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In vivo evaluation of the antioxidant potential of dicaffeoylquinic acid isolated from *Geigeria alata*

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Geigeria alata (DC) Oliv. & Hiern. (Asteraceae) is an aromatic medicinal plant used in traditional Sudanese medicine, with a number of biological activities including suggested antidiabetic potential. The objective of this study was to evaluate the antioxidant properties of 3,5-dicaffeoylquinic acid (diCQA), the major compound isolated from *Geigeria alata* roots extract in an experimental model of streptozotocin-induced type 2 diabetes in male Wistar rats. Diabetes results in severe organ pathology which main pathophysiological mechanisms are related to oxidative stress, discerned by increased production of malondialdehyde (MDA) and disturbance in both non-enzymatic (GSH) and enzymatic (GPx, GR, GST) antioxidant defense. DiCQA (5 mg/kg/po) administered for 21 days to control and diabetic Wistar rats ameliorated the activity of the antioxidant enzymes and the levels of the cellular protector GSH, as well as reduced the production of MDA. It also exerts antidiabetic effect in diabetic rats. On the basis of these results, as well as knowing that formation of ROS is considered to be one of the mechanisms in the pathogenesis of diabetes we concluded that diCQA isolated from *Geigeria alata* possesses antioxidant properties which most probably determined its *in vivo* antidiabetic activity.

Keywords: 3,5-Dicaffeoylquinic acid, Diabetes, Oxidative stress, Rats

INTRODUCTION

Oxidative stress now appears to be one of the fundamental mechanisms underlying a number of human disorders, like neurological, endocrine and others. In relation to this knowledge, antioxidants, preferably from natural sources, are used both to prevent the development of such disorders or to support their conventional treatment. Among the perspective biologically active compounds with antioxidant potential are carotenoids, phenolic acids, flavonoids, phenolic diterpenes and others [1]. Acylquinic acids, often called chlorogenic acids, are a group of esters formed between trans-cinnamic acids and (-)-quinic acid. Multiple acylquinic acid isomers usually co-exist in plants, most notably in some species from the Asteraceae family [2]. They demonstrate a variety of biological activities: enhance the accumulation of bile and reinforce the secretion of pancreatic enzymes, slow the aging process, regulate the lipid metabolism and weight gain, have anti-inflammatory and high antioxidant potential [3].

Geigeria alata (DC) Oliv. & Hiern is an aromatic plant belonging to the Asteraceae family found in northern and central Sudan. The roots and leaves are reputed in Sudanese folk medicine to be effective against epilepsy, pneumonia, and rheumatism. In addition, aerial parts are used for the treatment of cough and intestinal complaints. *G. alata* also showed α-glucosidase inhibitory potential with which the antidiabetic effect of an aqueous-methanol roots extract in streptozotocin-induced diabetic rats was explained [4].

As a part of our ongoing investigation of Sudanese medicinal plants, we reported the isolation of acylquinic acids from *G. alata* roots and their contribution to antioxidant and antimicrobial plant capacity [5]. Our experiments, using high-resolution mass spectrometry (LC-HRMS) revealed that the main compounds in *G. alata* roots extract belong to the group of phenolic and cafeoilquinic acids (mono-, di- and tricaffeoylquinic acids, *p*-coumaroylquinic, caffeoylsinapoylquinic, caffeoylferuloylquinic and feruloylquinic acids). 3,5-dicaffeoylquinic acid was the most abundant acylquinic acid in the hroots, being present at 25.96 ± 2.08 mg/g dry weight. The *in vitro* free radical scavenging potential of 3,5-diCQA was investigated using antiradical scavenging activity against (2,2-diphenyl-1-picrylhydrazyl) radical, ABTS and ferric reducing antioxidant power (FRAP) methods. The 3,5-diCQA demonstrated strong radical-scavenging

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activity, higher than that of 5-chlorogenic acid (5-CQA) [5].

On the basis of this information the aim of the current study was to investigate the *in vivo* antioxidant potential of 3,5-diCQA in an experimental model of streptozotocin-induced type 2 diabetes in rats.

MATERIALS AND METHODS

Plant material and isolation of the 3,5-dicaffeoylquinic acid (diCQA)

Geigeria alata roots were collected in July 2011 from 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.

Air-dried roots were stored at room temperature and protected from the light. Powdered dried roots (300 g) of *G. alata* were extracted with aqueous methanol (80 %, v/v) by ultrasound assisted extraction (2×15 min). Sample-solvent ratio was 1:10 (w/v). The combined extracts were concentrated under vacuum at 40°C. The crude extract was purified by solid-phase extraction (SPE) on Vac Elut 10 vacuum manifold (Varian, Walnut Creek, CA, USA). Aliquots of the crude extract (1 g) were fractionated on cartridges Strata C18-E, 10 g/60 ml (Phenomenex, USA). After loading samples on previously conditioned cartridges, and washing with 10 ml of water, the elution step was accomplished consequently with 30%, 70% and 100% methanol. Eluates obtained with methanol concentration of 30% were purified by repeated low-pressure liquid chromatography as described earlier to yield 0.950 g of 3,5-dicaffeoylquinic acid (diCQA) [5].

Liquid chromatography–mass spectrometry (LC-MS)

The identity of diCQA was verified by ultrahigh-performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS). LC-MS analysis was performed on Q Exactive mass spectrometer (ThermoScientific Co, Waltham, MA, USA) equipped with heated electrospray ionization module IonMax® (ThermoScientific Co, Waltham, MA, USA) and TurboFlow Ultra High Performance Liquid Chromatography (UHPLC) system (ThermoScientific Co, Waltham, MA, USA).

The chromatographic analysis was carried out by Syncronis® C18 column (2.1 mm × 50 mm i.d., 1.7 μm) using as eluents: (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile at a flow rate of 300 μl/min. The following binary gradient was used: 10% B for 1 min; 10-60% B for 8.0 min; 60-100% B for 2.0 min; 100% B for 1 min and 100-10% B for 2.0 min. Spray voltage at 4.2 kV, sheath gas flow rate 35 AU, auxiliary gas flow 8 AU, capillary temperature 320º C, probe heater temperature 300º C and S-lens level 50 were adjusted for the interface. Full-scan mass spectra over the *m/z* range 150-1800 were acquired in negative ion mode at resolution settings of 140 000. Targeted MS^2 mode at resolution settings of 17 500 and 1.0 amu isolation window of precursor ions was used for structural elucidation study. Data were processed using XCalibur® (ThermoScientific Co, Waltham, MA, USA) instrument control/data handling software.

Deprotonated molecule $[M-H]^-$ was observed at *m/z* 515.12. The MS/MS spectrum of diCQA gave the fragment ions as follows: 191.06 (100% relative abundance), 179.03 (80.04), 135.04 (14.89), 353.09 (8.58), 173.04 (6.50), 161.02 (4.70), 155.03 (1.56), 335.08 (0.85).

Animals

Male Wistar rats (body weight 200–250 g) were used. The rats were housed in plexiglass cages (3 per cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature 20°C ± 2°C and humidity 72% ± 4%) with free access to water and standard pelleted rat food 53-3, produced according to ISO 9001:2008. The animals were purchased from the National Breeding Center, Sofia, Bulgaria. Seven days acclimatization was allowed before the commencement of the study and a veterinary physician monitored the health of the animals regularly. Vivarium (certificate of registration of farm № 0072/01.08.2007) was inspected by the Bulgarian Drug Agency in order to check the husbandry conditions (№A-11-1081/03.11.2011). All performed procedures were approved by the Bulgarian Food Safety Agency (BFSA) and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) [6] were strictly followed during the experiment.

Chemicals

All the reagents used were of analytical grade. Streptozotocine, beta-nicotinamide adenine dinucleotide 2-phosphate reduced tetrasodium salt (NADPH), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (fraction V), 2,2-dinitro-5,5-dithiodibenzoic acid (DTNB) were obtained from Merck (Darmstadt, Germany).

Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), and cumene hydroperoxide were purchased from Sigma Chemical Co. (Taufkirchen, Germany).

Induction of diabetes

Prior to induction of diabetes, the rats were fasted for at least 16 hours. Diabetes was induced in rats by intraperitoneal injection of streptozotocine (STZ) (40 mg/kg body weight) [7], dissolved in 0.1M citrate buffer, pH 4.5. Another group of rats which served as control was injected with citrate buffer alone without STZ. Forty eight hours after STZ injection, diabetes was confirmed by measuring blood glucose concentrations (using an Accu-Chek Glucometer, Roche, Germany) in blood samples taken from tail vein. Rats with blood glucose levels of 9 mmol/L or more were considered to be diabetic and included in the study.

Design of the experiment

The animals were divided into eight groups (n=6) as follows:

Group 1 (C): Control animals, treated with the saline vehicle, administered by gavage at 5 mL/kg bw/day for 21 days. On day 7 of the experiment the animals received an i.p. injection with citrate buffer.

*Group 2 (*CQA*):* Animals treated with the positive control chlorogenic acid (5 mg/kg bw/day, oral-gavage) for 21 days.

*Group 3 (*3,5-diCQA*):* Animals treated with 3,5-dicaffeoylquinic acid at 5 mg/kg bw/day, oral-gavage for 21 days.

Group 4 (DM): Animals challenged with 40mg/kg bw, i.p. streptozotocin (STZ) dissolved in 0.1M citrate buffer, pH 4.4.

*Groups 5 (DM+CQA) and 6 (DM+*3,5-diCQA*):* Up to day 7th the animals were treated the same way as groups 2 and 3. On day 7th the animals were challenged with STZ (40 mg/kg bw, i.p.) and after that continued to be treated with chlorogenic acid at 5 mg/kg bw/day (group 5) and 3,5-diCQA at 5 mg/kg bw/day (group 6) for additional 14 days.

On the 22nd day of the experiment, blood has been collected from the tail vain of all animals and the glucose levels have been measured. After that, the animals in all groups were sacrificed and the livers were taken to assess the oxidative stress biomarkers – MDA, GSH, and 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.

Preparation of liver homogenate for MDA assessment

Lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS) (expressed as malondialdehyde (MDA) equivalents) described by Polizio and Peña [8] with slight modifications. One volume of homogenate was mixed with 1 mL of 25% trichloroacetic acid (TCA) and 1 mL of 0.67% thiobarbituric acid (TBA). Samples were then mixed thoroughly, heated for 20 min in a boiling water bath, cooled and centrifuged at 4000 rpm for 20 min. The absorbance of supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. MDA concentration was calculated using a molar extinction coefficient of $1.56\ 10^5\ M^{-1}\ cm^{-1}$ and expressed in nmol/g wet tissue.

Preparation of liver homogenate for GSH assessment

GSH was assessed by measuring non-protein sulfhydryls after precipitation of proteins with TCA, using the method described by Bump *et al.* [9]. Tissues were homogenized in 5% trichloroacetic acid (TCA) and centrifuged for 20 min at 4 000 ×g. The reaction mixture contained 0.05 mL supernatant, 3 mL 0.05 M phosphate buffer (pH = 8), and 0.02 mL DTNB reagent. The absorbance was determined at 412 nm and the results expressed as nmol/g wet tissue.

Assessment of antioxidant enzymes activity

The antioxidant enzymes activity was measured in the supernatant of 10% homogenates, prepared in 0.05M phosphate buffer (pH=7.4). The protein content of liver homogenate was measured by the method of Lowry [10]. Glutathione peroxidase activity (GPx) was assessed by NADPH oxidation, using a coupled reaction system consisting of glutathione, GR, and cumene hydroperoxide [11]. Glutathione reductase activity (GR) was measured spectrophotometrically at 340 nm according to the method of Pinto *et al*. [12] by following NADPH oxidation. GST was measured using CDNB as substrate [13].

Statistical analysis

Statistical analysis was performed using statistical programme 'MEDCALC'. Results are expressed as mean ± SEM for six rats in each group. The significance of the data was assessed using the non-parametric Mann–Whitney *U* test. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

Changes in blood glucose and body weight

All animals survived until the end of the treatment period. During treatment, there were no observed changes in behavior or in food and water consumption among the animals in either the control or treated groups. The initial and final body weights, as well as the body weight changes observed during treatment are presented in Table 1. The animals in the DM group had statistically significantly lower final body weight when compared to the control animals. In all other groups, the mean body weight was comparable to the control. Streptozotocin administration (DM group) resulted in increased blood glucose levels by 77 % ($p<0.05$). The results are presented in Table 1.

Table 1. Mean body weights and blood glucose levels – the effect of 3,5-dicafeoilquinic acid on control and diabetic rats.

Group	Mean body weight (g)			Blood glucose (mmol/L)
	Initial	Final	Change	
Control	205 ± 3	255 ± 5	50	5.2 ± 0.46
CQA	190 ± 3	258 ± 4	68	5.8 ± 0.40
3,5-diCQA	210 ± 4	260 ± 4	50	5.4 ± 0.39
DM	215 ± 3	235 ± 3*	20*	9.2 ± 0.69*
DM+CQA	205 ± 3	262 ± 6^{+}	57^{+}	5.9 ± 0.50^{+}
DM+ 3,5-diCQA	212 ± 3	258 ± 4^{+}	46^{+}	6.0 ± 0.49^{+}

Data are expressed as mean ± SEM of six rats (n = 6). For comparison between groups Mann—Whitney U test was performed. *$p<0.05$ *vs* control, $^{+}p<0.05$ *vs* DM

It is interesting to note that the treatment of the diabetic rats with both 3,5-diCQA and CQA resulted in a statistically significant decrease in blood glucose levels by around 35 % ($p<0.05$) when compared to the DM group.

Markers of oxidative stress

The quantities of the oxidative stress marker MDA and the levels of GSH are presented in Table 2.

Table 2. Effect of 3,5-dicafeoilquinic acid on MDA quantities and GSH levels in control and diabetic rats

Group	MDA (μmol/g wet tissue)	GSH (μmol/g wet tissue)
Control	1.27 ± 0.36	7.32 ± 0.36
CQA	1.35 ± 0.15	7.29 ± 0.54
3,5-diCQA	1.30 ± 0.16	7.05 ± 0.64
DM	4.80 ± 0.57*	4.57 ± 0.28*
DM + CQA	2.65 ± 0.45^{+}	5.35 ± 0.35^{+}
DM + 3,5-diCQA	2.70 ± 0.43^{+}	6.01 ± 0.38^{+}

Data are expressed as mean ± SEM of six rats (n = 6). For comparison between groups Mann—Whitney U test was performed. *$p<0.05$ *vs* control, $^{+}p<0.05$ *vs* DM

In the DM group rats, the MDA production was nearly three times higher and GSH levels were reduced by 37 % ($p<0.05$) when compared to the control. 3,5-dCQA administered to diabetic rats normalizes the levels of both parameters to those of the control. Compared to diabetic rats, 3,5-diCQA decreased the production of MDA by 44 % ($p<0.05$) and increased the GSH levels by 31 % ($p<0.05$). The effect of 3,5-diCQA was comparable to that of CQA, used as positive control.

Changes in antioxidant enzymes

In this study the activity of the antioxidant enzymes related to GSH turnover was measured. The results are shown in Table 3. Compared to control animals, the activity of GPx, GR and GST was statistically significantly ($p<0.05$) decreased by 26 %, by 38 % and by 18 %, respectively, in the rats from the DM group. Compared to the DM group, 3,5-diCQA treatment significantly restored the enzymatic activity as follows: GPx activity was increased by 21 % ($p<0.05$), GR activity – by 25 % ($p<0.05$) and GST activity – by 14 % ($p<0.05$). The effect was commensurable with that of CQA.

Table 3. Effect of 3,5-dicafeoilquinic acid on the activity of the antioxidant enzymes: GR, GPx and GST in control and diabetic rats

Group	GR (μmol/mg/min)	GPx (μmol/mg/min)	GST (μmol/mg/min)
Control	0.26 ± 0.031	0.46 ± 0.03	1.53 ± 0.088
CQA	0.27 ± 0.016	0.48 ± 0.02	1.56 ± 0.056
3,5-diCQA	0.25 ± 0.033	0.43 ± 0.05	1.54 ± 0.102
DM	0.16 ± 0.022*	0.34 ± 0.03*	1.25 ± 0.062*
DM + CQA	0.20 ± 0.015^{+}	0.42 ± 0.02^{+}	1.48 ± 0.035^{+}
DM + 3,5-diCQA	0.19 ± 0.015^{+}	0.41 ± 0.01^{+}	1.43 ± 0.070^{+}

Data are expressed as mean ± SEM of six rats (n = 6). For comparison between groups Mann—Whitney U test was performed. *p<0.05 *vs* control, $^{+}$p<0.05 *vs* DM

DISCUSSION

Type 2 diabetes is characterized by insulin resistance and inability of the beta cell to sufficiently compensate. One of the discussed mechanisms in the pathogenesis of the secondary complications of diabetes is oxidative stress. The evidence for oxidative damage in diabetic patients has been reported as far back as 1979 by Sato *et al.* [14] who reported that the average level of lipid peroxide in plasma is higher in diabetic patients than in healthy controls. It is proved that hyperglycemia generates reactive oxygen species (ROS) by several mechanisms. Giacco and Brownlee [15] showed that persistent hyperglycemia can enhance the oxidative stress by increasing glucose auto-oxidation, nonenzymatic protein glycation, and activation of polyol pathway. The oxidative cell damage ultimately results in vascular complications as a secondary damage in diabetes.

Along with the conventional drug therapy of diabetes, recently, attention has been directed towards nutraceuticals originating from plants that are rich in antidiabetic phyto-constituents and antioxidants. In this context, the effect of antioxidant supplementation on oxidative stress in diabetes has been extensively studied [16]. In this study, our attention was focused on *Geigeria alata*, Asteraceae, a Sudanese plant, used for the management of diabetes in Sudanese traditional medicine. Its effective use was supported by the observed antidiabetic effect of an aqueous-methanol roots extract in streptozotocin-induced diabetic rats [4]. *Geigeria alata* roots extract is rich in phenolic acids, mainly cafeoilquinic acids. 3,5-diCQA is the major component for which a potent free radical scavenging potential *in vitro* has been proven [5]. In order to extend our studies on the antioxidant activity of 3,5-diCQA, the aim of the current study was to investigate its antioxidant potential, applying a model of streptozotocin-induced diabetes in rats.

In experimental toxicology the induction of type 2 diabetes in rodents is an appropriate model used to investigate the effects of the diabetes alone, as well as to test drugs and therapies which main mechanism is related either to decrease the insulin resistance or to ameliorate some of the secondary mechanisms involved in the complications of this disease, such as the ROS formation. Streptozotocin is a common chemical of choice for inducing diabetes in experimental animals due to its irreversible damage to pancreatic β-cells [18]. In the scientific literature a dose range from 20 up to 200 mg/kg bw has been reported. In our study we clearly demonstrated that streptozotocin at a dose of 40 mg/kg was able to induce a sustained hyperglycemia in rats, discerned by significant increase of the blood glucose levels (see Table 1). The induced hyperglycemia was accompanied by oxidative stress, judged by increased production of MDA and depletion of GSH levels (see Table 2). Our results are in good agreement with the effects of diabetes reported in the scientific literature. Bandeira *et al*. [19] reported an increased MDA quantity in plasma, serum, and many tissues in diabetic patients. Decreased antioxidant defense, enzymatic and non-enzymatic alike is also reported as one of the main characteristics of diabetes [20-22]. In our study, STZ administration resulted in decreased activity of antioxidant enzymes: GR, GPx and GST (see Table 3). The treatment of the diabetic rats with 3,5-diCQA resulted in decreased production of MDA and normalized levels of GSH and antioxidant enzymes. These results proved the antioxidant potential of the studied compound. What is interesting to be noted is that 3,5-diCQA exerted an antidiabetic effect, discerned by decreased level of plasma glucose in STZ-induced diabetic rats (see Table 1). The effect was statistically significant, compared to the diabetic group and was commensurable with that of the chlorogenic acid, for which experimental data about its hypoglycemic activity are available [23, 24].

CONCLUSION

In the current study the *in vivo* antioxidant activity and possible antidiabetic potential of 3,5-

diCQA, the major compound isolated from *Geigeria alata* roots extract were investigated using a model of streptozotocin-induced type 2 diabetes in rats. Under the conditions of this study we could conclude that 3,5-diCQA showed potent *in vivo* antioxidant potential which confirms and supports our previous studies on the *in vitro* free radical scavenging activity of 3,5-diCQA. The tested compound also exerted a hypoglycemic activity, which is most probably due to its antioxidant properties. This study provides support for the use of natural antioxidants, in our case 3,5-diCQA, isolated from *G. alata*, in the supplementation therapy for reducing the level of oxidative stress and slowing or preventing the development of complications associated with diabetes.

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In vivo ОЦЕНКА НА АНТИОКСИДАНТНИЯ ПОТЕНЦИАЛ НА ДИКАФЕОИЛХИНОВА КИСЕЛИНА, ИЗОЛИРАНА ОТ *Geigeria alata*

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(Резюме)

Geigeria alata (DC) Oliv. & Hiern. (Asteraceae) е ароматно медицинско растение, използвано в традиционната суданска медицина с широк кръг от биоактивности, включително предполагаем антидиабетен потенциал. Целта на настоящото изследване е да се оценят антиоксидантните свойства на 3,5-дикафеоилхиновата киселина (diCQA), която е основният компонент, изолиран от екстракт от корените на *Geigeria alata* в експериментален модел на диабет тип 2, индуциран посредством стрептозотоцин в мъжки Wistar плъхове. Диабетът причинява тежка органна патология, чиито основни патофизиологични механизми са свързани с оксидативен стрес, характеризиращ се с повишено производство на малонов дианхидрид (MDA) и нарушение както на неензимната (GSH), така и на ензимната (GPx, GR, GST) антиоксидантна защита. DiCQA (5 mg/kg/po), прилагана в продължение на 21 дни на контролните и на диабетните плъхове, подобрява активността на антиоксидантните ензими и нивата на клетъчния протектор GSH, както и намалява производството на MDA. DiCQA има също антидиабетно действие при диабетни плъхове. На основата на тези резултати и имайки пред вид, че образуването на реактивни форми на кислорода (ROS) е един от механизмите на патогенезата на диабета, ние правим извода, че diCQA, изолирана от *Geigeria alata*, притежава антиоксидантни свойства, които най-вероятно определят нейната *in vivo* антидиабетна активност.