



The methanolic fraction of *Centratherrum anthelminticum* seed downregulates pro-inflammatory cytokines, oxidative stress, and hyperglycemia in STZ-nicotinamide-induced type 2 diabetic rats

Aditya Arya^{a,b,*}, Shiau Chuen Cheah^a, Chung Yeng Looi^a, Hairin Taha^a, Mohd. Rais Mustafa^a, Mustafa Ali Mohd^a

^a Department of Pharmacology, Faculty of medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

^b Department of Pharmacy, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

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ABSTRACT

This study aimed to ascertain the potential of *Centratherrum anthelminticum* seeds methanolic fraction (CAMFs) for the management of type 2 diabetes and its associated complications. CAMFs was initially tested on β-TC6 cells for H₂O₂-induced nuclear factor-κB (NF-κB) translocation effects. The result displayed that CAMFs significantly inhibited NF-κB translocation from cytoplasm into the nucleus, dose-dependently. Furthermore, a 12-week sub-chronic CAMFs study was carried out on streptozotocin (STZ)-nicotinamide-induced type 2 diabetic rat model to evaluate glycemia, essential biochemical parameters, lipid levels, oxidative stress markers, and pro-inflammatory cytokines level. Our study result showed that CAMFs reduced hyperglycemia by increasing serum insulin, C-peptide, total protein, and albumin levels, significantly. Whereas, elevated blood glucose, glycated hemoglobin, lipids and enzyme activities were restored to near normal. CAMFs confirmed antioxidant potential by elevating glutathione (GSH) and reducing malondialdehyde (MDA) levels in diabetic rats. Interestingly, CAMFs down-regulated elevated tumor necrosis factor α (TNF-α), interleukin (IL)-1β and IL-6 in the tissues and serum of the diabetic rats. We conclude that CAMFs exerted apparent antidiabetic effects and demonstrated as a valuable candidate nutraceutical for insulin-resistant type 2 diabetes and its associated complications such as dyslipidemia, oxidative stress, and inflammation.

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1. Introduction

Despite the great strides that have been made in the understanding and management of diabetes, the incidence of the disease

and its complications are increasing unabated. A combination of insulin resistance and an inadequate compensatory insulin secretory response accounts for non-insulin-dependent type 2 diabetes mellitus (DM2). It is the most prevalent disease in the world, affecting 7% of the population, or 285 million people worldwide. If untreated, DM2 may lead to insulin-dependent type 1 diabetes.

Clinical, preclinical, and epidemiological studies indicate an association between oxidative stress and inflammation in the development of DM2 and its complications (Zozulinska and Wierusz-Wysocka, 2006). In DM2, production of reactive oxygen species (ROS) is increased due to insulin resistance and hyperglycemia (Brownlee, 2001). Compared to healthy subjects, DM2 patients have a lower ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), a major endogenous antioxidant. In contrast, malondialdehyde (MDA), a highly toxic by-product generated partially by lipid oxidation and ROS, is increased in patients with diabetes (Evans, 2007). The generated ROS create oxidative stress and exert major effects on signaling pathways, which further affect cellular metabolism and trigger a low-grade inflammatory reaction (Dominiczak, 2003). Lipid accumulation in adipose tissue and expansion of the fat mass in the liver initiate steatosis that

Abbreviations: γ-GT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; ALT, alanine transaminase; ANOVA, analysis of variance; AST, aspartate transaminase; ATCC, American Type Culture Collection; bw, body weight; CA, *Centratherrum anthelminticum*; CAMFs, crude methanolic fraction of *C. anthelminticum* seeds; CRP, C-reactive protein; DM2, type 2 diabetes; DMEM, Dulbecco's modified Eagle medium; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; FBS, fetal bovine serum; FFA, free fatty acid; GSSG, oxidized glutathione; HbA1c, glycated hemoglobin; HCS, high content screening; HDL-C, high-density lipoprotein cholesterol; IKK-β, NF-κB regulatory protein kinase; IL, interleukin; IRS, insulin receptor substrate; LCMS-MS, liquid chromatography–tandem mass spectrometry; LDL-C, low-density lipoprotein cholesterol; NMR, nuclear magnetic resonance; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PKC, protein kinase C; ROS, reactive oxygen species; SD, standard deviation; SH, sulfhydryl; SMC, smooth muscle cell; STZ, streptozotocin; TC, total cholesterol; TG, triglycerides; TNB, 5-thio-2-nitrobenzoic acid.

* Corresponding author at: Department of Pharmacy, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. Tel.: +603 7967 5749; fax: +603 7967 4964.

E-mail addresses: aditya@um.edu.my, adityaarya18@gmail.com (A. Arya).

promotes low-grade inflammation via activation of nuclear factor- κ B (NF- κ B) (Arkan et al., 2005) and provokes an inflammatory process accompanied by local production and secretion of pro-inflammatory cytokines and chemokines (Hotamisligil et al., 1995; Jager et al., 2007). It has been hypothesized that DM2 is a manifestation of an ongoing acute-phase response that is primarily characterized by alterations of the so-called acute-phase proteins, such as C-reactive protein (CRP) (Pickup and Crook, 1998; Pickup et al., 1997), with other cytokines that are central mediators of inflammatory reactions, such as interleukin (IL) 6, IL-1 β , or tumor necrosis factor α (TNF- α). It is well established that cytokines operate as a network in stimulating the production of acute-phase proteins. For example, the effects of IL-6 on CRP synthesis largely depend on its interaction with IL-1 β (Joachim, 2003). The pro-inflammatory cytokine TNF- α reduces insulin sensitivity in muscle tissue and stimulates hepatic lipogenesis and hyperlipidemia (Franckhauser et al., 2008). However, it appears that treatments aimed at reducing the degree of oxidative stress and the production of pro-inflammatory cytokines in DM2 is warranted.

Centratherum anthelminticum (L.) Kuntze (bitter cummin) is a member of the Asteraceae family, an important plant of great significance and usage in Ayurvedic medicine. The records from traditional healers and ethno-botanists state that it is useful in alleviating diabetes. Experimental studies have proven the pharmacological potential of this plant in diverse biological activities, some of which are anti-diabetic, anti-cancer with anti-oxidant and anti-inflammatory activity (Ani and Naidu, 2008; Fatima et al., 2010; Arya et al., 2012a,b,c). Nevertheless, researchers have yet to investigate the hypoglycemic action of sub-chronic administration of *C. anthelminticum* seeds defatted crude methanolic fraction (CAMFs) or the plant's other healing properties, some of which might act against other inflammatory processes and oxidative stress associated with DM2.

Therefore, we attempted to gain a better understanding of the effect of CAMFs on ROS-induced oxidative stress associated with insulin resistance signaling pathway in H₂O₂-induced NF- κ B activation on mouse pancreatic β -TC6 cells. Subsequently, we carried out *in vivo* studies to determine whether long-term administration of CAMFs for 12-weeks exerts anti-hyperglycemic, anti-hyperlipidemic, anti-oxidant, and inflammatory cytokines inhibitory effects in STZ-nicotinamide-induced type 2 diabetic rats.

2. Materials and methods

2.1. Preparation of CAMFs

2.1.1. Collection of plant material

Dried *C. anthelminticum* seeds were procured from the medicinal plant cultivation zone of Amritum Bio-Botanica Herbs Research Laboratory Pvt. Ltd., (Madhya Pradesh, India) in April 2008. The seeds were botanically classified and authenticated by the company's quality control department. Voucher specimens (CA-9) were deposited with the company and with the Department of Pharmacology in the Faculty of Medicine at the University of Malaya.

2.1.2. Extraction and fractionation

Two kilogram of seeds were coarsely powdered and first extracted with 100% n-hexane using hot extraction with a Soxhlet extractor for 24 h. Further fractionation of the obtained defatted residue was carried out using 100% chloroform, and lastly with 100% (absolute) methanol. The solvents from each crude fractions were dried by rotary evaporation under reduced pressure at a maximal temperature of 40 °C. The final fraction was then freeze-dried to yield a crude methanolic fraction (CAMFs), that was stored at -20 °C until further use. Thereafter, CAMFs was subjected to mass spectrometry analysis by using LCMS-MS, for the qualitative analysis of major compounds, as well as evaluated for the total phenolic and flavonoid contents.

2.1.3. Phytochemical analysis of CAMFs by LCMS-MS

Phytochemical analysis of the major compounds in CAMFs was carried out with liquid chromatography-tandem mass spectrometry (LCMS-MS). A triple quadrupole mass spectrometer equipped with a turbo ion spray source (AB Sciex QTrap 5500, Ontario, Canada) was used to obtain the MS/MS data in negative ion mode.

The mobile phase consists of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (ACN), were eluted by gradient elution at a flow rate of 0.4 mL/min with an injection volume of 20 μ L. Separation of the compounds was performed using a Luna 3- μ m RP C18 column (100 \times 2.00 mm; Phenomenex). The turbo ion source settings were as follows: capillary voltage, -4000 V; dry gas flow (N₂), 9 L/min; nebulizer pressure, 35 psi; and capillary temperature, 365 °C. A full scan of the mass spectra was recorded from m/z 50 to m/z 1000. The acquisition data was processed with Analyst Software version 1.5.1. Compounds were characterized based on their UV spectra and MS² and MS³ fragmentations spectra data by correlation with previous reports (Table 1). Whereas, compounds F, G, H, I and J in the table are unknown compounds.

2.1.4. Determination of total phenolic content

The total phenolic content in CAMFs was determined by adapting the method as published in our previous article (Arya et al., 2012a,b). In brief, CAMFs was initially prepared in methanol with concentration of 10 mg/mL. From this solution 5 μ L was transferred to 96-well microplate (TPP, USA). To this, 80 μ L of Folin-Ciocalteu reagent (1:10) were added and mixed thoroughly. After 5 min, 160 μ L of sodium bicarbonate solution (NaHCO₃ 7.5%) were added and the mixture was allowed to stand for 30 min with intermittent shaking. Absorbance was measured at 765 nm using microplate reader (Molecular Devices, Sunnyvale, USA). The TPC was expressed as gallic acid equivalent (GAE) in mg/g fraction, obtained from the standard curve of gallic acid.

The gallic acid standard curve was established by plotting concentration (mg/mL) versus absorbance (nm) ($y = 0.001x + 0.045$; $R^2 = 0.9975$), where y is absorbance and x is concentration in GAE ($n = 3$).

2.1.5. Determination of total flavonoid content

The total flavonoid content in CAMFs was determined by following the method published in our previous article (Arya et al., 2012a,b). In brief, 5 mL of 2% aluminum trichloride was mixed with the same volume of CAMFs. Absorbance readings at 415 nm were taken after 10 min against a blank sample consisting of 5 mL of sample solution and 5 mL of methanol without aluminum trichloride. The total flavonoid content was determined using a standard curve of mg Quercetin (Q) equivalents. The average of three readings was used and then expressed as quercetin equivalents (QE) on a dry weight (DW) basis.

2.2. *In vitro* assay

2.2.1. Cell culture

Mouse pancreas β -TC6 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in 15% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM). Cultures were maintained at 37 °C in 5% CO₂ in a humidified incubator. The growth medium was changed every 3 days.

2.2.2. NF- κ B translocation assay

We seeded 1.5×10^4 cells/mL onto a 96-well plate. The cells were pre-treated for 1 h with 6.25, 12.5, or 25 μ g/mL CAMFs, or were left untreated. The cells were then stimulated for NF- κ B translocation with 50 μ M of H₂O₂ for 30 min. NF- κ B staining was performed according to the manufacturer's instructions with an NF- κ B activation kit (Cellomics Inc., Pittsburgh, PA, USA). We used the ArrayScan high content screening (HCS) system (Cellomics Inc., Pittsburgh, PA, USA) to quantify the difference between the intensity of nuclear and cytoplasmic NF- κ B-associated fluorescence.

2.3. Preclinical studies

2.3.1. Experimental animals

We obtained Sprague-Dawley rats weighing 180–200 g from the Animal Care Unit of the University Malaya Medical Centre (Kuala Lumpur, Malaysia) and maintained them under pathogen-free conditions in the animal housing unit in a temperature (23 \pm 2 °C) and light-controlled (12-h light/dark cycle) room with 35–60% humidity. The animals were acclimatized for 10 days prior to the experiments and were provided rodent chow and water *ad libitum*.

The animal experiments were performed in accordance with the guidelines for animal experimentation issued by the Animal Care and Use Committee at the University of Malaya (Ethics Number: FAR/10/11/2008/AA[R]) and was conducted in accordance with internationally accepted principles for laboratory animal use and care.

2.3.2. Oral acute toxicity studies

CAMFs oral acute toxicity tests were carried out according to the guidelines of the Organization for Economic Co-operation and Development (OECD). For these test, we used healthy adult Sprague Dawley rats of either sex (180–200 g). These rats were fasted overnight, divided into 6 groups ($n = 6$), and orally fed with CAMFs in doses of 10, 20, 50, 100 and 500 mg/kg; the control group was given distilled water. We observed the rats for 1 h continuously and then hourly for 4 h for any

Table 1
Characterization of phenolic compounds in CAMFs by LC–MS/MS.

	Tentative compounds	[M–H] [−] (m/z)	Major fragment ions (m/z)	UV λ _{max} (nm)	References
A	Quercetin glycoside C ₂₁ H ₂₀ O ₁₂	463	301, 287, 271	220, 300	Lin and Harnly (2007)
B	3,4–O-Dicaffeoylquinic acid C ₂₅ H ₂₄ O ₁₂	515	353, 191, 179, 135	240, 300	Gouveia and Castilho (2011) and Lin and Harnly (2008)
C	Caffeic acid C ₉ H ₈ O ₄	179	135	220, 325	Luo et al. (2003), Lin and Harnly (2008) and Plazonic et al., 2011
D	Naringenin-7–O-glucoside C ₂₁ H ₂₂ O ₁₀	433	271, 153	200, 300	Charrouf et al., (2007)
E	Kaempferol C ₁₅ H ₁₀ O ₆	287	287, 151	220, 340	Sun et al. (2007)
F	Unknown	377	253, 215, 135	200,300	–
G	Unknown	303	181, 167, 135	220,300	–
H	Unknown	341	303, 167, 135	200,300	–
I	Unknown	539	503, 377, 221	200,300	–

changes in the blood glucose levels and finally after every 24 h up to 14 days for any physical signs of toxicity, such as writhing, gasping, palpitation and decreased respiratory rate or for any lethality.

2.3.3. Induction of DM2

DM2 was induced by following the methods of Masiello et al. (1998) with slight modifications. In brief, after standardization of STZ (Sigma-Aldrich, St. Louis, MO, USA) doses, single intraperitoneal injection of freshly prepared STZ (55 mg/kg b.wt) in 0.1 M citrate buffer (pH 4.5) in a volume of 1 ml/kg b.wt was injected to overnight fasted normal male rats, 15 min after i.p administration of nicotinamide (210 mg/kg). Hyperglycemia was confirmed by elevated blood glucose levels, determined at 96 h after the STZ-nicotinamide administration. Rats with fasting blood glucose range of 11–14 mmol/L were considered as type 2 diabetic and further used for the study.

2.3.4. Experimental procedure

The rats were divided into the following 6 groups (9 or 10 rats per group): normal control rats, diabetic control rats, diabetic rats treated with 50 mg/kg bw of glibenclamide (a standard drug), and diabetic rats treated with 50, 25, and 10 mg/kg bw of CAMFs, respectively. All groups were fed their respective doses of CAMFs or glibenclamide once daily for 12 weeks. After the 12-week treatment period, all 6 groups were fasted for 12 h and then anesthetized using pentobarbital; the blood was collected into heparinized tubes. Any residual blood was removed by perfusion using phosphate-buffered saline (PBS; pH 7.4) through the abdominal aorta. The blood was centrifuged at 2000 rpm for 10 min and the serum was collected and stored at –80 °C until analysis. The liver, kidney, and pancreas were removed, washed in ice-cold isotonic saline, and blotted individually on ash-free filter paper; the organs were weighed and tissues were collected and fixed in 10% formalin for histology and markers estimation. Tissues were then homogenized in ice-cold 5% metaphosphoric acid or PBS (pH 7.4). The homogenates collected were used for the enzyme estimations before being centrifuged at 4500 rpm for 30 min at 4 °C, and the supernatant was collected for the analysis of oxidative stress markers and pro-inflammatory cytokines.

2.3.5. Assessment of biochemical parameters

Glycemia in fasted animals with free access to water was quantified weekly from tail vein blood using a standardized glucometer (Accu-Chek; Roche, Mannheim, Germany) until the end of the treatment period, as well as intake of food was also monitored on daily basis. Serum insulin and C-peptide levels were measured using a radioimmunoassay kit (Packard, USA) according to the manufacturer protocol. Glycated hemoglobin (HbA1c) was estimated by a DCA 2000 device (Bayer, Sunnyvale, CA, USA). Serum total protein, albumin and lipids, i.e., triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and free fatty acid (FFA) levels were measured in triplicate using an automatic biochemical analyzer (Beckman-700, Fullerton, CA, USA). The aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and γ -glutamyl transpeptidase (γ -GT) enzymes in serum and in liver and kidney tissues were analyzed according to the method in King and Armstrong (1988), and Rosalki and Rau (1972).

2.3.6. Assessment of oxidative stress markers

The supernatants collected after centrifugation of the liver, kidney, and pancreas homogenates were used to determine the GSH and MDA levels. GSH was measured as described in Draper and Hadley (1990) using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent), which produces a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The absorbance of TNB at 412 nm provides an accurate estimation of the level of GSH in a sample. A lipid peroxidation assay was used to determine the amount of MDA, an end product of fatty acid peroxidation that reacts with thiobarbituric acid to form a colored complex with a maximum absorbance at 532 nm, as stated in Shain and Gusmuslu (2007).

2.3.7. Assessment of pro-inflammatory cytokines

Serum and the supernatant collected after centrifugation of the pancreas and kidney homogenates were used for the measurement of TNF- α , IL-1 β , and IL-6 levels with a rat TNF- α , IL-1 β , and IL-6 ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer protocol.

2.4. Statistical analysis

All values are expressed as mean \pm standard deviation (SD). The significant differences between the means of the experimental groups was determined with analysis of variance (ANOVA), followed by a Tukey–Kramer multiple comparisons test (Graph Pad version 5.0; Graph Pad Software Inc., San Diego, CA, USA).

3. Results

3.1. Total phenolic and flavonoid contents in CAMFs

The final yield of the obtained CAMFs was 12.6% w/w. The total phenolic and flavonoid contents in CAMFs was determined to be (665.3 \pm 188.8 mg GAE/g, and 98.2 \pm 27.6 mg Quercetin/g).

3.2. CAMFs analysis by LCMS–MS

The LCMS–MS phytochemical analysis detected quercetin glycoside, 3,4–O-dicaffeoylquinic acid, caffeic acid, naringenin-7–O glucoside and kaempferol as the major compounds in CAMFs, as well as with other unknown compounds (Table 1 and Fig. 1). The fragmentation patterns of known compounds were in agreement with those documented in the literatures as reported in our other study (Arya et al., 2012c).

3.3. Effect of CAMFs on NF- κ B activation

We tested CAMFs for its *in vitro* inhibitory effects against H₂O₂-induced NF- κ B translocation. NF- κ B was detected in the cytoplasm, but not in the nucleus of non-H₂O₂-induced cells. There was a significant increase in NF- κ B staining in the nucleus area when cells were stimulated with H₂O₂ alone, suggesting that NF- κ B translocated from the cytoplasm into the nucleus. However, treatment with 25 μ g/mL CAMFs significantly inhibited H₂O₂-induced NF- κ B translocation (Fig. 2).

3.4. Acute toxicity study

No lethality or toxic reactions were observed at any graded doses of CAMFs up to a dosage of 500 mg/kg. Hence, produced no alterations in the blood glucose levels, and on the general behavior or appearance of the rats during the whole experimental period. Based on these findings, the concentration was fixed as 50, 25 and 10 mg/kg bw; which were used as the maximum doses for

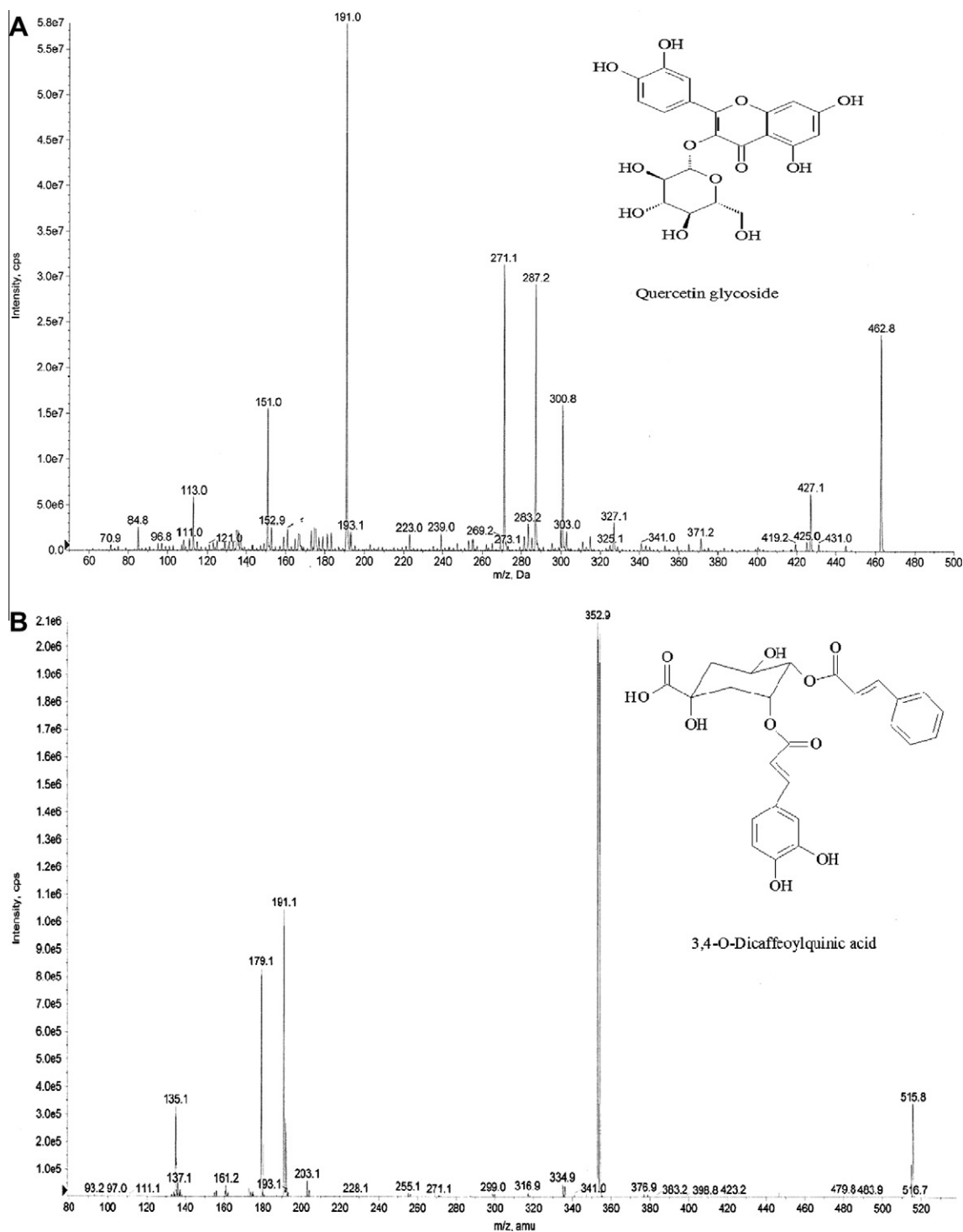


Fig. 1. (A) The mass spectrometric characterization of compound A indicates the presence of quercetin glycosides. (B) The fragmentation pattern of compound B displays an ion peak at m/z 515, indicating the loss of the first caffeoyl, loss of the second caffeoyl at m/z 353, and loss of the third caffeoyl at m/z 191 to yield quinic acid, a loss of caffeic acid at m/z 179, and another caffeoyl at m/z 135. Thus, the compound was characterized as 3,4-O-dicaffeoylquinic acid. (C) Compound C exhibits fragment ions at m/z 179 and m/z 135, the characteristic ions of caffeic acid. (D) Compound D was identified as naringenin-7-O-glucoside based on mass fragment ions at m/z 433 and at m/z 271 and 153. (E) Compound E was identified as kaempferol based on mass fragments at m/z 287 and m/z 151.

the subsequent experiments involving oral administration as reported in our other study on CAMFs (Arya et al., 2012c).

3.5. Effect of CAMFs on blood glucose levels

From the first day up until the end of the 12-week treatment period, monitoring of the rats' fasting blood glucose levels revealed a significant reduction in the elevated blood glucose levels of

diabetic rats treated with CAMFs compared to that of untreated diabetic rats (Table 2). At weeks 3 and 6, the inhibition percentage of blood glucose levels in CAMFs-treated diabetic rats was lower compared to that of glibenclamide-treated diabetic rats. At weeks 9 and 12, the percentage inhibition in rats treated with a higher concentration of CAMFs was greater than that in glibenclamide-treated rats. At the end of the treatment period, the percentage inhibition effected by 50, 25, and 10 mg/kg bw CAMFs was

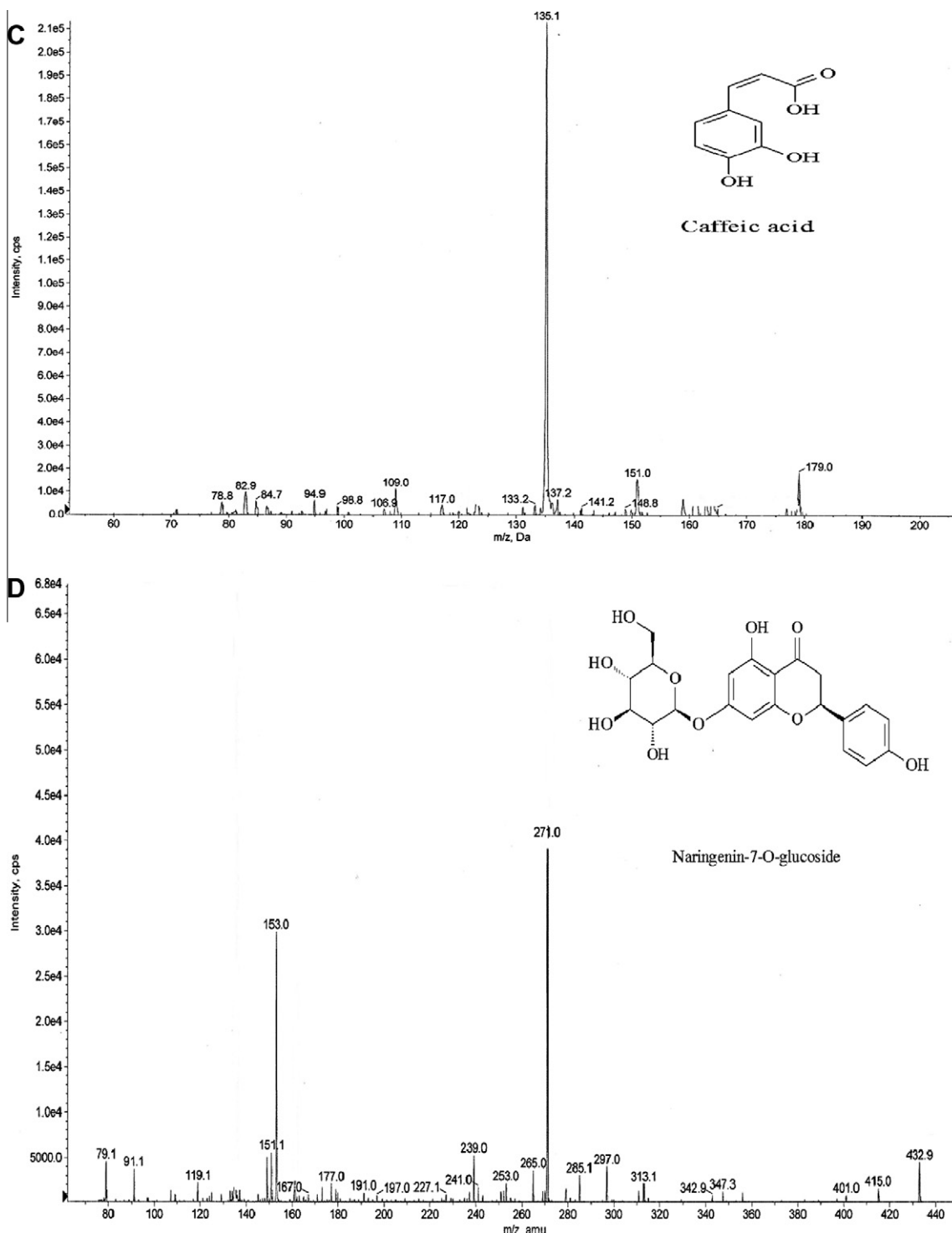


Fig. 1. (continued)

75.86%, 69.82%, and 65.08%, respectively, compared to that of glibenclamide (68.53%). The blood glucose levels of normal rats were not greatly altered after week 12 when compared to that of week 0, whereas that of the untreated diabetic rats increased to 23.2 mmol/L after 12 weeks as compared to 12.3 mmol/L at week 0.

3.6. Effect of CAMFs on serum biochemical parameters

After the 12-week treatment period, serum insulin, C-peptide, total protein, and albumin levels in diabetic untreated rats were significantly reduced, while their HbA1c levels were significantly

elevated compared to that of the normal control group. Upon treatment with CAMFs or glibenclamide, serum insulin, C-peptide, total protein, and albumin levels in diabetic rats were significantly elevated, and CAMFs significantly inhibited HbA1c levels in a dose-dependent manner compared to that of the diabetic control rats (Fig. 3), indicating significant improvement in glycemic control by CAMFs in diabetic rats.

In addition, there was a significant improvement in the overall body weights of CAMFs-treated diabetic rats compared to untreated diabetic rats at the end of the treatment period, and we recorded weight recovery in the liver and kidney. Whereas

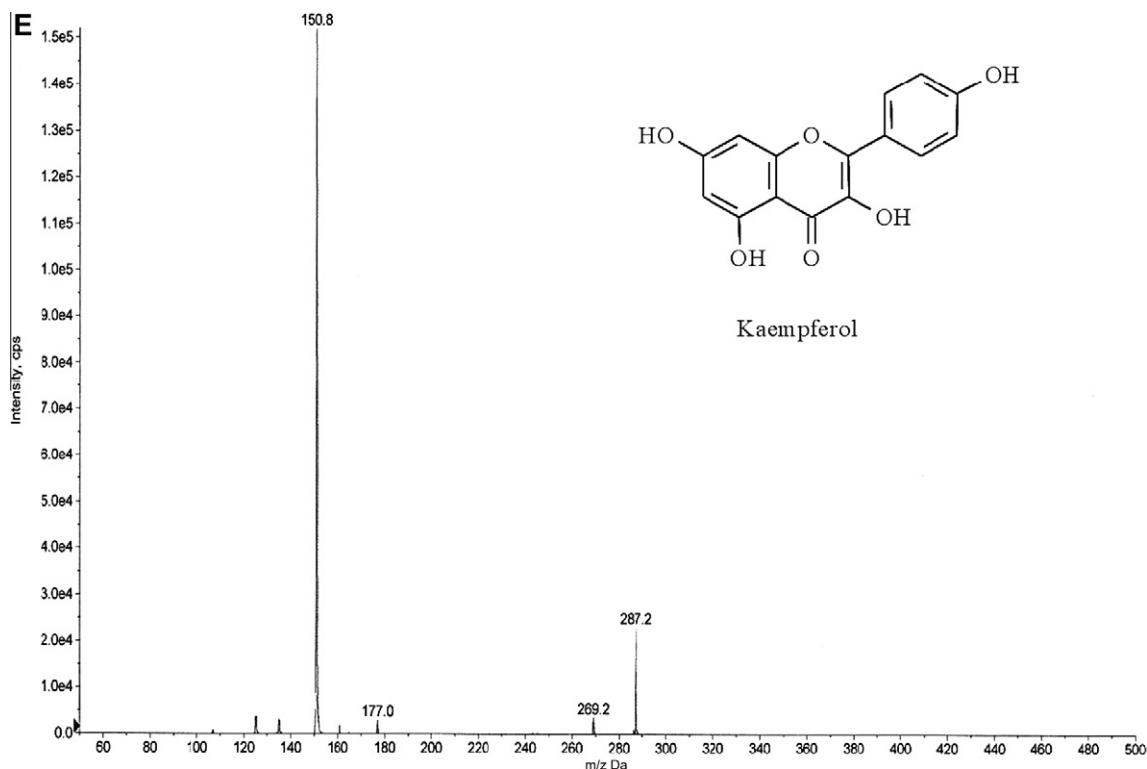


Fig. 1. (continued)

there was a significant reduction in the daily consumption of food by diabetic treated rats (Fig. 4).

3.7. Effect of CAMFs on hyperlipidemia

Fig. 5 depicts the effect of CAMFs and glibenclamide on serum TG, TC, HDL-C, LDL-C, and FFA activity in treated and untreated diabetic rats and in normal control rats. Serum TG, TC, LDL-C, and FFA levels in untreated diabetic rats were significantly elevated when compared to that of normal control rats, while the HDL-C levels in untreated diabetic rats were significantly decreased compared to those in normal rats.

Serum TG, TC, LDL-C, and FFA levels were significantly reduced after treatment with glibenclamide and CAMFs; CAMFs effected a dose-dependent reduction, and HDL-C levels were significantly elevated compared to that of untreated diabetic rats after the 12-week treatment period. Evidently, continuous treatment with CAMFs decreased these lipid parameters in diabetic rats to near normal levels.

3.8. Effect of CAMFs on enzyme markers

Table 3 illustrates the effects of CAMFs on AST, ALT, ALP, and γ -GT enzyme activities in the serum, liver, and kidney of treated and untreated diabetic rats and in that of normal rats. The enzyme activities in the serum and liver of the untreated diabetic rats were significantly increased; ALP and γ -GT activities in the kidney were increased, while AST and ALT activities were not affected. Near-normalization of the AST, ALT, ALP, and γ -GT activities in the serum, liver, and kidney of diabetic rats was achieved after 12-week administration of glibenclamide and CAMFs; CAMFs effected normalization dose-dependently.

3.9. Effect of CAMFs on oxidative stress markers

GSH levels in untreated diabetic rats were reduced while MDA levels were significantly elevated compared to that of normal control rats (Fig. 6). Compared to untreated diabetic rats, GSH levels

in the liver and pancreas of diabetic rats were significantly elevated upon administration of glibenclamide and CAMFs; CAMFs effected elevation dose-dependently, although not much change was observed in kidney GSH levels. There was maximum reduction of MDA levels in the liver of diabetic rats treated with glibenclamide and CAMFs compared to that of untreated diabetic control rats; reduction was effected dose-dependently by CAMFs, while there was a significant reduction of MDA in the pancreas following administration of glibenclamide and 50 mg/kg bw CAMFs. In the kidney, only glibenclamide suppressed MDA levels significantly, with CAMFs effecting a slight reduction in a dose-dependent manner.

3.10. Effect of CAMFs on pro-inflammatory cytokines level

The levels of pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 in the pancreas, kidney, and serum of diabetic untreated rats were significantly elevated compared to that of normal control rats (Fig. 7). Compared to untreated diabetic rats, the levels of TNF- α in the pancreas, kidney and serum of diabetic rats were significantly down-regulated by daily administration of glibenclamide and CAMFs after the 12-week period; CAMFs down-regulated these parameters dose-dependently. In the same manner, glibenclamide and CAMFs significantly reduced the IL-1 β and IL-6 levels in the pancreas, with non-significant reduction being observed in the kidney and serum after the 12-week study period.

4. Discussion

In an effort to uncover novel and effective treatment from plants traditionally used in India for the management of diabetes mellitus and its associated complications, we evaluated the hypoglycemic effects of a number of plants in an *in vivo* experiment and selected the most potent plant, *C. anthelminticum*, (Arya et al., 2012c) whose seed yields a crude fraction that exerts maximum glycemic control. To the best of our knowledge, this is the first

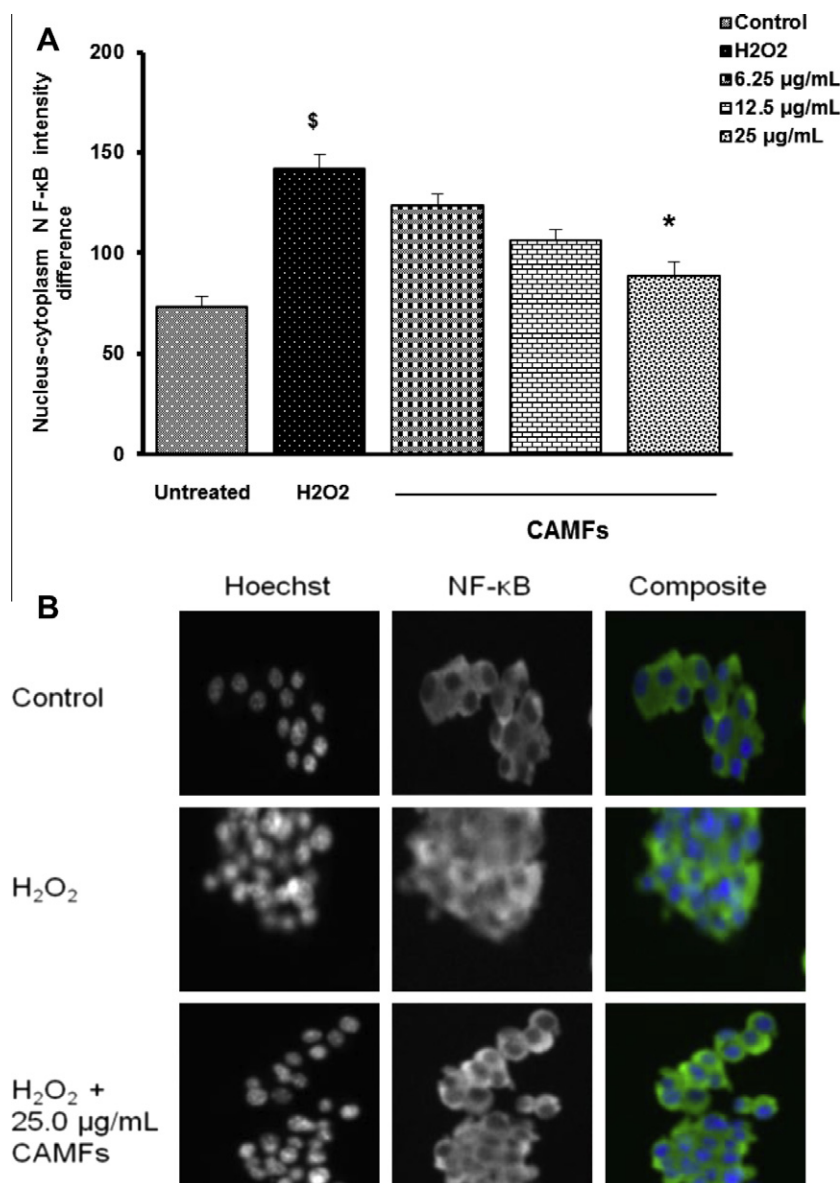


Fig. 2. Stained β -TC6 cells were treated with different concentrations of CAMFs for 1 h and stimulated for 30 min with 50 μ M H₂O₂ (NF- κ B activation). (A) Dose-response histogram of CAMFs-treated β -TC6 cells for quantitative image analysis of NF- κ B translocation. (B) Representative images of control, H₂O₂, and H₂O₂ pre-treated cells treated with 25.0 μ g/mL CAMFs. ^aSignificant difference compared to non-H₂O₂-treated cells ($P < 0.05$). ^{*}Significant difference compared to H₂O₂-treated cells ($P < 0.05$).

Table 2

Effects of CAMFs and glibenclamide on blood glucose levels of normal, diabetic control, and diabetic treated rats.

Group	Fasting blood glucose level (mmol/L)				
	Pretreatment period	Treatment period			
		Week 0	Week 3	Week 6	Week 9
Normal control	3.9 ± 0.23	3.4 ± 0.26	4.2 ± 0.32	4.1 ± 0.39	4.6 ± 0.37
Diabetic control	12.3 ± 0.44	14.9 ± 0.64	17.8 ± 0.89	19.9 ± 1.09	23.2 ± 2.03
Glibenclamide (50 mg/kg)	12.2 ± 0.65	6.9 ± 0.33 ^a (53.69)	6.6 ± 0.48 ^a (62.92)	7.0 ± 0.76 ^a (64.82)	7.3 ± 0.79 ^a (68.53)
CAMFs (50 mg/kg)	11.9 ± 0.53	7.8 ± 0.34 ^a (47.65)	6.7 ± 0.32 ^a (62.35)	6.2 ± 0.48 ^a (68.84)	5.6 ± 0.46 ^a (75.86)
CAMFs (25 mg/kg)	12.1 ± 0.46	8.8 ± 0.65 ^a (40.93)	7.5 ± 0.47 ^a (57.85)	7.1 ± 0.65 ^a (64.32)	7.0 ± 0.57 ^a (69.82)
CAMFs (10 mg/kg)	12.3 ± 0.51	9.8 ± 0.43 ^a (34.22)	8.7 ± 0.77 ^a (51.12)	8.9 ± 0.70 ^a (55.27)	8.1 ± 0.83 ^a (65.08)

Values denote mean ± SD, $n = 9-10$.

Values in parentheses denote that group's percentage decrease in blood glucose level when compared to the diabetic control group in that week.

^a Mean values that are significantly different from diabetic control group as revealed by the Tukey-Kramer multiple comparisons test ($P < 0.05$).

in vitro cell-based and *in vivo* preclinical study on the crude methanolic fraction of the seed (CAMFs), which demonstrated great therapeutic utility in the management of complications associated with type 2 diabetes.

We evaluated the effects of CAMFs in an animal model of insulin resistance, STZ-nicotinamide-induced DM2, which produces greatly similar features to DM2 in rats (Like and Rossini, 1976; Shima et al., 1998). Daily administration of varying concentrations

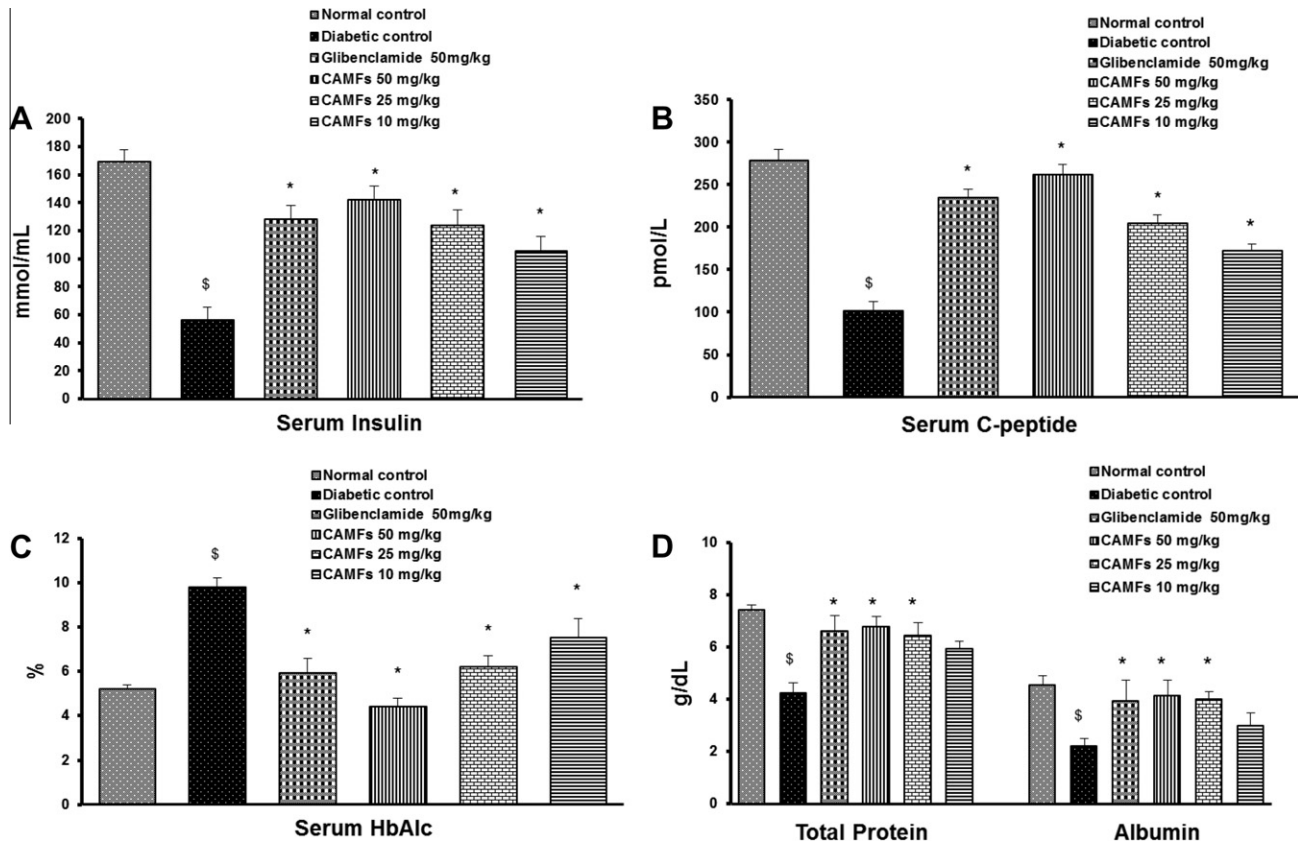


Fig. 3. Effects of CAMFs on serum biochemical parameters of STZ-nicotinamide-induced diabetic rats in comparison with normal and diabetic control rats after the 12-week treatment period. At the end of the treatment, rats were fasted for 12 h and blood was drawn to collect the serum. Panels denote (A) insulin, (B) C-peptide, (C) HbA1c, and (D) total protein and albumin levels. Data are presented as means \pm SD ($n = 9-10$). $^{\$}$ Significant difference compared to normal control group ($P < 0.05$). * Significant difference compared to diabetic control ($P < 0.05$).

of CAMFs to diabetic rats for 12 weeks significantly reduced blood glucose levels, thereby increased insulin and C-peptide levels. Elevated insulin levels in diabetics usually normalize the serum and tissue proteins by increasing protein synthesis, decreasing protein degradation or protein glycosylation, supporting our study (Almdal and Vilstrup, 1988). In addition, we observed a reduction in HbA1c level. High HbA1c level has been linked to micro- and macrovascular diabetes complications (Selvin et al., 2004), while decline in HbA1c level reduced morbidity and mortality (Wagner et al., 2001). The recovery of body and organ weights observed in the CAMFs-treated diabetic rats could be due to an improvement in insulin secretion and glycemic control produced by CAMFs. In line with the normalized serum lipid levels, CAMFs restored elevated levels of the enzyme markers AST, ALT, ALP, and γ -GT to normal in the serum, liver, and kidney of diabetic rats. This suggests that CAMFs can be helpful in preventing hepatocellular damage and tissue necrosis through suppression of gluconeogenesis.

Furthermore, we studied the role of ROS in the development of DM2 by activating stress signaling pathways known to participate in insulin signaling pathways, such as the ROS-induced NF- κ B activation pathway (Bierhaus et al., 2001; Mohamed et al., 1999; Schreck et al., 1992). Our study result demonstrated that CAMFs inhibited the H_2O_2 -induced NF- κ B translocation from the cytoplasm into the nucleus in β -TC6 cells (Fig. 2). Moreover, in line with the inhibitory effects of NF- κ B translocation, we confirmed antioxidant effects by investigating oxidative stress markers, i.e., GSH and MDA levels in CAMFs treated and untreated diabetic rats, which showed that CAMFs dose-dependently augmented GSH production in the pancreas, kidney, and liver of diabetic rats, with reduction

caused in MDA levels, suggesting CAMFs antioxidant ability. It is well established that GSH is an intracellular antioxidant with several biological functions, such as cellular protection against oxidation, which is one of the more important GSH functions because its sulfhydryl (SH) group is a strong nucleophile that confers antioxidant protection and protects DNA, proteins, and other biomolecules from ROS (Fang et al., 2002). In this regard, an increased level of GSH implicates augmentation of the antioxidant capacity and reduced peroxidation of membrane lipids, whose principal end product is MDA, which is a marker of damage caused by oxidative stress (Johansen et al., 2005; Pastore et al., 2003). However, we should not disregard the fact that the antioxidant potential of CAMFs may be, in part, a result of the reduction in hyperglycemia and elevated pro-inflammatory cytokines levels as observed in the diabetic rats.

As we know, DM2 is linked with oxidative stress resulting from free radicals/ROS. ROS act as intercellular second messengers downstream of many signaling molecules, including transcription factors (NF- κ B), which mediate vascular smooth muscle cell (SMC) growth/migration and the expression of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 (Reuter et al., 2010; Touyz, 2004). These elevated pro-inflammatory cytokines possess antagonistic properties to insulin because of their ability to augment insulin receptor substrate (IRS) phosphorylation, leading to insulin resistance (Emanuelli et al., 2000; Senn et al., 2003; Steinberg, 2007; Tataranni and Ortega, 2005). Therefore, inhibition of H_2O_2 -induced NF- κ B translocation in β -TC6 cells and ameliorating oxidative stress in diabetic rats explains an associative relationship between the inflammatory cytokines and type 2 diabetes, as shown by our study results on the elevated levels of TNF- α , IL-1 β , and IL-6

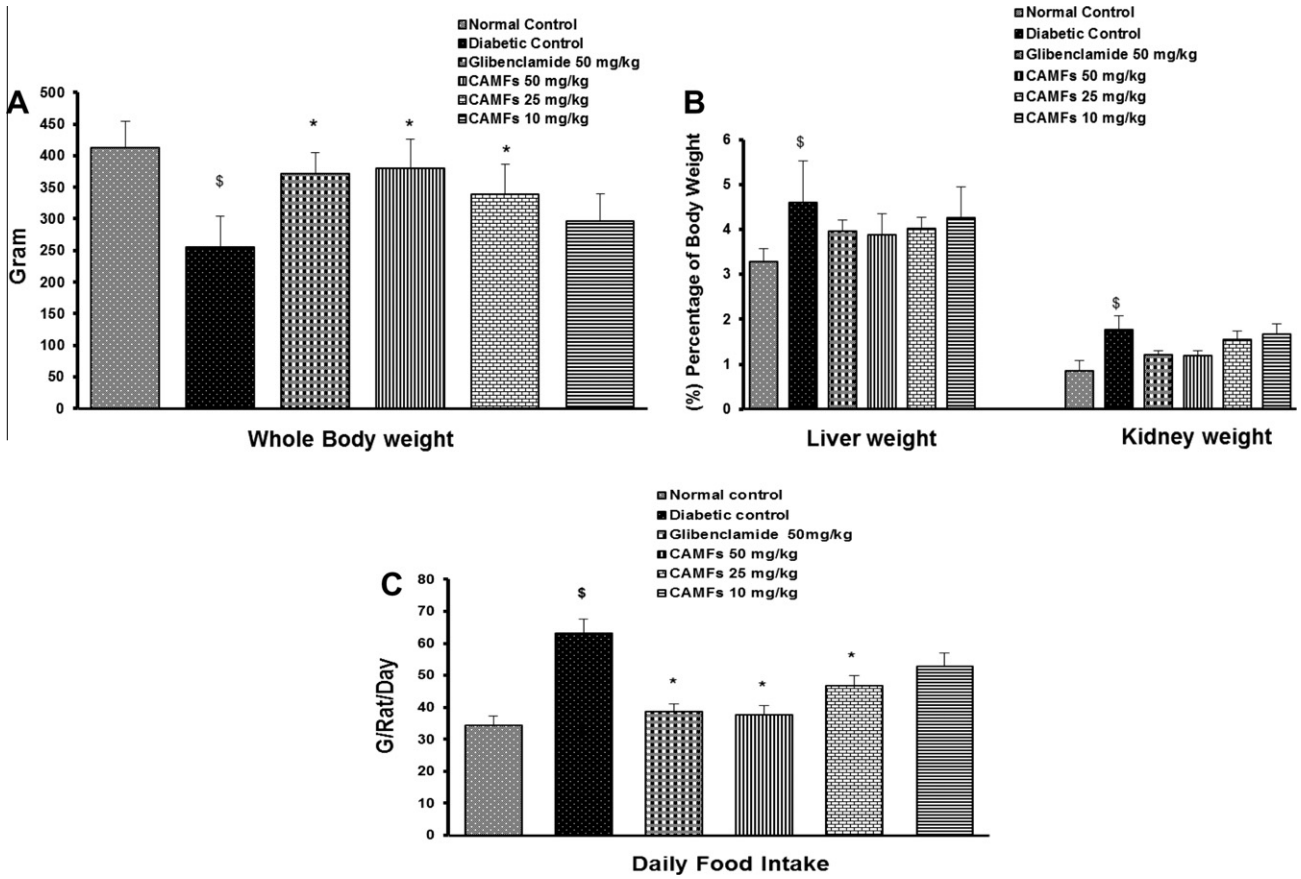


Fig. 4. Effects of CAMFs on (A) whole body weight (B) weight of liver and kidney and (C) daily food intake of STZ-nicotinamide-induced diabetic rats after 12 weeks in comparison with normal and diabetic control rats. Data are presented as means \pm SD ($n = 9-10$). ^{\$}Significant difference compared to normal control group ($P < 0.05$). ^{*}Significant difference compared to diabetic control ($P < 0.05$).

in the serum and tissues of CAMFs treated diabetic rats, suggesting beneficial anti-inflammatory effect of CAMFs on insulin resistance in DM2.

Taken together, our data indicate that 50 mg/kg bw of CAMFs possessed effects that were highly similar to the same dose of glibenclamide. Interestingly, this might be due to the presence of

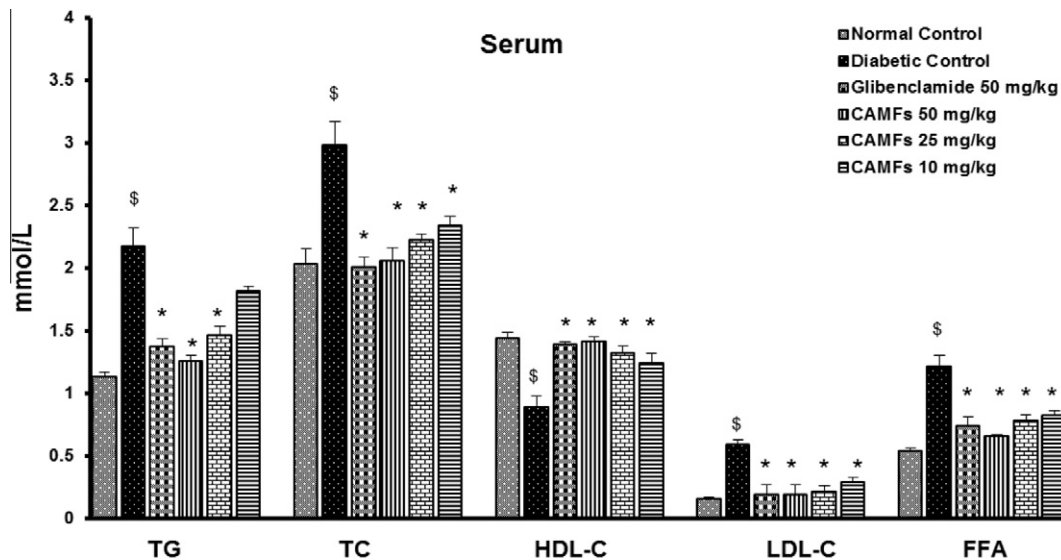


Fig. 5. Effects of CAMFs on serum lipid profiles of STZ-nicotinamide-induced diabetic rats in comparison with normal and diabetic control rats after the 12-week treatment period. At the end of the treatment, rats were fasted for 12 h and blood was drawn to collect the serum. Figure represents TG, TC, HDL-C, LDL-C, and FFA levels in 6 groups. Data are presented as means \pm SD ($n = 9-10$). ^{\$}Significant difference compared to normal control group ($P < 0.05$). ^{*}Significant difference compared to diabetic control ($P < 0.05$).

Table 3
Effect of CAMFs and glibenclamide on enzymes marker in the serum, liver, and kidney of STZ-nicotinamide-induced diabetic rats in comparison with normal and diabetic control rats after the 12-week treatment period.

Group	AST ^a	ALT ^b	ALP ^c	γ-GT ^d
<i>Serum</i>				
Normal control	49.9 ± 3.66 ^e	43.7 ± 2.64 ^e	67.1 ± 3.14 ^e	19.7 ± 1.63 ^e
Diabetic control	87.5 ± 3.73	72.2 ± 3.65	107.3 ± 4.49	33.4 ± 1.05
Glibenclamide (50 mg/kg)	55.4 ± 2.92 ^e	48.6 ± 2.03 ^e	69.4 ± 4.23 ^e	21.1 ± 1.01 ^e
CAMFs (50 mg/kg)	52.3 ± 2.53 ^e	44.2 ± 1.36 ^e	57.6 ± 2.07 ^e	19.8 ± 1.75 ^e
CAMFs (25 mg/kg)	62.2 ± 3.23 ^e	57.8 ± 2.02 ^e	77.4 ± 5.32 ^e	25.7 ± 1.26
CAMFs (10 mg/kg)	73.7 ± 3.12	63.7 ± 2.47	81.2 ± 6.41 ^e	30.1 ± 2.73
<i>Liver</i>				
Normal control	723.7 ± 13.6 ^e	883.8 ± 17.25 ^e	0.19 ± 0.52 ^e	3.11 ± 0.26 ^e
Diabetic control	937.2 ± 20.18	1259.6 ± 26.31	0.36 ± 0.21	5.53 ± 1.21
Glibenclamide (50 mg/kg)	754.3 ± 17.38 ^e	905.2 ± 16.42 ^e	0.22 ± 0.23	3.31 ± 0.87
CAMFs (50 mg/kg)	757.5 ± 18.23 ^e	876.8 ± 22.43 ^e	0.20 ± 0.13	3.01 ± 0.13 ^e
CAMFs (25 mg/kg)	807.2 ± 19.36	923.4 ± 21.41	0.29 ± 0.76	3.82 ± 0.17
CAMFs (10 mg/kg)	893.4 ± 22.46	1033.5 ± 21.32	0.33 ± 0.26	4.23 ± 0.72
<i>Kidney</i>				
Normal control	848.2 ± 19.31	935.7 ± 21.67	0.43 ± 0.16 ^e	2.96 ± 0.87 ^e
Diabetic control	839.4 ± 18.36	929.4 ± 21.61	0.98 ± 0.56	4.21 ± 0.74
Glibenclamide (50 mg/kg)	852.4 ± 24.26	927.3 ± 24.18	0.50 ± 0.32 ^e	3.02 ± 0.21
CAMFs (50 mg/kg)	846.3 ± 22.43	944.5 ± 20.78	0.39 ± 0.13 ^e	2.79 ± 0.32 ^e
CAMFs (25 mg/kg)	826.1 ± 18.21	903.2 ± 20.13	0.58 ± 0.34	3.16 ± 0.56
CAMFs (10 mg/kg)	818.9 ± 21.23	896.5 ± 22.79	0.68 ± 0.23	3.53 ± 0.19

Values denote mean ± SD, *n* = 9–10.

Units of measurement (per L) for AST and ALT: μmol of pyruvate liberated/h; ALP: μmol of phenol liberated/min; γ-GT: mol of p-nitroaniline liberated/min.

^a Aspartate transaminase.

^b Alanine transaminase.

^c Alkaline phosphatase.

^d γ-Glutamyltranspeptidase.

^e Mean values that are significantly different from diabetic control in the same group, as revealed by the Tukey–Kramer multiple comparisons test (*P* < 0.05).

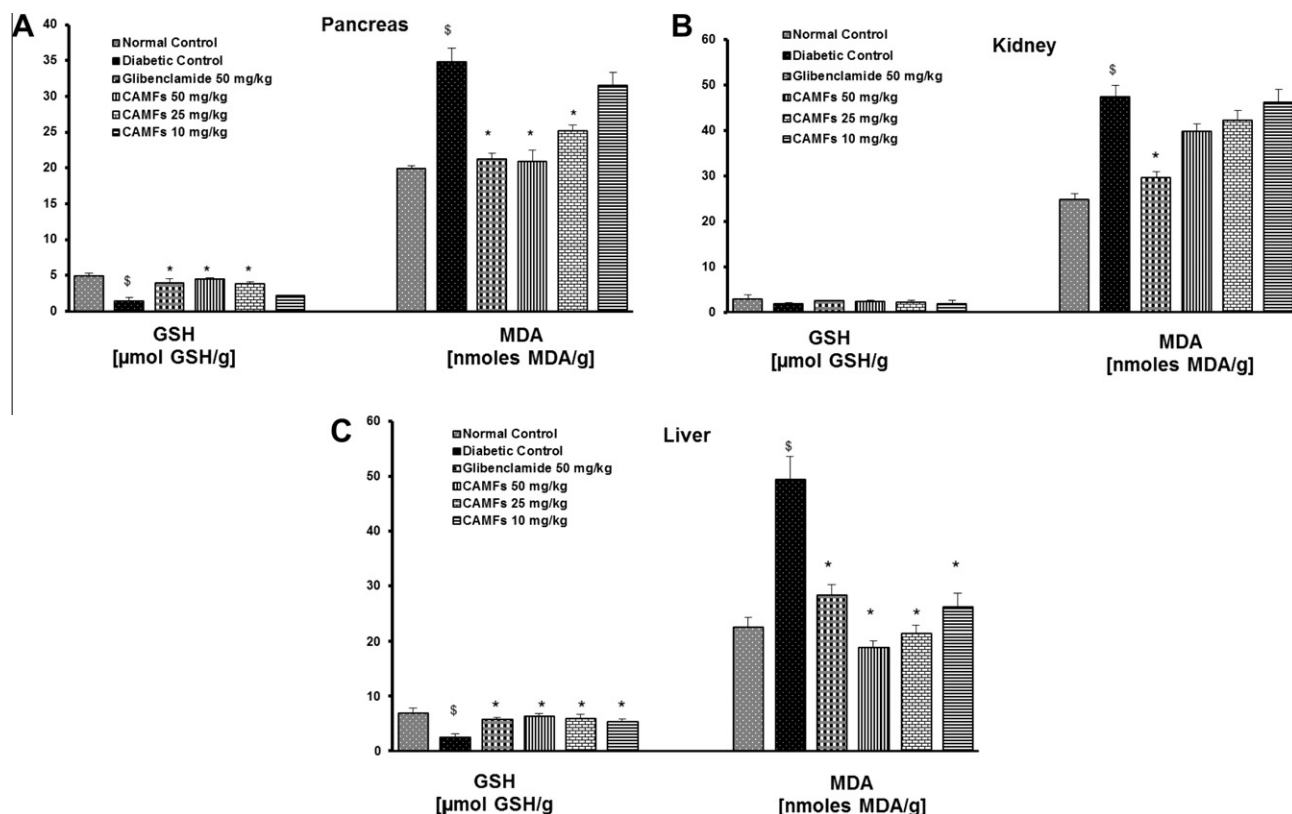


Fig. 6. Antioxidant effect of CAMFs on the pancreas, kidney, and liver of STZ-nicotinamide-induced diabetic rats in comparison with normal and diabetic control rats after the 12-week treatment period. At the end of the treatment, the organs were removed, homogenized, and centrifuged to collect the tissue supernatant. Panels denote (A) pancreas, (B) kidney, and (C) liver, GSH and MDA levels respectively. Data are presented as means ± SD (*n* = 9–10). \$Significant difference compared to the normal control group (*P* < 0.05). *Significant difference compared to the diabetic control (*P* < 0.05).

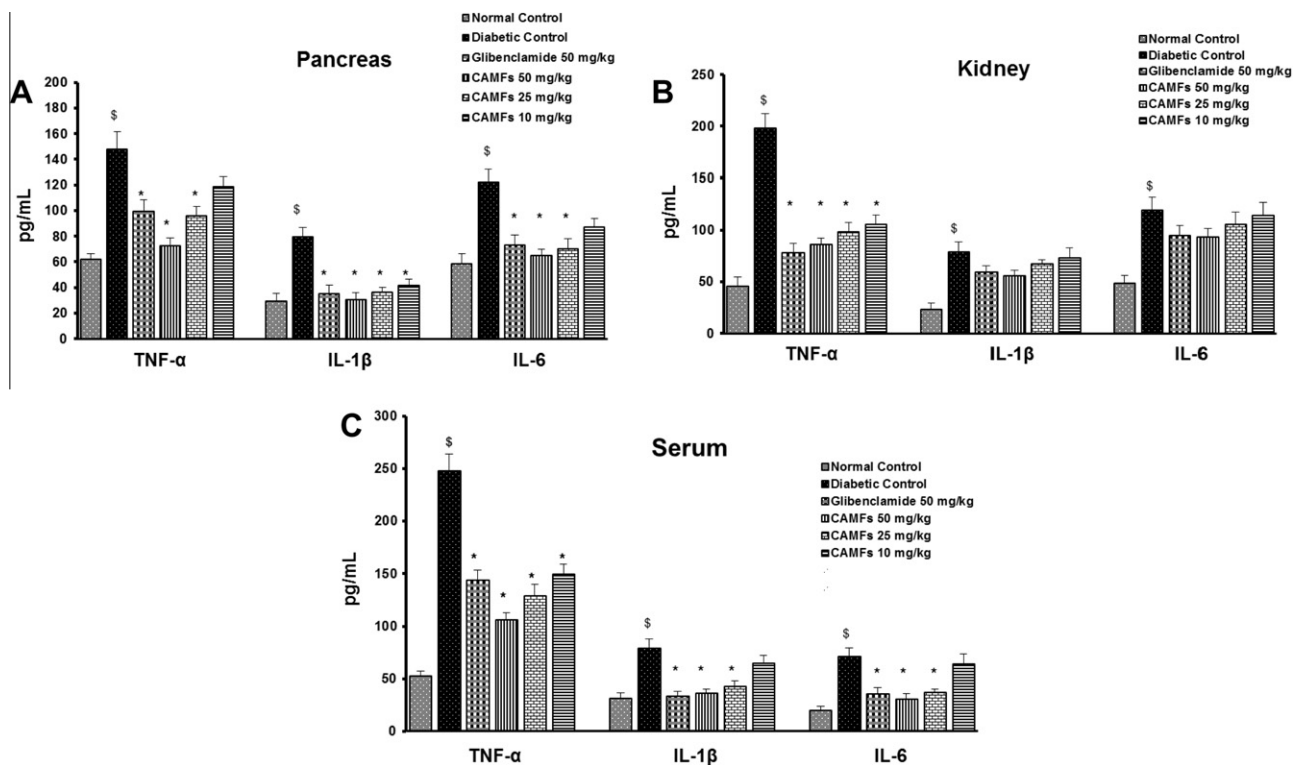


Fig. 7. Anti-inflammatory effect of CAMFs and glibenclamide in STZ-nicotinamide-induced diabetic rats in comparison with normal and diabetic control rats after the 12-week treatment period. At the end of the treatment, the blood and organs were removed and centrifuged to collect serum from the blood and tissue supernatant from the homogenized organs. Panels denote (A) pancreas, (B) kidney, and (C) serum, respectively, illustrating TNF- α , IL-1 β , and IL-6 levels. Data are presented as means \pm SD ($n = 9-10$). [§]Significant difference compared to normal control group ($P < 0.05$). ^{*}Significant difference compared to the diabetic control ($P < 0.05$).

the phytochemicals in CAMFs, such as quercetin glycoside, 3,4-dicaffeoylquinic acid, caffeic acid, naringenin-7- β -D-glucoside and kaempferol. Several studies on these compounds have demonstrated antidiabetic, antioxidant and anti-inflammatory properties (Jung et al., 2006; Ortiz-Andrade et al., 2008; Shih et al., 2012). Thus, the combination of these compounds in CAMFs may be responsible for the synergistic effects observed in STZ-nicotinamide induced type 2 diabetic rats.

5. Conclusion

The crude methanolic fraction of *C. anthelminticum* seeds (CAMFs) displayed inhibitory effects on NF- κ B translocation in H₂O₂-stimulated β -TC6 cells. This observation was corroborated with the beneficial effects of CAMFs in attenuating hyperglycemia, by down-regulating elevated levels of pro-inflammatory cytokines, oxidative stress and hyperlipidemia in an animal model of type 2 diabetes. These findings pave the way for a novel approach to potential treatments of insulin-resistant type 2 diabetes and propose CAMFs as a valuable candidate nutraceutical for the type 2 diabetic complications.

Conflict of Interest

The authors disclose no conflicts of interest.

Role of the funding source

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