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Centella asiatica ameliorates diabetes-induced stress in rat tissues *via* influences on antioxidants and inflammatory cytokines

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ABSTRACT

Centella asiatica (L.) Urban (Family: Apiaceae) is a perennial herb that has been used to elevate mood, improve memory, treat wounds and manage kidney-related ailments in African traditional medicine practice. This study evaluated the potential benefits of *C. asiatica* (CA) on diabetes-induced stress in kidney and brain of rats. Following the induction of *diabetes mellitus* (DM), rats were orally treated with vehicle, CA or Metformin daily for 14 days. After treatment, renal and brain levels of inflammatory cytokines, TNF-α, IFN-γ, IL-4, and IL-10 were assessed. Oxidant and antioxidant biomarkers were also evaluated. Phyto-compounds in the crude methanol extract of CA were analyzed by Gas Chromatography–Mass Spectroscopy. Diabetes increased malondialdehyde (MDA) concentration by 39%; elevated levels of TNF-α (44%) and IFN-γ (20%); and reduced the antioxidant status in the kidney in comparison to normal control rats. In the brain, diabetic control rats had significantly greater levels of MDA, TNF-α, and IFN-γ (182%, 40%, and 20%, respectively) in addition to the lowered antioxidant status when compared to normal control rats. However, treatment with CA significantly reduced the renal levels of MDA (33%), TNF-α (78%), and IFN-γ (42%) while that of IL-10 increased by 18% when compared to diabetic control rats. In the brain, CA treatment elicited significant reductions in MDA (37%), TNF-α (30%), and IFN-γ (37%) levels while those of IL-4 and IL-10 increased by 94% and 20% respectively. In addition, the renal and brain antioxidant status was significantly boosted by CA treatment. Several medicinal compounds including ascorbic acid, asiatic acid, oleanolic acid, stevioside, stigmasterol, and α-humulene were identified in the crude extract of CA. Findings from this study suggest CA may protect diabetic tissues from stress *via* antioxidant and anti-inflammatory mechanisms that can be useful in the management of diabetic complications.

1. Introduction

Globally, diabetic nephropathy and neuropathy are significant causes of diabetes-related end-stage renal failure and non-traumatic amputations respectively [1,2]. Therapeutic agents that have the capacity to ameliorate or prevent the deleterious consequences resulting from the prolonged oxidative stress and inflammatory processes in *diabetes mellitus* (DM) may be highly beneficial to diabetic patients [3]. Chronic hyperglycemia, a hallmark of DM, results in increased generation of reactive oxygen species (ROS) through various molecular pathways leading to a decline in the activities of enzymatic antioxidants as well as levels of non-enzymatic cellular antioxidants [4]. The resulting oxidative stress coupled with the over-activation of pro-inflammatory processes within tissues are major recurring themes proposed to be responsible for the progression of DM and associated life-limiting complications [4]. In DM, the elevated ROS results in damage to membrane lipids, proteins, and DNA that terminate in tissue damage with consequent development of diabetic complications such as nephropathy and neuropathy [5].

Diabetic nephropathy and neuropathy have been considered inflammatory diseases since accumulated basic and clinical data have suggested the important roles played by inflammatory processes such as recruitment of immune cells, expression of pro-inflammatory chemokines, cytokines and cell adhesion molecules in the development of these complications [1,6]. Elevated production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ together with the reduced production of anti-inflammatory cytokines like IL-4 and IL-10, not only enhance systemic stress in diabetics but also worsen the harmful effects of diabetic complications [7]. Moreover, inflammation and oxidative stress have been termed essential partners since the prolongation of inflammatory processes can lead to an increase in the generation of ROS and vice versa [8]. Therefore, the

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regulation of excess ROS production may inhibit tissue damage that is initiated by inflammatory processes.

The lack of an ideal cure for DM and reduced effectiveness of current oral medications on disease progression is a cause for concern. While these medications are crucial in the management of DM, the decline in their efficacy in regulating glucose homeostasis over time as well as associated side effects have limited their use [9]. Therefore, the discovery of new interventions that overcome these limitations to achieve durable glycemic control is warranted. In this regard, plant extracts and plant-derived compounds with antioxidant properties may be used as complimentary or alternative treatments to slow and/or prevent the inherent complications of DM [10]. *Centella asiatica* (L.) Urban (CA), also known as "Gotu kola" in India, "Pohekula" in Hawaii, "Indian pennywort" in USA or "Icudwane" in South Africa is a plant with many medicinal properties including those of wound healing and improvement of cognition [11]. It is commonly used in South African traditional medicine to treat different ailments such as wounds and sores, fever, leprosy, syphilis, gastric complaints, *etc*. [12]. It has been suggested that the neuroprotective action of CA in animal models of diseases is due to its antioxidant effects [13]. CA has also demonstrated several pharmacological actions such as anti-hyperglycemic effects in obese diabetic rats [14]; anti-inflammatory and analgesic effects based on chemically-induced animal behavior tests [15]; and restoration of the activities of liver and kidney enzymes involved in glucose and amino acid oxidation following their alteration by diabetes [16]. Recently, it was shown that CA has the beneficial modulation of liver antioxidant status and cytokine activity in type II diabetic rats [17]. There is still limited knowledge of the effects of CA in DM and associated complications. Based on this background, the aim of the present study is to evaluate the potential effects of the crude extract of CA leaves in diabetes-induced oxidative and inflammatory stress in kidney and brain of rats.

2. Materials and methods

2.1. Chemicals

Chemicals and biochemicals of analytical grade were used in this study. They were purchased from Sigma-Aldrich (St. Louis, MO) through Capital Labs, New Germany, South Africa or Merck (Darmstadt, Germany) through Merck, Halfway House, South Africa, except Isofor which was purchased from Safeline Pharmaceuticals (Johannesburg, South Africa) and the Bio-plex Pro-magnetic bead-based Luminex kit which was sourced from BioRad Laboratories (Hercules, CA, USA through BioRad, Johannesburg, South Africa).

2.2. Extraction of plant material

C. asiatica leaves were collected in the Wild specifically within the lush vegetation of Westville Campus of University of KwaZulu-Natal, South Africa and authenticated by Prof. A. Nicholas (Discipline of Biological Sciences) in the same University. A voucher specimen (Dladla 02) was deposited in the Ward Herbarium at University of KwaZulu-Natal. The fresh leaves were rinsed with water and dried at room temperature before being pulverized in an electric grinder. Thereafter, 100 g of the powder was soaked in 1 L of methanol with continuous stirring for 72 h after which the mixture was filtered and the filtrate evaporated to dryness at 60 °C. This gave about 19% yield dry weight which was stored at −20 °C until needed.

2.3. Animal handling and induction of DM

Male Sprague-Dawley rats (150–180 g) were obtained from the Biomedical Resource Unit (BRU) at Westville campus of the University of KwaZulu-Natal, South Africa. The animals were enclosed two rats per cage in a temperature and humidity controlled room (23 ± 1 °C, 40–60% humidity) with a set 12 h light-dark cycle and fed standard rat chow diet (Meadows, Pietermaritzburg, South Africa) and water *ad libitum* for the duration of the study. This study was approved by the University of KwaZulu-Natal Animal Ethics Committee (Reference 024/15/Animal) and animals were handled humanely throughout the experimental period adhering to the guidelines of Laboratory Animal Care of the National Society of Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (National Institutes of Health (NIH) publication no. 85-23, revised in 1985).

The animals were acclimatized for 7 days following which DM was induced by having them drink 10% fructose solution *ad libitum* for 14 days followed by a single intraperitoneal (*ip*) injection of streptozotocin (STZ, 40 mg/kg) dissolved in freshly prepared 0.1M citrate buffer (pH 4.5). This model of DM induction in rats has been demonstrated to induce insulin resistance coupled with insufficient insulin secretion thus closely mimicking the characteristics of type II DM observed in human diabetic patients [18]. Diabetes was confirmed in rats 7 days after STZ injection by obtaining blood through a once-off tail prick and glucose levels determined using Accu-Chek Glucometer (Roche, Basel, Switzerland). Only diabetic rats having a fasting blood glucose values between 12– 18 mM were used for the study.

2.4. Experimental design

Experimental animals were divided randomly into 5 treatment groups containing 6 rats each as designated as follows: NC: Normal control rats treated with distilled water, DC: Diabetic control rats treated with distilled water, DCA and D1000: Diabetic rats treated with *C. asiatica* extract (500 and 1000 mg/kg body weight respectively) dissolved in distilled water and DME: Diabetic rats treated with the standard antidiabetic drug, Metformin (300 mg/kg body weight) dissolved in distilled water. The NC group was given water *ad libitum* for 14 days prior to a single *ip* injection of freshly prepared citrate buffer. All treatment regimens started 8 days after injection with STZ and continued daily for 14 days. The dose of CA (500 mg/kg body weight) was chosen based on its pharmacological activities reported previously in the literature [19]. The authors used doses of 250, 500 and 1000 mg/kg of an ethanolic extract of CA and the most effective doses in lowering blood glucose and glucose absorption from the intestine were the latter two doses. In our model, the blood glucose lowering effect was evaluated at 500 and 1000 mg/kg. Our result (Fig. 1) showed that the 500 mg/kg dose was more effective in lowering fasting blood glucose in

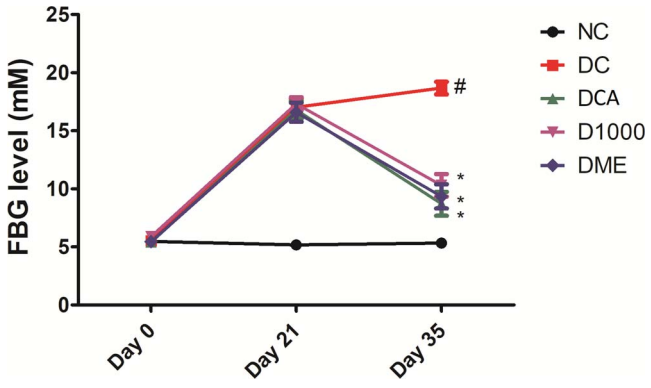


Fig. 1. Effect of *C. asiatica* on fasting blood glucose in diabetic rats. The blood glucose levels were determined using an Accu-Chek® glucometer to evaluate the effects of treatments on hyperglycemia. Day 0 = Start of study; Day 21 = Confirmation of diabetes; Day 35 = After 14 days of treatment; FBG = Fasting blood glucose. NC (Normal Control); DC (Diabetic Control); DCA and D1000 (Diabetic rats treated with *C. asiatica* extract at doses of 500 and 1000 mg/kg body weight, respectively); DME (Diabetic rats treated with Metformin at 300 mg/kg body weight). Data are presented as mean ± standard error of mean; n = 6. $^{\#}p < 0.05$ *versus* NC and $^{*}p < 0.05$ *versus* DC.

diabetic rats hence was used in subsequent experiments as we have done previously [17].

2.5. Preparation of tissue samples

At the end of the treatment period, all rats were fasted overnight and euthanized humanely through an overdose of gas inhalant Isofor in an anesthetic chamber followed by immediate dissection. Whole kidney and brain were carefully excised from each rat, rinsed with physiological saline, blotted-dried, weighed and snap-frozen in liquid nitrogen then stored in −20 °C freezer until needed. Tissue samples (kidney and brain) were homogenized (1:5 w/v) in 50 mM Tris–HCl buffer (pH 7.4) containing 1.15% KCl and centrifuged at 10,000 × *g* for 15 min at 4 °C using an Avanti J26-XPI centrifuge (Beckman Coulter, California, USA). The supernatants obtained were used to determine MDA concentration and antioxidant status. For the evaluation of cytokines, tissue samples were homogenized (1:10 w/v) in 10 mM phosphate buffered saline (PBS, pH 7.2) and centrifuged twice at 15,000 × *g* for 15 min at 4 °C to obtain the supernatants.

2.6. Determination of protein and MDA concentration

Protein concentration in tissues was determined by the Biuret method as described by Gornall and colleagues [20]. The concentration of malondialdehyde (MDA), a product of lipid peroxidation was determined by the method described by Varshney and Kale [21].

2.7. Analysis of antioxidant status

The ferric reducing antioxidant power (FRAP) assay was performed using the method of Benzie and Strain [22] that measures the reduction of ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to the ferrous (Fe^{2+}) form by antioxidants at low pH, monitored by the change in absorption at 593 nm in a Multiskan Spectrum plate reader (ThermoFischer Scientific, Waltham, MA, USA). Trolox equivalent absorbance capacity (TEAC) assay measures antioxidants ability to scavenge ABTS (2,2′-azino-di-3-ethylbenzthialozine sulphonate) radical cation and was performed as described by Miller et al. [23]. The oxygen radical absorbance capacity (ORAC) of tissue samples was determined by a fluorescence spectrophotometer (Fluoroskan Ascent, ThermoFischer Scientific) using a slightly modified method of Ou et al. [24]. Reduced glutathione (GSH) concentration was measured spectrophotometrically at 412 nm according to the method described by Jollow and others [25]. Glutathione *S*-transferase (GST) and glutathione peroxidase (GPx) activities were determined by the method of Habig et al. [26] and Rotruck et al. [27] respectively.

2.8. Analysis of pro- and anti-inflammatory cytokines

The concentrations of IL-4, IL-10, IFN-γ, and TNF-α in tissues were analyzed on the Bio-plex platform using a Bio-plex Pro-magnetic bead-based Luminex kit. Each undiluted supernatant was reacted with a mixture of internally dyed magnetic beads bound to specific anti-cytokine primary antibodies to allow for the attachment of specific cytokine to the bead with its equivalent antibody. The complex thus formed binds to a biotinylated anti-cytokine secondary antibody which was visualized when fluorescent phycoerythrin-conjugated streptavidin was added. Bead acquisition and final analysis were done with the aid of Bio-plex Manager software version 6.1 (BioRad Laboratories, USA).

2.9. Gas chromatography–mass spectroscopy (GC–MS) analysis

The GC–MS analysis was performed using GC–MS 2010 QP-plus (Shimadzu, Kyoto, Japan) equipped with a 30 m × 0.25 mm i.d. 0.25 µm film thickness capillary column containing a stationary phase of 5% phenyl and 95% methyl polysiloxane. The injection was carried out in a current transformer (CT) split mode at an injector temperature of 250 °C. Helium gas was used as a carrier gas with a flow rate of 0.68 mL/min. The oven temperature was initially set at 60 °C and then increased to 300 °C at a rate of 10 °C/min held for 16 min. The ion source and transfer line temperature were 200 °C and 250 °C, respectively. Mass spectra were taken at 70 eV; a scan interval of 0.3 s and fragments from 50 to 800 *m/z*. Compounds were identified by comparing their mass spectra with the NIST library installed within the instrument.

2.10. Statistical analysis

Data are expressed as the mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to test for significant differences between experimental groups followed by unpaired Student's *t*-test to compare differences between two groups. GraphPad prism 5 and InStat 3 software (Graph-Pad Software, San Diego, CA, USA) were used to plot graphs and analyze results. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Effect of C. asiatica on blood glucose, body weight and organ weight in diabetic rats

The result in Fig. 1 show that treatments of diabetic rats with CA (500 and 1000 mg/kg body weight) and Metformin (300 mg/kg body weight) significantly lowered blood glucose levels ($p < 0.05$) by 47%, 41%, and 44%, respectively, when compared to pre-treatment levels. A CA dose of 500 mg/kg body weight was, therefore, most effective in lowering blood glucose within the treatment period of 14 days and was used in subsequent experiments.

The percentage weight gains by rats induced with DM significantly decreased in comparison with normal control, however, significant increases of 24% and 33% in weight gain were seen in CA and Metformin-treated diabetic rats respectively (Table 1). The kidney weight and

Table 1
Effects of *C. asiatica* on body and organ weights in diabetic rats.

Group	Body Weight			Organ Weight			
	Initial body weight (g)	Final body weight (g)	Weight gain (%)	Kidney weight (g)	Kidney/bw ratio (%)	Brain weight (g)	Brain/bw ratio (%)
NC	163.38 ± 5.53	281.88 ± 8.97	72.65 ± 6.49	0.93 ± 0.05	0.33 ± 0.02	1.83 ± 0.05	0.65 ± 0.04
DC	161.00 ± 12.73	241.00 ± 12.41	49.69 ± 6.82#	1.02 ± 0.12	0.42 ± 0.05#	1.86 ± 0.03	0.77 ± 0.06#
DCA	170.25 ± 20.00	275.25 ± 13.45	61.67 ± 9.78*	1.02 ± 0.12	0.37 ± 0.05	1.81 ± 0.03*	0.66 ± 0.04*
DME	151.40 ± 14.22	251.40 ± 21.93	66.05 ± 10.92*	0.91 ± 0.08	0.36 ± 0.02*	1.84 ± 0.06	0.73 ± 0.03

NC (Normal Control); DC (Diabetic Control); DCA (Diabetic rats treated with *C. asiatica* extract at 500 mg/kg body weight); DME (Diabetic rats treated with Metformin at 300 mg/kg body weight). Data are presented as mean ± standard deviation; n = 6.

# $p < 0.05$ *versus* NC.

* $p < 0.05$ *versus* DC.

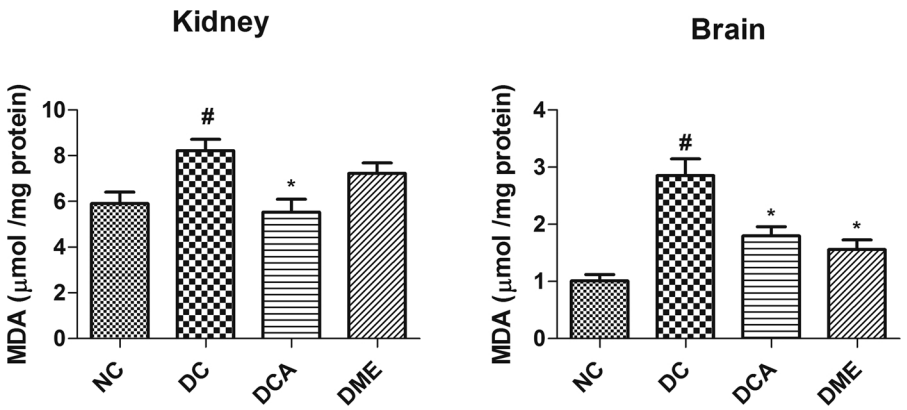


Fig. 2. Effect of *C. asiatica* on the formation of MDA in kidney and brain in diabetic rats. MDA (malondialdehyde) was measured to determine the extent of lipid peroxidation in tissues as an index of oxidative stress following diabetes induction. NC (Normal Control); DC (Diabetic Control); DCA (Diabetic rats treated with *C. asiatica* extract at 500 mg/kg body weight); DME (Diabetic rats treated with Metformin at 300 mg/kg body weight). Data are presented as mean ± standard error of mean; n = 6. $^{\#}$ $p < 0.05$ *versus* NC and * $p < 0.05$ *versus* DC.

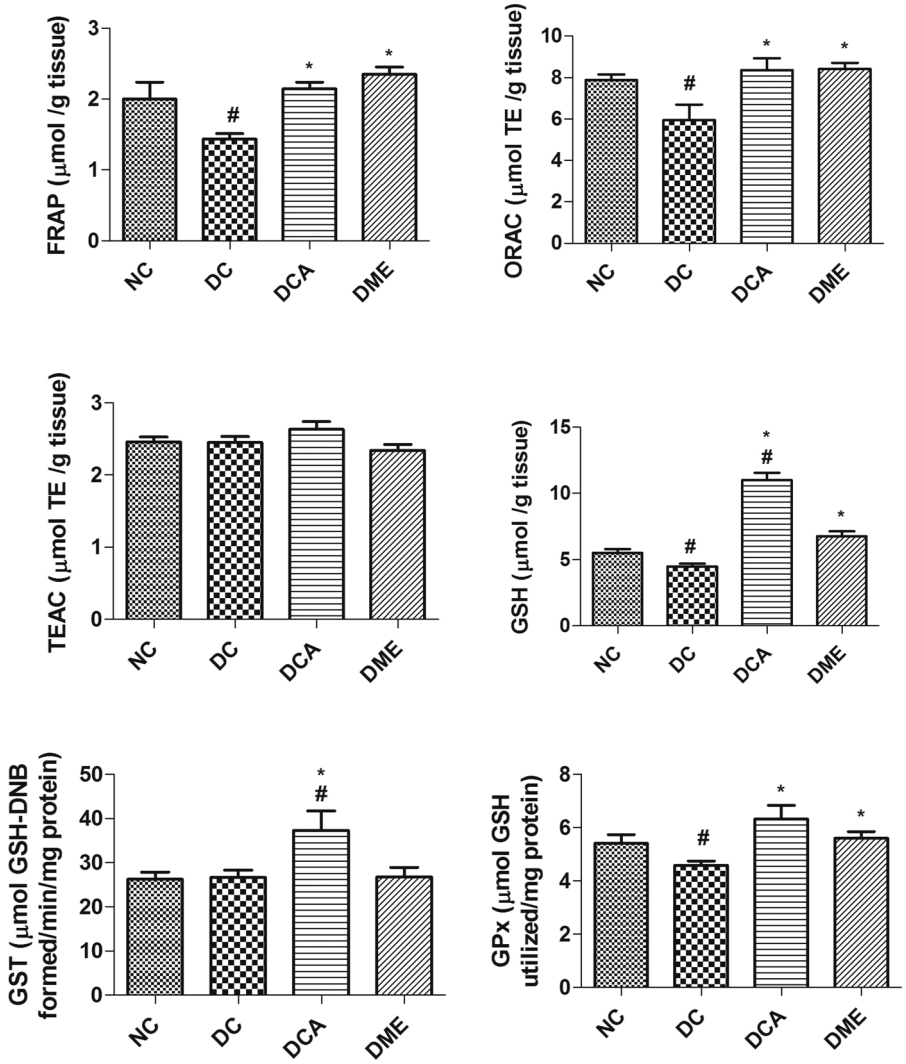


Fig. 3. Effect of *C. asiatica* on renal antioxidant status in diabetic rats. The antioxidant capacity of renal tissues to withstand oxidative stress was quantified using the FRAP (ferric reducing antioxidant power), ORAC (oxygen radical absorbance capacity), TEAC (trolox equivalent antioxidant capacity), GSH (reduced glutathione), GST (glutathione *S*-transferase) and GPx (glutathione peroxidase) assays. NC (Normal Control); DC (Diabetic Control); DCA (Diabetic rats treated with *C. asiatica* extract at 500 mg/kg body weight); DME (Diabetic rats treated with Metformin at 300 mg/kg body weight); TE (trolox equivalents). Data are presented as mean ± standard error of mean; n = 6. $^{\#}$ $p < 0.05$ *versus* NC and * $p < 0.05$ *versus* DC.

kidney weight/body weight ratios in diabetic control rats were significantly ($p < 0.05$) increased by 10% and 27% respectively when compared to normal control rats. Kidney weight in CA-treated rats remained unchanged when compared with diabetic control rats while the kidney weight/body weight ratio was reduced by 12%. Diabetes did not cause any significant change in brain weight when compared to normal control rats in contrast to the significant ($p < 0.05$) increase in the brain weight/body weight ratio. Treatment of diabetic rats with CA significantly reduced the brain weight/body weight ratio by 14% to almost the value seen in normal control rats. The treatment of diabetic rats with Metformin led to 11% and 14% decreases in kidney weight and kidney weight/body weight ratio, respectively compared to

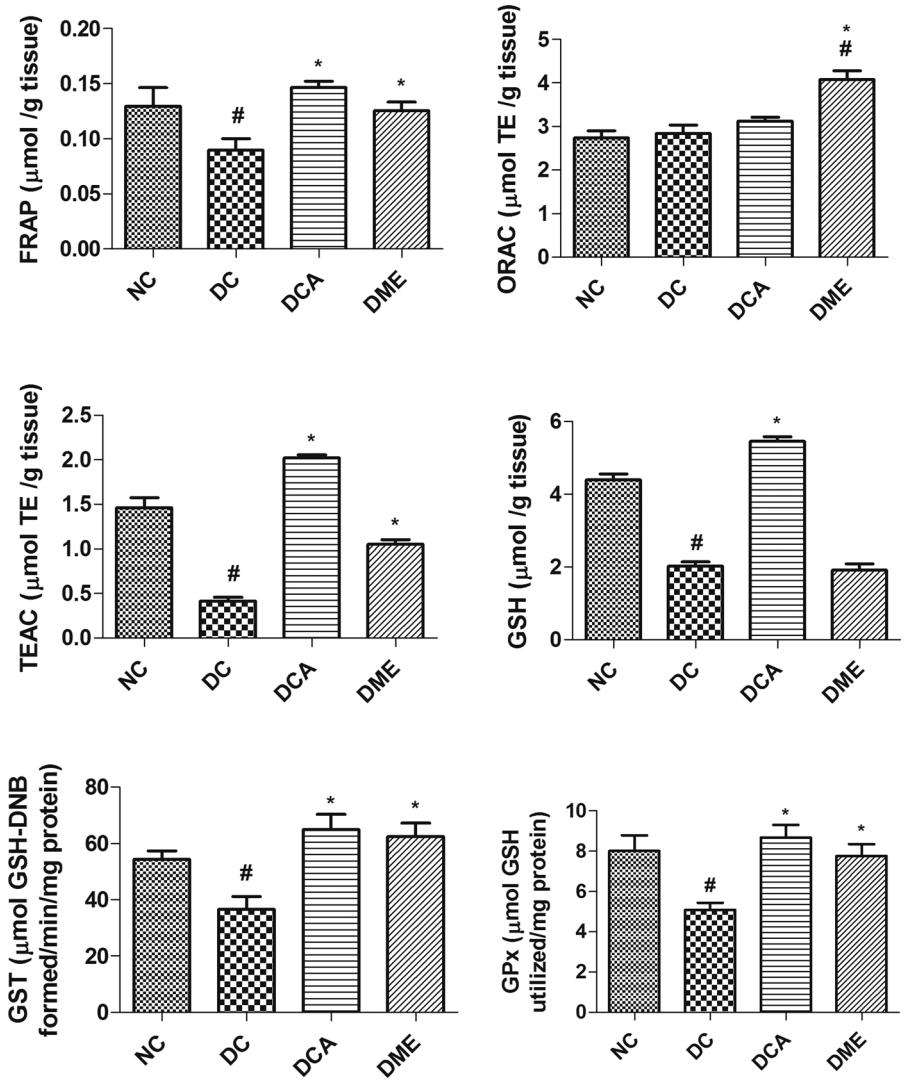


Fig. 4. Effect of *C. asiatica* on brain antioxidant status in diabetic rats. The antioxidant capacity of brain tissues to withstand oxidative stress was quantified using the FRAP (ferric reducing antioxidant power), ORAC (oxygen radical absorbance capacity), TEAC (trolox equivalent antioxidant capacity), GSH (reduced glutathione), GST (glutathione *S*-transferase) and GPx (glutathione peroxidase) assays. NC (Normal Control); DC (Diabetic Control); DCA (Diabetic rats treated with *C. asiatica* extract at 500 mg/kg body weight); DME (Diabetic rats treated with Metformin at 300 mg/kg body weight); TE (trolox equivalents). Data are presented as mean ± standard error of mean; n = 6. $^{\#}p < 0.05$ *versus* NC and $^{*}p < 0.05$ *versus* DC.

diabetic control rats however these parameters remained largely unchanged for the brain.

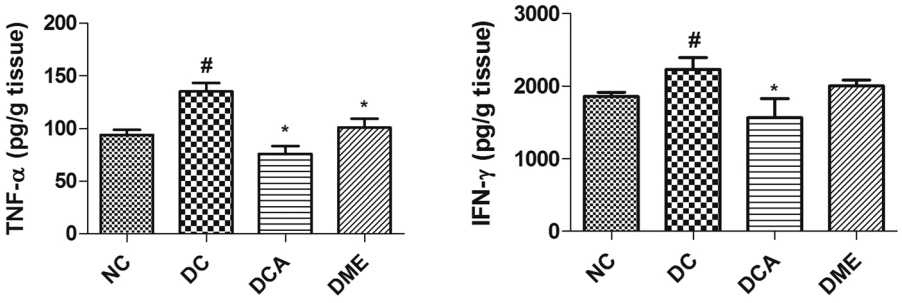
3.2. Effect of C. asiatica on the formation of MDA in the kidney and brain in diabetic rats

The result in Fig. 2 shows the formation of MDA, an end-product of extensive lipid peroxidation, in tissues investigated. In the kidney and brain, MDA was significantly ($p < 0.05$) increased by 39% and 182% respectively in diabetic control rats when compared to normal control rats. However, treatment of the diabetic animals with CA lowered the MDA levels in kidney and brain significantly ($p < 0.05$) by 33% and 37%, respectively. In addition, treatment of the diabetic animals with Metformin decreased MDA levels but this decrease was only significant ($p < 0.05$) in the brain.

3.3. Effect of C. asiatica on renal and brain antioxidant status in diabetic rats

Figs. 3 and 4 show the effects of CA on renal and brain antioxidant status in diabetic rats after treatment for 14 days. In the kidney, diabetes caused significant ($p < 0.05$) reductions of 28%, 25%, 19% and 15% in renal FRAP, ORAC, GSH and GPx values respectively in diabetic control rats when compared to the normal control rats (Fig. 3). The treatment of diabetic rats with CA resulted in significant ($p < 0.05$) increases in renal levels of FRAP (49%), ORAC (40%), GSH (146%) and activities of GST (40%) and GPx (38%) when compared to diabetic control rats. Interestingly, the increases in renal GSH level and GST activity in DCA rats were higher (99% and 42% respectively) than the values seen in NC rats. In addition, treatment of diabetic animals with Metformin significantly (< 0.05) increased the renal levels of FRAP, ORAC, GSH and GPx activity by 64%, 41%, 51% and 22%, respectively, in comparison to values in diabetic control animals.

In the brain, diabetes caused significant ($p < 0.05$) decreases in FRAP (31%), TEAC (71%), GSH (54%), GST (33%) and GPx (36%) values in diabetic control rats when compared to those of normal control rats (Fig. 4). However, treatment with CA significantly ($p < 0.05$) increased brain FRAP, TEAC, GSH, GST and GPx values by 63%, 385%, 169%, 78% and 71%, respectively when compared levels found in DC animals. In addition, DME treated animals had significant increases of 40%, 44%, 152%, 71% and 52% in FRAP, ORAC, TEAC, GST and GPx values, respectively, in comparison to values in diabetic



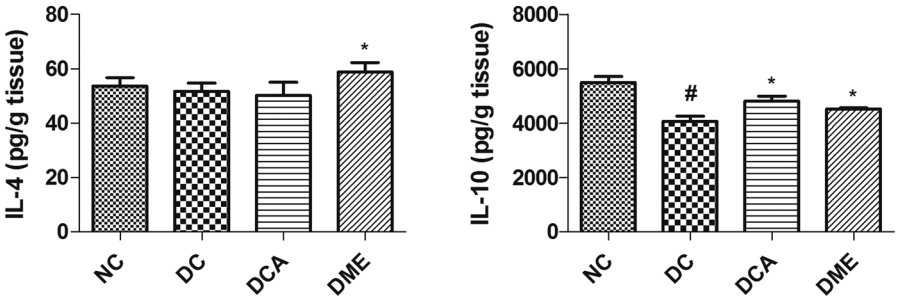


Fig. 5. Effect of *C. asiatica* on renal inflammatory cytokines in diabetic rats. The inflammatory stress in renal tissues was assessed by the levels of cytokines; TNF (tumor necrosis factor)-α, IFN (interferon)-γ, IL (interleukin)-4, 10 using the Luminex xMAP technology. NC (Normal Control); DC (Diabetic Control); DCA (Diabetic rats treated with *C. asiatica* extract at 500 mg/kg body weight); DME (Diabetic rats treated with Metformin at 300 mg/kg body weight). Data are presented as mean ± standard error of mean; n = 6. $^{\#}$ $p < 0.05$ *versus* NC and * $p < 0.05$ *versus* DC.

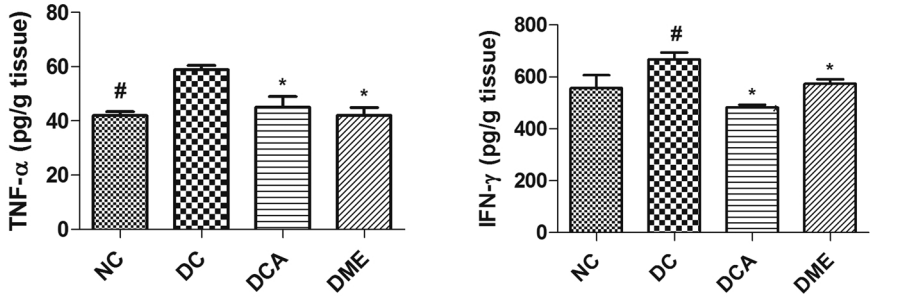
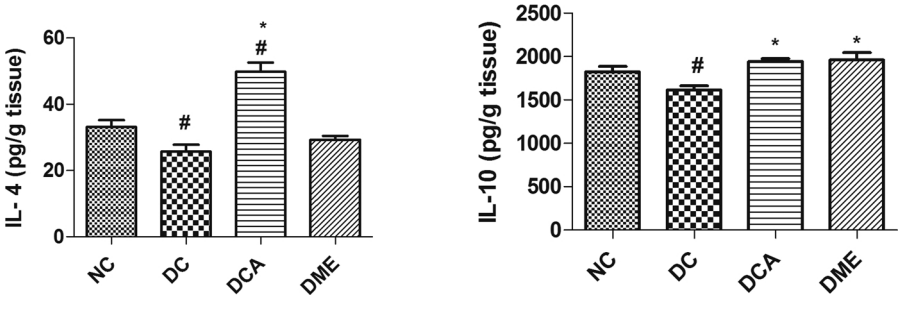


Fig. 6. Effect of *C. asiatica* on brain inflammatory cytokines in diabetic rats. The inflammatory stress in brain tissues was assessed by the levels of cytokines; TNF (tumor necrosis factor)-α, IFN (interferon)-γ, IL (interleukin)-4, 10 using the Luminex xMAP technology. NC (Normal Control); DC (Diabetic Control); DCA (treated with *C. asiatica* extract at 500 mg/kg body weight); DME (Diabetic rats treated with Metformin at 300 mg/kg body weight). Data are presented as mean ± standard error of mean; n = 6. $^{\#}$ $p < 0.05$ *versus* NC and * $p < 0.05$ *versus* DC.



animals. Curiously, the brain ORAC value observed in DME treated animals was 49% higher than that of NC group.

3.4. Effects of C. asiatica on renal and brain inflammatory cytokines in diabetic rats

The results obtained on the selected inflammatory cytokines in kidney and brain of diabetic rats treated with CA and Metformin for 14 days are illustrated in Figs. 5 and 6. There were significant ($p < 0.05$) increases in renal levels of TNF-α (44%) and IFN-γ (20%) in diabetic control rats when compared to normal control rats (Fig. 5). However renal IL-10 level was significantly ($p < 0.05$) reduced in DC rats to 74% of the value in NC rats while that of IL-4 remain unchanged. Treatment of diabetic rats with CA significantly ($p < 0.05$) reduced the renal levels of TNF-α and IFN-γ by 78% and 42%, respectively while the level of IL-10 increased by 18% when compared with the values measured in diabetic control rats (Fig. 5). Metformin treatment of diabetic rats significantly ($p < 0.05$) reduced the renal level of TNF-α (25%)

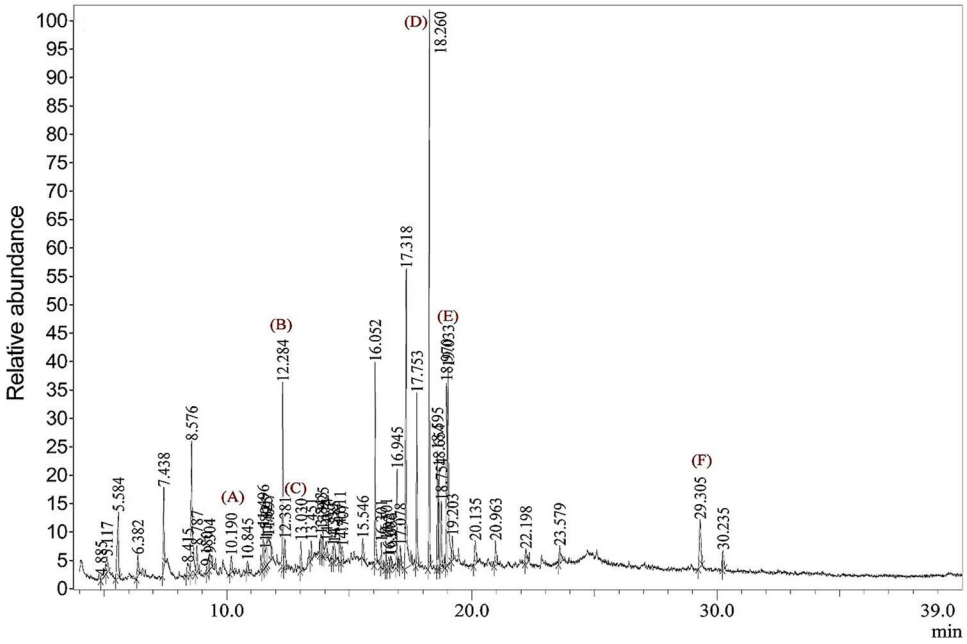


Fig. 7. The GC–MS chromatogram of crude methanol extract of*C. asiatica*. GC–MS (gas chromatography–mass spectroscopy) analysis was done to identify the different chemical constituents of the crude methanol extract of *C. asiatica*. Mass spectra were taken at 70 eV; a scan interval of 0.3 s and fragments from 50 to 800 *m/z*. The peaks with retention times of the selected active compounds; A (asiatic acid), B (stevioside), C (alpha-humulene), D (ascorbic acid), E (oleanolic acid), and F (stigmasterol) identified in the extract are highlighted.

and elevated those of IL-4 (14%) and IL-10 (11%) when compared to diabetic control rats.

In the brain, diabetic control rats had significant ($p < 0.05$) increases of 40% and 20% in levels of TNF-α and IFN-γ, respectively in comparison to normal control rats (Fig. 6). In addition, DM induction caused 29% and 13% significant ($p < 0.05$) decreases in brain IL-4 and IL-10 levels, respectively in rats. However, the levels of brain TNF-α and IFN-γ in CA-treated rats were significantly ($p < 0.05$) less (30% and 37%, respectively) than the values obtained in the DC group (Fig. 6). Treatment with CA also elicited significant ($p < 0.05$) elevation in the levels of brain IL-4 (94%) and IL-10 (20%) when compared to diabetic control rats. Notably, the brain IL-4 level in the DCA group was 50% greater than that of the NC group. In addition, Metformin treatment significantly ($p < 0.05$) reduced brain TNF-α and IFN-γ levels in diabetic rats by 29% and 14%, respectively; and increased IL-10 level by 21% in comparison with diabetic control rats.

3.5. GC–MS analysis of crude extract of C. asiatica

The GC–MS chromatogram of the crude extract of leaves of CA revealed the presence of peaks representing over 50 compounds with matching similarity index (SI) greater than 70% in the National Institute of Standards and Technology (NIST) library as shown in Fig. 7. The fragmentation pattern and mass spectra of two newly identified compounds in the crude extract of CA are shown in Fig. 8; these being ascorbic acid and stevioside. Additionally, the fragmentation pattern and mass spectrums of asiatic acid, alpha-humulene, oleanolic acid, and stigmasterol were also obtained (data not shown). Previous studies have identified the presence of asiatic acid [28,29], alpha-humulene [30], oleanolic acid and stigmasterol [31] among other compounds in CA plant parts hence, justifying their selection in the present study. This is also the first study reporting the presence of ascorbic acid and stevioside in the methanol extract of CA leaves identified by the GC–MS analysis. The peak number, retention times (RT) and percentage composition (area) of these compounds are presented in Table 2.

4. Discussion

The present study was designed specifically to investigate the potential effects of crude methanol extract of *C. asiatica* (CA) leaves on oxidative and inflammatory stress resulting from *diabetes mellitus* (DM) using a rat model that exhibits both insulin resistance and insufficiency. The characteristics of this rat model of DM closely resemble the human type II DM which occurred within a quicker induction period [18] thus offering comparative advantages over previous non-genetic diabetic rodent models. This study is important so that the traditional medical uses of CA are continually supported by empirical pre-clinical evidence which may also expand its clinical utility and commercialization. In this study, common symptoms of DM such as excessive thirst, urination, and hunger were observed among diabetic rats. However, despite the reduction in mean body weight of diabetic control rats observed in the present study, the whole organ weights and organ/bodyweight ratios of kidney and brain were increased compared to normal control rats (Table 1). The increase in the weights of kidney and brain are probably due to hypertrophy of the glucose-sensitive organs in response to persistent hyperglycemic states [32]. The administration of CA, however, ameliorated the change in brain weight of diabetic rats but that of the kidney remained unaffected in contrast to organ/body weight ratio which was reduced in both organs because of CA treatment.

Complications associated with DM including nephropathy and neuropathy are mediated through the release of excess ROS caused by acute or chronic excursions of blood glucose into tissues [33]. Hyperglycemia leads to the excess formation of ROS such as superoxide anions and hydrogen peroxide (H_2O_2) by the mitochondrial electron transport chain [5]. These ROS may then trigger a cascade of oxidative reactions including lipid peroxidation that ultimately culminates in the formation of lipid peroxidation end-products such as malondialdehyde (MDA) and 4-hydroxynonenal [34]. MDA is a highly reactive aldehyde which has been documented as a primary marker of the free radical mediated destruction of membrane lipids and has the capacity to cause DNA damage by forming adducts with DNA bases [34]. In the present study, elevated levels MDA were observed in the kidney and brain of diabetic rats (Fig. 2) suggesting an increased state of cellular oxidative injury. Treatment with CA significantly reduced the production of this ROS-induced lipid peroxide in the kidney and brain of diabetic rats thereby limiting oxidative damage to these tissues.

We also evaluated the effects of DM and treatments on the concentration of non-enzymatic antioxidant, reduced glutathione (GSH) and activities of antioxidant enzymes; glutathione *S*-transferase (GST) and glutathione peroxidase (GPx) in rat kidney and brain. The tripeptide GSH is a prominent antioxidant that plays major roles in restoring other antioxidants such as vitamins C and E to active reduced states,

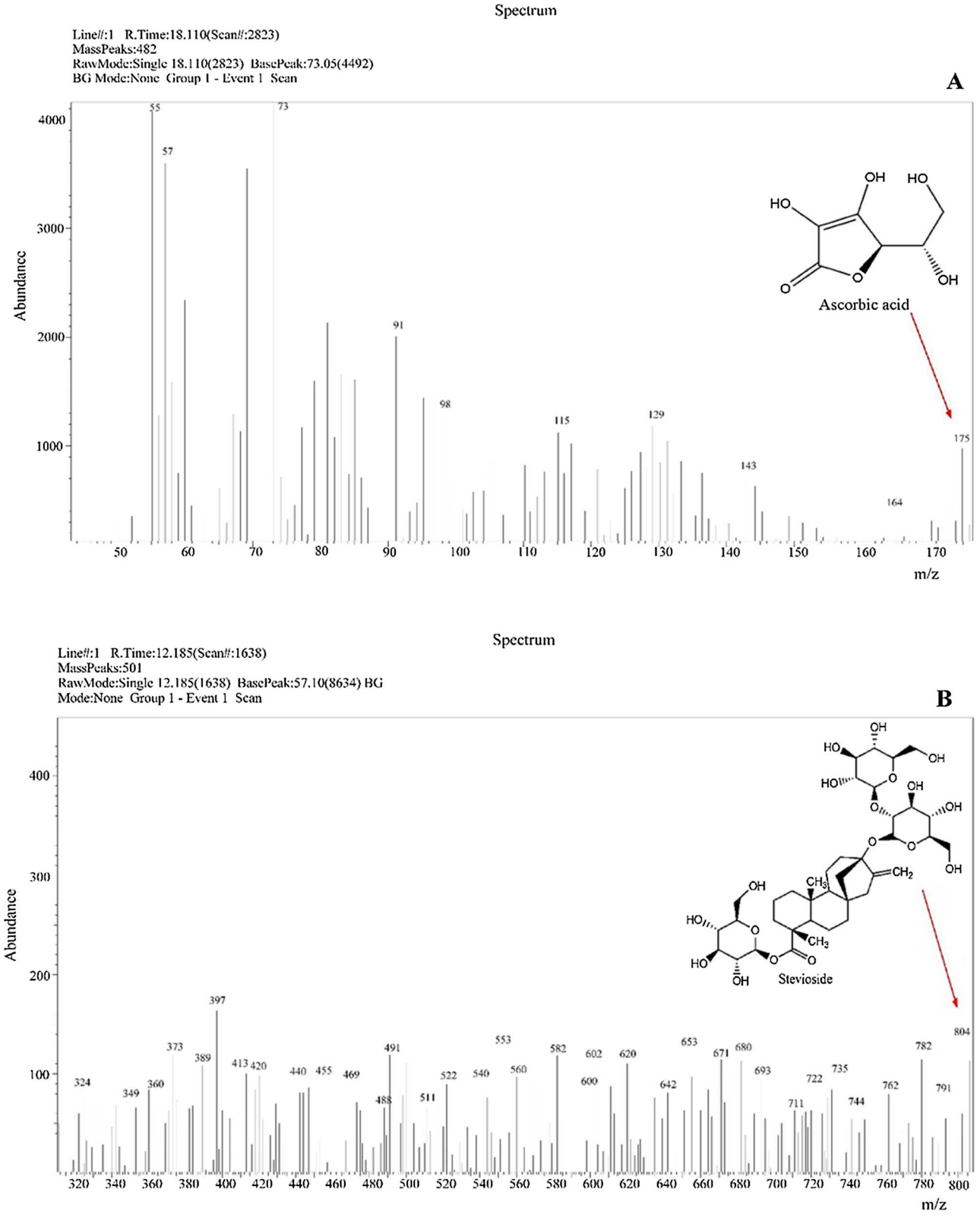


Fig. 8. Mass spectra of ascorbic acid (A) and stevioside (B) identified in the crude extract of *C. asiatica*. The fragmentation patterns of the two new compounds identified in the crude methanol extract of *C. asiatica* are shown.

and directly inactivate ROS like hydrogen peroxide in a reaction catalyzed by GPx or indirectly detoxify lipid peroxides by the catalytic actions of GST [35]. Therefore, the maintenance of GSH level is very critical to the antioxidant defense mechanisms of a tissue. In the present study, reductions in GSH level and GPx activity were observed in kidney and brain tissues of diabetic rats (Figs. 3 and 4). These effects suggest an increased utilization of GSH against excess free radicals such as lipid peroxides that are greater than its synthesis. The decreased activity of GPx due to diabetes may also lead to increased oxidative stress resulting from H_2O_2 and lipid peroxides. However, treatment of

Table 2
Selected compounds identified in crude extract of *C. asiatica*.

Peak number	Retention time	Area (%)	Name of compound	Molecular weight (g/mol)
11	10.33	0.70	Asiatic acid	488.70
17	12.18	4.70	Stevioside	804.87
18	12.41	1.25	Alpha-humulene	204.36
38	18.11	11.56	Ascorbic acid	176.12
41	18.80	1.99	Oleanolic acid	456.71
49	29.35	2.28	Stigmasterol	412.69

diabetic rats with CA restored both antioxidants to near-normalcy indicating that oxidative stress in both tissues was reduced. Interestingly, the level of GSH increase in CA-treated rats was significantly higher than that in normal untreated rats. This suggests that CA has the capacity to activate GSH synthesis to the point of overcompensating its depletion that results from diabetes-induced oxidative stress, a finding we have previously also observed in liver tissue [17]. The increase in the activity of GST due to CA treatment in both tissues suggests an increased rate of conversion of excess highly reactive metabolites to less toxic, more hydrophilic conjugates that are easily excreted from the body. In addition, the increase in the activity of GPx seen in CA-treated rats may help tissues convert excess H_2O_2 to water and effectively halting the propagation of oxidative reactions. A recent study also showed that ethanol extract of CA protected cultured neuronal cells against amyloid-β-induced oxidative stress *via* the activation of antioxidant defense systems [28].

Furthermore, the capacity of tissues to scavenge ROS and reduce oxidized metals involved in the Fenton reaction as assessed by ORAC, TEAC and FRAP assays can serve as an indicator of the ability to ameliorate the harmful consequences of excess oxidative stress in DM [36]. In this study, the decline in renal FRAP and ORAC values were associated with the induction of DM in rats although TEAC value remained unchanged (Fig. 3). On the other hand, FRAP and TEAC values were reduced while changes in ORAC value in the brain of diabetic control rats was not significant when compared to normal rats (Fig. 4). Thus, our results from ORAC, TEAC and FRAP assays are further evidence in support of the antioxidant activity of CA since an improvement in antioxidant capacity to withstand the onslaught of ROS in DM, was observed in diabetic rats. It may also be noted that reductions in ORAC value have been observed in diabetic patients [37] and rats [38] when compared to their non-diabetic counterparts.

There is compelling evidence that inflammatory processes, a constant companion of oxidative stress participate centrally in the pathogenesis of DM and associated complications [39]. In addition to the decrease in the antioxidant status in diabetic rats, the data obtained in the present study revealed marked elevation in the levels of TNF-α and INF-γ in the kidney and brain of diabetic animals (Figs. 5 and 6). TNF-α contributes to renal damage through the induction of local generation of ROS, increased endothelial cell permeability, alterations in intraglomerular hemodynamics, recruitment of infiltrating cells into the kidney, and induction of apoptosis [40]. TNF-α has also been implicated to play a key role in insulin resistance *via* reduction of insulin receptor activity and the depression of the insulin receptor substrate (IRS)-1 and glucose transporter (GLUT)-4 [41]. INF-γ has been reported to have harmful effects on pancreatic β-cell viability through the activation of several molecular signaling pathways such as signal transducer and activator of transcription-1 (STAT-1) and has been shown to upregulate inducible nitric oxide synthase (iNOS) expression *via* IL-1β activation in insulin-producing cells [42]. The serum concentration of INF-γ in type II diabetic patients with complications was previously shown to be significantly elevated in comparison to controls [7]. Therefore, decreases in the levels of TNF-α and INF-γ in kidney and brain in diabetic rats upon treatment with CA as revealed in this study, may reduce insulin resistance and pancreatic β-cell destruction in DM. The effects of pro-inflammatory cytokines may be countered by the activities of anti-inflammatory cytokines such as IL-4 and IL-10 [43]. In this study, lower level of IL-10 observed in the brain and kidney of experimentally-induced diabetic rats was significantly increased after CA-treatment. Although the renal level of IL-4 did not differ significantly in CA-treated diabetic rats from those of diabetic control rats, an increase in the level of this anti-inflammatory cytokine was observed in the brain suggesting a difference in tissue responses to CA treatment in type II diabetic rats. Therefore, anti-inflammatory activities of CA as observed in the present investigation may be a mechanism to protect tissues from cytokine-mediated toxicity that can be of therapeutic relevance in halting the development of diabetic complications.

Plant-derived compounds possessing therapeutic potential have been leading the way in the search for novel antidiabetic drugs. Previously, high performance liquid chromatography (HPLC) and liquid chromatography–mass spectroscopy (LC–MS) techniques have revealed the presence of triterpenes (asiatic acid, asiaticoside, madecassic acid, madecassoside, centellasaponins), phenolic (quercetin-3-*O*-b-D-glucuronide) and flavonoid (kaempferol) in the crude aqueous-methanolic extract of CA [29]. GC–MS analysis of the crude methanol extract of CA leaves used in this study identified ascorbic acid, asiatic acid, oleanolic acid, stevioside, stigmasterol and α-humulene among several other compounds. While other studies have reported the presence of asiatic acid, oleanolic acid, stigmasterol, and α-humulene in different extracts of CA [28–31], this is the first study reporting the presence of ascorbic acid and stevioside in the methanol extract of CA leaves identified by the GC–MS analysis. Asiatic acid has been reported to reduce blood glucose by modulating key enzymes of carbohydrate metabolism in diabetic rats [44]. In addition, stevioside and stigmasterol isolated from *Dillenia indica* have been previously reported to exhibit potent antidiabetic and antioxidant properties in animal models of DM [45,46]. Ascorbic acid and oleanolic acid are potent antioxidants that have been utilized as a supplementary or alternative medicine in the management of type II DM [47,48]. Furthermore, α-humulene isolated from *Cordia verbenacea* was shown to possess anti-inflammatory activities in lipopolysaccharide-induced inflamed rat [49]. Therefore, we suggest that the presence of a mixture of these medicinal compounds in the crude

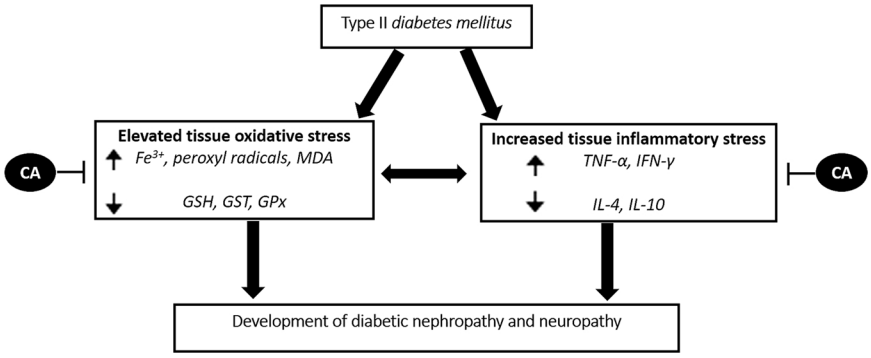


Fig. 9. A proposed pathway for the protective effects of *C. asiatica* in renal and brain tissues of diabetic rats. The administration of CA (*C. asiatica*) extract reduced the over-production of free radicals and pro-inflammatory cytokines in diabetic rats that may be important in halting the development of diabetic nephropathy and neuropathy. Fe^{3+} (ferric ions), MDA (malondialdehyde) GSH (reduced glutathione), GST (glutathione *S*-transferase), GPx (glutathione peroxidase), TNF (tumor necrosis factor), IFN (interferon), IL (interleukin).

extract of the leaves of CA may be responsible for the pharmacological effects observed in the CA-treated diabetic rats in this study.

5. Conclusion

This is the first study demonstrating the *in vivo* effects of *C. asiatica* (CA) on inflammatory cytokines in rat kidney and brain in a diabetic rat model that exhibits both insulin insufficiency and resistance. Data obtained from the present study revealed the promising potential of CA in the management of DM and associated complications that are comparable to the antidiabetic effects of the 'standard' orthodox drug–Metformin. The pathway for the proposed beneficial effects CA against diabetes-induced stress is illustrated in Fig. 9. The modulatory effects on inflammatory cytokines through a reduction in the levels of TNF- α and INF-γ and elevation in levels of IL-4 and IL-10 may be important mechanisms by which CA elicits its tissue protective effects in DM. The protective effect of CA against oxidative stress that is evidenced by an elevation in the synthesis and activities of antioxidants in the tissues of diabetic rats cannot be over-emphasized. These pharmacological actions of CA may be attributed to the presence a plethora of phyto-compounds within its leaves. The combination of these compounds that act with different but synergistic cellular mechanisms may offer improved therapeutic alternatives to diabetic individuals.

6. Competing interests

The authors declare no competing interest.

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