR[b]	0.548 ± 0.045	0.524 ± 0.056	0.378 ± 0.037[a]	0.385 ± 0.018[a]	0.460 ± 0.015[abc]
GST[b]	1.51 ± 0.11	1.25 ± 0.07[a]	1.18 ± 0.06[a]	1.36 ± 0.17	1.38 ± 0.09[b]

Treatment: as described in the experimental design section.
Data are expressed as mean ± SEM of four rats ($n = 4$). For comparison between groups, Mann—Whitney U test was performed.
[a]$p < 0.05$ vs SHR control group; [b]$p < 0.05$ vs diabetic SHR group; [c]$p < 0.05$ vs diabetic SHR + acarbose.
[a] nmol/g.
[b] μmol/min/mg protein.

Table 5
Acute oral toxicity of 3,5-diCQA in male SHRs.

Compound	1st phase		2nd phase	
	Doses mg/kg p.o.	Mortality	Doses mg/kg p.o.	Mortality
3,5-diCQA	10	0/3	1600	0/3
	100	0/3	2900	1/3
	1000	0/3	5000	3/3

intracapilar glomerulosclerosis and hyalinosis of afferent arteries (a sign of arterial hypertension (Fig. 2C). The kidneys from acarbose and 3,5-diCQA treated diabetic SHRs (Fig. 2D–E) showed normal glomeruli with normal tubules.

The Haematoxylin-eosin stained structure of pancreas section was morphologically regular in the control SHRs. The complete islets were uniformly arranged with substantial pancreatic β-cells (Fig. 2A). In contrast, in the diabetic SHRs, the pancreatic tissue atrophy, accompanied by the decreased pancreatic β-cell counts, replaced by a fatty tissue was observed (Fig. 2C). Recovered pancreatic structure in the acarbose treated animals was seen but still with dissolved and deformed cells (Fig. 2D). Following the 3,5-diCQA administration, the islets morbid structure was effectively reversed with an increased amount of pancreatic β-cells (Fig. 2E).

4. Discussion

In the current study, the antihyperglycemic, antioxidant and antihypertensive potential of 3,5-diCQA acid, the major acylquinic acid in *G. alata*, was evaluated using a model of streptozotocin-induced type 2 diabetes in SHRs.

Diabetes and hypertension frequently occur together, which makes their effects more detrimental than those with either condition alone (Kaur et al., 2002). There is a substantial overlap between diabetes and hypertension in the etiology and disease mechanisms, as free radical formation and lipid peroxidation play an important role in both disorders (Cheung and Li, 2012). The common mechanism of both pathologies is associated with the over-activity of the Renin-Angiotensin-Aldosterone System (RAS) leading to increased production of lipid peroxides that augment blood pressure and insulin resistance (Henriksen, 2019).

In our experimental model of diabetes and hypertension, the sustained hyperglycemia was discerned by a significant increase in the serum blood glucose levels (Fig. 1A). However, the induced hyperglycemia was accompanied by reduced body weight, increased MDA production and decreased non-enzymatic (GSH) and enzymatic (GPx, GR, GST) antioxidant defence in the liver (Fig. 1C, Table 4). Regarding this model of STZ action, natural agents with antioxidant potential could also exert antihyperglycemic activities (Anaya-Eugenio et al., 2014). From this *in vivo* study it was found that 3,5-diCQA from *G. alata* exerted antihyperglycemic, antioxidant and antihypertensive activity in experimental diabetes in spontaneously hypertensive animals. Indeed, *G. alata* root extract (250 mg/kg b.w. administered 14 days) displayed an antihyperglycemic effect in streptozotocin-induced diabetic rats (Hafizur et al., 2012). The authors explained this effect with the α-glucosidase inhibitory potential of the extract and/or the enhancement of the insulin secretion from the β-cells. In line with the previously published data on the *G. alata* crude extract, the substantial decrease in the blood glucose levels was observed after the administration of 3,5-diCQA (Fig. 1A). Caffeoylquinic acids, widely spread in Asteraceae family, have been shown to exhibit *in vitro* inhibitory activity against α-glucosidase (Chen et al., 2014; Nurul Islam et al., 2013). The literature survey showed that caffeoylquinic acids are responsible for the antihyperglycemic activity of *Cichorium intybus* formulation (Ferrare et al., 2018), *Artemisia ludoviciana* infusion in NA-STZ-induced diabetes animal model (Anaya-Eugenio et al., 2014), and *Artemisia herba-alba* in alloxan-induced diabetic rats (Boudjelal et al., 2015). Evidence came from both decreased blood glucose levels and the lowering trend in oral glucose tolerance tests. Yin et al. (2018) highlighted the effects of *Gynura divaricata* methanol-aqueous extract, rich in 3,5-/4,5-dicaffeoylquinic acid, on the expression of key proteins involved in the regulation of pancreatic function and cell apoptosis in STZ-induced diabetes in mice (Yin et al., 2018). The authors suggested that caffeoylquinic acids could alleviate the insulin resistance and enhance insulin sensitivity.

The structure-activity relationship study suggested that the number and binding position of caffeoyl moieties are important for both α-glucosidase and protein tyrosine phosphatase 1B (PTP1B) inhibitory potential (Chen et al., 2014). Thus, dicaffeoylquinic acids exerted a much higher α-glucosidase inhibitory activity when compared to monocaffeoylqunic acids (Chen et al., 2014; Nurul Islam et al., 2013). Concerning PTP1B, which is a negative regulator of the insulin signaling pathway (Xu et al., 2016). 3,5-diCQA has been reported to show a strong inhibitory effect (Chen et al., 2014). For this reason, the α-glucosidase and α-amylase inhibitor acarbose was used as a positive control in the study. Our results showed that 3,5-diCQA was a more potent α-glucosidase inhibitor than acarbose.

The observed dyslipidemia (Table 2), could result in an increased production of pro-oxidant molecules leading to the impairment of both, insulin action and glucose homeostasis (Henriksen, 2019). As highlighted in a recent review article, 5-caffeoylquinic acid (chlorogenic acid), the most common monocaffeoylquinic acid, plays an important role in regulating both glucose and lipid metabolism (Meng et al., 2013). It has been reported that chlorogenic acid reduces the LDL levels and LDL-oxidation *in vitro*, and therefore can reduce the risk of cardiovascular disease (Meng et al., 2013). In line with the reports of Heidarian and Soofiniya (2011) on the hypolipidemic effect of *Cynara scolimus*, well known for dicaffeoylquinic acids content, our results indicated that 3,5-diCQA attenuated the cholesterol and triglycerides levels in diabetic SHRs (Table 2).

Free radical-generated β-cell destruction is the key factor underlying the diabetogenic effect of STZ (Szkudelski, 2001). The pancreatic

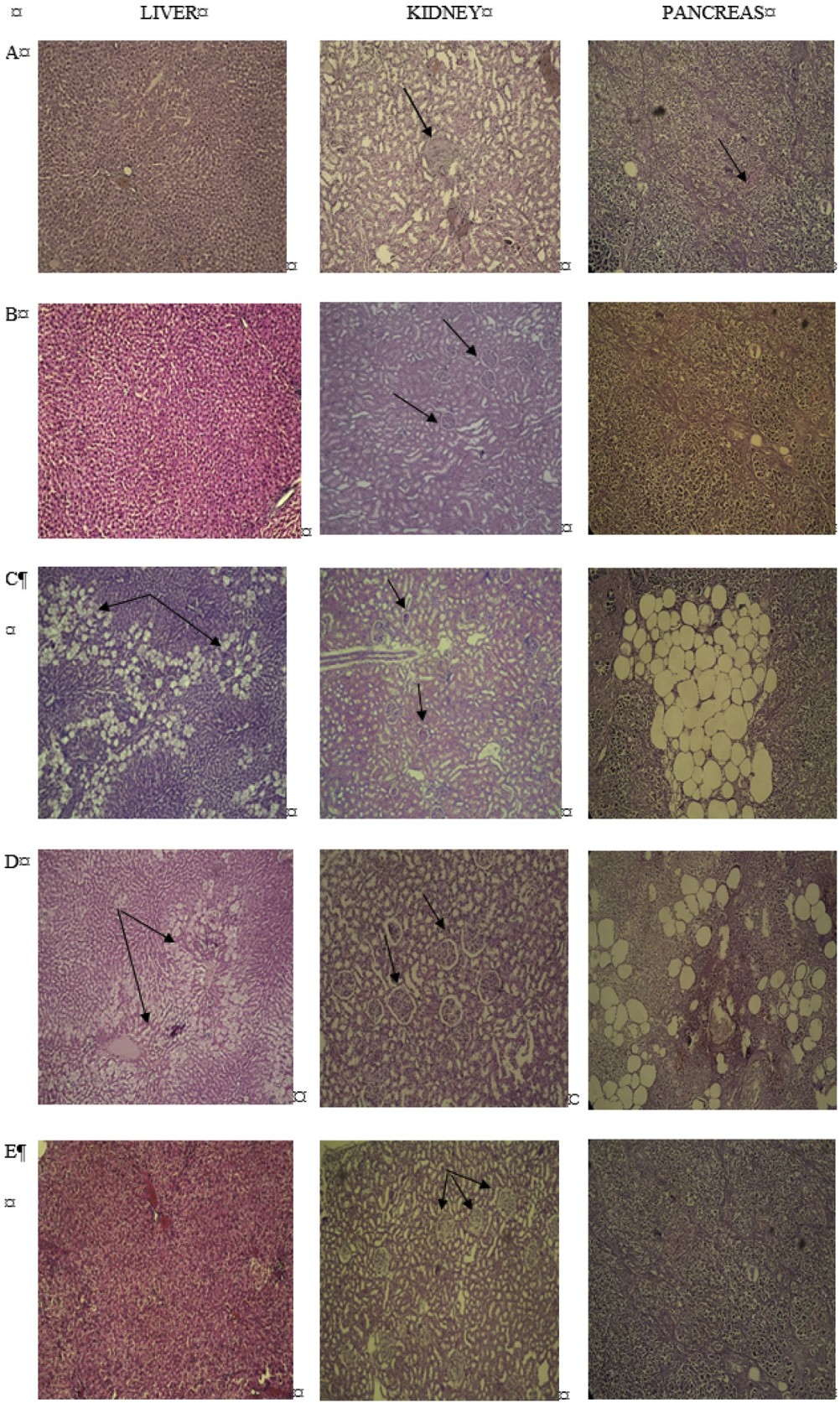

Fig. 2. Histopathological evaluation of liver, kidney and pancreas in control and diabetic SHRs. A – SHR control animals: normal liver structure; hypertensive kidney – renal tubules changed by the hypertension; normal pancreas. B – SHRs treated with 3,5-diCQA: liver and pancreas without changes; kidney - normal glomeruli and tubules. C – SHRs challenged with NA-STZ: liver showed sinusoidal dilatation and congestion with portal inflammation, small droplets focal steatosis (toxic type, due to STZ and hypertension); kidney - dilated tubules with cloudy swelling, diabetic intracapilar glomerulosclerosis and hyalinosis of afferent arteries (sign of arterial hypertension); pancreas - dissolved and deformed cells, necrosis and apoptosis, and was accompanied by the decreased pancreatic beta cell counts. D – diabetic SHRs, treated with acarbose (5 mg/kg, oral-gavage) for 21 days after induction of diabetes. Liver with less pronounced small focal hepatosteatosis; kidney with normal glomeruli and normal tubules; pancreas – recovered pancreatic structure but still with dissolved and deformed cells. E – diabetic SHRs, treated with 3,5-diCQA for 7 days. On day 7th the animals were challenged with NA-STZ and after that continued to be treated with 3,5-diCQA for another 3 weeks: liver and kidney with almost normal structure; pancreas – recovered pancreatic structure with an increased amount of pancreatic beta cells.

β-cells possess both low transcription rate and enzymatic antioxidants levels which may lead to disruption of cellular function by induction of lipid peroxidation and oxidative membrane damage (de Souza et al., 2016).

In the present study, 3,5-diCQA had a discernible impact on antioxidant enzyme activity and oxidative stress markers (Table 4). Hyperglycemia enhances oxidative stress and caused an increase in MDA concentration, as a noxious biomarker for lipid peroxidation in diabetic

rats through activation of NADPH oxidase (Honari et al., 2018). We demonstrated that 3,5-diCQA prevented the STZ-induced MDA elevation and GSH depletion in the liver. We also showed the reduced activity of antioxidant enzymes in diabetic SHRs. It has been shown that caffeoylquinic acids have radical-scavenging activity (Heidarian and Soofiniya, 2011) which improves the antioxidant capacity of STZ-treated rats. Chen et al. (2014) deduced that caffeoylquinic acid's antioxidant potential is related to the pancreatic β-cells protection or stimulation of undamaged cells to secrete more insulin.

Moreover, considering that the oxidative stress plays a pivotal role in hypertension, the bioantioxidants are also suggested to have a hypotensive potential and could exert a beneficial effect in this co-morbidity. 3,5-diCQA decreased the systolic blood pressure in both control and diabetic SHRs, explained by the higher diuresis it produced (Table 3). The diuretic effect of chlorogenic acids is due to the increased excretion of sodium (Angappan et al., 2018). It is well known that caffeoylquinic acids are metabolized to caffeic acid and quinic acid (Gonthier et al., 2003); the latter is further metabolized to hippuric acid by the microflora and subsequent conjugation with glycine in the liver and kidney. The authors suggested that the hippuric acid, a catabolite of caffeoylquinic acids, was responsible for inducing diuresis and it could be used as a biomarker to detect the polyphenols induced diuretic activity. It is worth noting that caffeoylquinic acids and their metabolites enhance endothelial vasodilatation evidenced by reduced NAD(P)H-dependent superoxide production in the aorta of SHRs (Zhao et al., 2012). Thus, the observed 3,5-diCQA antihypertensive activity could be also related to improved endothelial function.

Diabetes and hypertension are characterized also by histopathological changes of pancreas, kidney and liver (Fig. 2C). Administration of STZ resulted in disease-induced changes and a decrease in pancreatic β-cell counts. The fatty changes in the liver and pancreas are supported by the increased cholesterol and triglyceride levels detected in the diabetic SHRs serum (Table 2). These changes could be due to the increased influx of fatty acids entering the liver, induced by hypoinsulinemia and the low excretion capacity of liver lipoproteins, as a result of a deficiency of apolipoprotein B synthesis (Ohno et al., 2000). In both control and diabetic SHRs, we observed the so-called “hypertensive kidney”, characterized by dilated tubules with cloudy swelling (Fig. 2A–C). It is interesting to be noted that the organs of the diabetic SHRs, treated with 3,5-diCQA showed almost normal architecture (Fig. 2). These results proved that 3,5-diCQA ameliorates not only the oxidative stress biomarkers and improves the antioxidant defence of the cells, but prevents the histopathological changes related to diabetes and hypertension.

In addition to evoking an antihyperglycemic response, 3,5-diCQA was beneficial for attenuating AST activity and creatinine level in diabetic animals (Table 2). These biochemical markers are associated with the liver and kidney function and their increase could be a predictor for abnormalities. In contrast, acarbose did not affect the higher transaminase activity in the diabetic animals. Several reports of acarbose-induced hepatotoxicity have been described (Hsiao et al., 2006) which means that the use of acarbose should be monitored for signs of hepatotoxicity.

5. Conclusion

For the first time, *in vivo* administration of 3,5-diCQA from *G. alata* roots to hypertensive diabetic rats improved their glycemic and liver biochemical and antioxidant status. The studied compound also exerted an antihypertensive effect when administered to control and diabetic SHRs. Even if it has been accepted that the complications, resulting from co-morbidity of diabetes and hypertension are more difficult to be influenced, the current research provides support to the use of natural antioxidant 3,5-diCQA in their supplementation therapy. The antihyperglycemic, antioxidant and antihypertensive effects of 3,5-diCQA on streptozotocin-induced diabetic hypertensive rats may support the traditional claims regarding the use of *G.alata* roots for the management of diabetes. It would be interesting to examine further the 3,5-diCQA mechanism of action and its metabolites on the sensitivity of cell insulin receptors, on proinflammatory cytokines production linked to these pathologies, and nitric oxide bioavailability in the arterial vasculature.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110678.

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