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Anti-diabetic effects of *Centratherum anthelminticum* seeds methanolic fraction on pancreatic cells, β-TC6 and its alleviating role in type 2 diabetic rats

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ABSTRACT

Ethnopharmacological relevance: Seeds of *Centratherum anthelminticum* (Asteraceae) have been popularly used in Ayurvedic medicine to treat diabetes and skin disorders. Folk medicine from Rayalaseema (Andhra Pradesh, India) reported wide spread usage in diabetes.

Aim of the study: To investigate the hypoglycemic properties and mechanism of the methanolic fraction of *C. anthelminticum* seeds (CAMFs) on mouse *β*-TC6 pancreatic cell line and streptozotocin (STZ)-induced diabetic rat models.

Materials and Methods: We investigated the crude methanolic fraction of *C. anthelminticum* seeds (CAMFs) on β-TC6 cell line and confirmed its effects on type 1 and type 2 diabetic rats to understand its mechanism in managing diabetes mellitus. CAMFs were initially tested on β-TC6 cells for cytotoxicity, 2-NBDG glucose uptake, insulin secretion and glucose transporter (GLUT-1, 2 and 4) protein expression. Furthermore, streptozotocin (STZ)-induced type 1 diabetic and STZ-nicotinamide-induced type 2 diabetic rats were intraperitoneally (i.p) injected or administered orally with CAMFs daily for 28 days. The effect of CAMFs on blood glucose and insulin levels was subsequently evaluated.

Results: In cell line studies, CAMFs showed non-cytotoxic effect on β-TC6 cell proliferation compared to untreated control cells at 50 μg/ml. CAMFs increased glucose uptake and insulin secretion dose-dependently by up-regulating GLUT-2 and GLUT-4 expression in these cells. Further *in vivo* studies on streptozotocin induced diabetic rat models revealed that CAMFs significantly reduced hyperglycemia by augmenting insulin secretion in type 2 diabetic rats. However, CAMFs displayed less significant effects on type 1 diabetic rats.

Conclusions: CAMFs demonstrated anti-diabetic potential on β-TC6 cells and type 2 diabetic rat model, plausibly through enhancing glucose uptake and insulin secretion.

1. Introduction

Diabetes mellitus (DM) is a complex metabolic disorder characterized by high blood glucose level due to defects in insulin secretion, insulin action or both. It affects the metabolism of carbohydrates, fats, proteins and electrolytes in the body. Type I diabetes (DM1) or insulin-dependent diabetes mellitus (IDDM) occurs due to immunological destruction of pancreatic β cells and consequent insulin deficiency. Type II diabetes (DM2) or non-insulin-dependent diabetes mellitus (NIDDM) is characterized by impaired insulin secretion or insulin resistance. DM2 is usually associated with obesity and hereditary disposition and it is the most common form of diabetes, affecting 90–95% of cases (Tamrakara et al., 2011). Resistance to insulin impairs the sensitivity of the main target organs (muscle, liver and adipose tissues) to the hormone, which increases circulating FFA concentrations, inhibits muscle cell's glucose uptake and enhances glucose

Abbreviations: 2-NBDG, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose; ATCC, American Type Culture Collection; bw, body weight; CA, *Centratherum anthelminticum*; CAMFs, methanolic fraction of *C. anthelminticum* seeds; CI, cell index; DM1, type 1 diabetes; DM2, type 2 diabetes; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; GC–MS, gas chromatography–mass spectrometry; GLUT, glucose transporter; FFA, free fatty acid; HCS, high content screening; HRP, horseradish peroxidase; IC_{50}, 50% inhibitory concentration; IDDM, insulin-dependent diabetes mellitus; IP, intraperitoneal; LCMS-MS, liquid chromatography–tandem mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NIDDM, non-insulin-dependent diabetes mellitus; OECD, Organization for Economic Co-operation and Development; OGTT, oral glucose tolerance test; PBS, phosphate-buffered saline; PBS-T, PBS-Tween 20; PPARs, peroxisome proliferator-activated receptors; PVDF, polyvinylidene fluoride; r^2, coefficient of determination; RTCA, Real-Time Cellular Analysis; SD, standard deviation; SDS, sodium dodecyl sulfate; STZ, streptozotocin; TMB, 3,3',5,5'-tetramethylbenzidine

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production by the liver. These conditions are considered as DM2 hallmarks (Reaven, 1988; Defronzo, 2009). Currently, the range of medication is limited. None of the available options is able to vigorously enhance insulin secretion and sensitivity simultaneously (Cohen and Horton, 2007). Thus, it is obvious that there is an urgent need for more effective therapeutic agents.

The plant kingdom holds great potential to meet this need. However, scientific testing and validation of the efficacy of most medicinal plants in alleviating DM1 and DM2 is rare. Thus, we have limited knowledge of their safety and efficacy, as most of the data is based on information obtained from traditional medicinal plant practitioners (Smirin et al., 2010). Folk medicine from Rayalaseema reports 26 plants for the management of diabetes. One such plant is *Centratherum anthelminticum* (L.) Kuntze, which belongs to the family Asteraceae (Nagaraju and Rao, 1990). Seed of this plant is being used by Indian traditional healers to treat diabetic conditions. The trade name of *C. anthelminticum* is wild cumin and it is also known as Kalizeeri in Hindi. *C. anthelminticum* is one of the major contents for the Ayurvedic formulation, e.g., Kayakalp, a preparation used for the whole-body rejuvenation. Pharmacological investigations of *C. anthelminticum* (CA) seeds demonstrated various biological activities, including anti-cancer, anti-diabetes and anti-inflammation (Ani and Naidu, 2008; Fatima et al., 2010; Arya et al., 2012). However, little is known on the anti-diabetic effect of the defatted crude methanolic fraction of *C. anthelminticum* seeds (CAMFs).

The present study was undertaken to scientifically investigate anti-diabetic potential of CAMFs using *in vitro* mouse pancreatic β-TC6 cells and *in vivo* diabetic rat models. We first studied cytotoxicity and cell proliferation assay on β-TC6 cells. Furthermore, we investigated CAMFs effects on *in vitro* glucose uptake in β-TC6 cells using 2-[*N*-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG) and insulin secretion. Next, we performed Western blotting to evaluate glucose transporter protein (GLUT-1, 2 and 4) expression after CAMFs treatment. Subsequently, we investigated the effect of CAMFs on glucose levels and insulin secretions in streptozotocin (STZ)-induced type 1 and STZ-nicotinamide-induced type 2 diabetic rat models.

2. Material and methods

2.1. Preparation of CAMFs

2.1.1. Collection of plant material

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

2.1.2. Extraction and fractionation

The seeds of *C. anthelminticum* (2 kg) were pounded using grinder and extracted with 100% *n*-hexane using a Soxhlet extractor for 24 h. The solvents were then removed under reduced pressure at a maximal temperature of 40 °C to get viscous greenish oil. Then, the obtained residue was further fractionated by 100% chloroform, to get semisolid fraction. Finally, the defatted residue was again fractionated with absolute methanol to get defatted crude methanolic fraction (CAMFs). The final fraction was then freeze-dried and stored at −20 °C until further use.

2.1.3. Phytochemical analysis of CAMFs by LCMS-MS

Analysis of the major compounds in CAMFs was done using liquid chromatography–tandem mass spectrometry (LCMS-MS). A triple quadrupole mass spectrometer equipped with a turbo ion spray source (AB SciexQTrap 5500, Ontario, Canada) was used to obtain the MS/MS data in negative ion mode. 0.1% formic acid in water and in acetonitrile (ACN) were used as mobile phase by eluting at a flow rate of 0.4 mL/min with an injection volume of 20 μL. Separation of the compounds was performed using a Luna 3-μm RP C18 column (100 mm × 2.00 mm; Phenomenex). The turbo ion source settings were as follows: capillary voltage, −4000 V; dry gas flow (N_2), 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 MS^2 and MS^3 fragmentations spectra data by correlation with the previous reports.

2.1.4. Phytochemical analysis of CAMFs by GC–MS

The analysis of CAMFs by GC/MS was carried out using a Shimadzu GC-17A network GC system coupled to a mass detector with a SGE BPX35 MS (25 m × 0.22 mm × 0.25 μm) capillary column. Helium gas was used as the carrier gas at a flow rate of 1 mL/min. The injection volume was 1 μL and the samples were injected in split mode as 10:1. The column temperature was initially set at 100 °C for 3 min, raised to 250 °C at the rate of 10 °C/min, and maintained at this temperature for 3 min. The injector and detector temperatures were set at 250 °C and 280 °C, respectively. The ionization voltage was 70 eV with the mass scan operating in the range of 40–450 amu. Identification of the major compounds was based on the comparison of mass spectra and retention times with those of standards from the database of National Institute Standards and Technology (NIST21 and NIST Wiley).

2.2. In vitro assays

2.2.1. Cell culture

Mouse 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% CO_2 in a humidified incubator. The growth medium was changed every 3 days.

2.2.2. Cellular viability

The β-TC6 cells were used to determine the effect of CAMFs on cell growth using the MTT assay. The MTT assay was performed as described by Mosmann (1983) with slight modification. Briefly, cells were seeded at a density of 1.5×10^4 cells/mL in a 96-well plate and incubated for 24 h at 37 °C in 5% CO_2. The next day, cells were treated with CAMFs and incubated for another 24 h, following which 2 mg/mL MTT solution was added for 1 h. Absorbance at 570 nm was measured and recorded using a Plate Chameleon V microplate reader (Hidex, Turku, Finland). Results are expressed as a percentage of control cells demonstrating percentage cell viability after 24-h exposure to CAMFs. The potency of cell growth was expressed. Viability was defined as the ratio (expressed as a percentage) of the absorbance of treated cells to that of the untreated cells.

2.2.3. Real-time cell proliferation

In vitro cell proliferation was assessed using an xCELLigence Real-Time Cellular Analysis (RTCA) system (Roche, Manheim, Germany). Briefly, background measurements were obtained after

adding 50 μL of the appropriate medium to the wells of the 16X E-plate, and 1.5×10^4 cells were added to each well. Cell attachment and proliferation were monitored every 5 min using the RTCA system. Approximately 17 h after seeding, when the cells were in the log growth phase, the cells were treated with 100 μL of CAMFs in concentrations of 3.125, 6.25, 12.5, 25, 50, and 100 μg/mL dissolved in cell culture media, and continuously monitored for up to 72 h. The cells were also treated with medium alone, which served as the vehicle control. Cell sensor impedance was expressed as an arbitrary unit called the cell index (CI).

2.2.4. 2-NBDG glucose uptake

The uptake of the fluorescent hexose 2-NBDG, a glucose analog, was assayed as described by Loaiza et al. (2003) with modifications. Briefly, 1.5×10^4 cells were seeded onto a 96-well plate and allowed to attach, spread, and proliferate to near confluence at 37 °C in 5% CO_2. The medium was then removed and the cells were washed with phosphate-buffered saline (PBS) twice. The PBS was then replaced with 2.5 mM glucose in basal medium comprising DMEM without glucose or pyruvate supplemented with L-glutamine and 15% (v/v) FBS (final serum glucose concentration of approximately 0.25 mM). Conditioning of the cells proceeded at 37 °C in 5% CO_2 for 60 min. The conditioning medium was then removed and replaced with 10 mM 2-NBDG (Invitrogen, Carlsbad, CA, USA) in basal medium in the presence or absence of CAMFs. The cells were then incubated at 37 °C in 5% CO_2 for 30 min to permit endocytosis of the 2-NBDG, with the selected concentration being the minimum concentration capable of producing an adequate signal-to-noise ratio. The 2-NBDG was then removed, the cells washed with PBS, and stained with the nucleic dye Hoechst 33342 for another 30 min. The cells were then observed for intra-cellular fluorescence at *Ex*/*Em* = 350 nm/461 nm and *Ex*/*Em* = 475 nm/550 nm for Hoechst 33342 and 2-NBDG, respectively. Plates were evaluated using the ArrayScan High Content Screening (HCS) system (Cellomics Inc., Pittsburgh, PA, USA) and analyzed with Target Activation BioApplication software (Cellomics Inc.).

2.2.5. In vitro insulin secretion

The β-TC6 cells (1.5×10^5 cells/mL) were seeded onto a 24-well plate and allowed to attach, spread, and proliferate at 37 °C in 5% CO_2. After reaching 80–90% confluence, the cells were washed with glucose-free Krebs/HEPES Ringer solution (115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM HEPES [pH 7.4]) twice and pre-incubated at 37 °C for 30 min with the glucose-free Krebs/HEPES Ringer solution. Following this, the cells were incubated in Krebs/HEPES Ringer solution containing 1 mg/mL bovine serum albumin and 6.25, 12.5, 25, or 50 μg/mL of glucose in the presence or absence of CAMFs for 60 min. An aliquot of the supernatant was collected for ELISA. The amount of insulin released was measured with a Mouse Insulin ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer protocol. Results (in pmol) are expressed as the means ± SE of at least two independent experiments.

2.2.6. Western blotting analysis

To determine protein expression, 1×10^6 cells/mL were seeded onto 25-cm^2 tissue culture flasks. Cells were treated with 6.25, 12.5, or 25 μg/mL of CAMFs for 24 h; whole cell extracts were prepared as described by Looi et al. (2011). Briefly, cells were collected and lysed in lysis buffer (20 mM Tris [pH 7.4], 250 mM NaCl, 0.1% Triton X-100, 2 mM EDTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 4 mM sodium orthovanadate, 1 mM DTT), and 60 μg of the protein was resolved on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to PVDF membranes (Millipore). The membranes were blocked with 5% non-fat dry milk in PBS-T (0.05% Tween 20) for 1 h at room temperature. Membranes were probed with primary goat anti-GLUT-1, GLUT-2, or GLUT-4 antibody followed by horseradish peroxidase (HRP)–conjugated secondary donkey anti-goat antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Protein-antibody complexes were detected with a ProteoQwest™ Colorimetric Western Blotting Kit with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO, USA). To ensure equal loading, membranes were stripped and re-probed with mouse anti-β-actin antibody (Santa Cruz Biotechnology Inc.).

2.3. In vivo studies

2.3.1. Experimental animals

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

We performed the animal experiments in accordance with the animal experimentation guidelines issued by the UM Animal Care and Use Committee (Ethics Number: FAR/10/11/2008/AA[R]) and in accordance with the internationally accepted principles for laboratory animal use and care.

2.3.2. In vivo acute oral and intraperitoneal toxicity studies

CAMFs oral and intraperitoneal acute toxicity tests were determined according to the guidelines of the Organization for Economic Co-operation and Development (OECD). We used healthy adult Sprague Dawley rats of either sex. The rats were fasted overnight, divided into 12 groups ($n = 6$), and injected intraperitoneally (IP) or orally fed CAMFs at doses 10, 20, 50, 100 and 500 mg/kg body weight (bw), CAMFs was completely dissolved in distilled water and filtered before injecting i.p to the animals; the control groups were given distilled water alone. We observed the rats for 1 h continuously and then hourly for 4 h 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 DM1

We induced DM1 in overnight-fasted normal male rats through intraperitoneal administration of 65 mg/kg of freshly prepared STZ (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M citrate buffer (pH 4.5) in a volume of 1 mL/kg bw. STZ-injected rats were given 20% glucose solution for 12 h to prevent initial drug-induced hypoglycemic mortality. Hyperglycemia was confirmed by elevation in blood glucose levels, determined at 96 h after the STZ administration. Rats with fasting blood glucose range of 22–26 mmol/L were considered DM1 and used for the study.

2.3.4. Induction of DM2

DM2 was induced (Masiello et al., 1998) with slight modifications, by using standardized dose of STZ. Intraperitoneal injection of freshly prepared STZ (55 mg/kg) in 0.1 M citrate buffer (pH 4.5) in a volume of 1 mL/kg was injected to overnight-fasted normal male rats, 15 min after i.p administration of nicotinamide (210 mg/kg). Hyperglycemia was confirmed by elevation in blood glucose levels, determined at 96 h after the STZ-nicotinamide administration. Rats with a fasting blood glucose range of

11–14 mmol/L were considered DM2 and subsequently used for the study.

2.3.5. Division of animals for type 1 and type 2 diabetic model study

The rats were divided into two segments: one each for the type 1 and type 2 diabetic model study. The type 1 segment was divided into the following groups, Group 1: normal control rats, Group 2: type 1 diabetic control rats, Group 3: type 1 diabetic rats treated with 6 U/kg of insulin (standard positive), Group 4: diabetic rats treated with 100 mg/kg of CAMFs, and Group 5: diabetic rats treated with 50 mg/kg of CAMFs. The type 2 segment was divided into the following groups, Group 1: normal control rats, Group 2: type 2 diabetic control rats, Group 3: type 2 diabetic rats treated with 50 mg/kg of glibenclamide (standard positive), Group 4: diabetic rats treated with 100 mg/kg of CAMFs, and Group 5: diabetic rats treated with 50 mg/kg of CAMFs. All groups were injected and fed once daily with the respective doses of CAMFs or drugs for 4 weeks (28 days). Administration was based on volume (2 mL/200 g bw). CAMFs was completely dissolved in distilled water and filtered before i.p. injection.

2.3.6. Experimental procedure

We collected blood samples from the tail by snipping; fasting blood glucose concentrations were determined weekly after the above treatments by a glucose oxidase-peroxidase enzymatic method using a standardized glucometer (Accu-Check Performa, Roche Diagnostic Germany). Changes in body weight, food intake, and water intake were recorded daily. After the 4-week treatment period, all groups from both segments were fasted overnight and sacrificed by cervical dislocation under mild anesthesia. Blood was collected upon decapitation into heparinized tubes and centrifuged at 2000 rpm for 10 min, the serum was collected and stored at $-80\,^{\circ}C$ until analysis.

2.3.7. Glucose challenge (glucose tolerance test)

We used the oral glucose tolerance test (OGTT) to evaluate the effectiveness of CAMFs that had demonstrated the highest glycemic control as studied in the type 1 and type 2 diabetic rat models described earlier. CAMFs (100 or 50 mg/kg) was administered to overnight-fasted rats in 2 mL/200 g bw doses. Fasting blood glucose concentrations were measured before the respective CAMFs administrations, following which oral glucose (3 g/kg) was administered and blood glucose levels were measured at 30, 60, 90, and 120 min, respectively.

2.3.8. Estimation of insulin and body weight

We determined serum insulin by using radioimmunoassay kit (Packard, USA) according to the manufacturer protocol. In addition, the whole body weight of type 1 and type 2 diabetic rats were examined at the end of study period in normal, treated, and untreated diabetic rats.

2.4. Statistical analysis

The results are expressed as mean ± standard deviation (SD). Significant differences between the means of the experimental groups were identified with analysis of variance (ANOVA), followed by the Tukey–Kramer multiple comparisons test (GraphPad version 5.0; GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $P<0.001$, $P<0.01$, $P<0.05$. We performed log IC_{50} calculations using the built-in algorithms for dose–response curves with variable slope. We set a fixed maximum value of the dose–response curve to each sample's maximum obtained value.

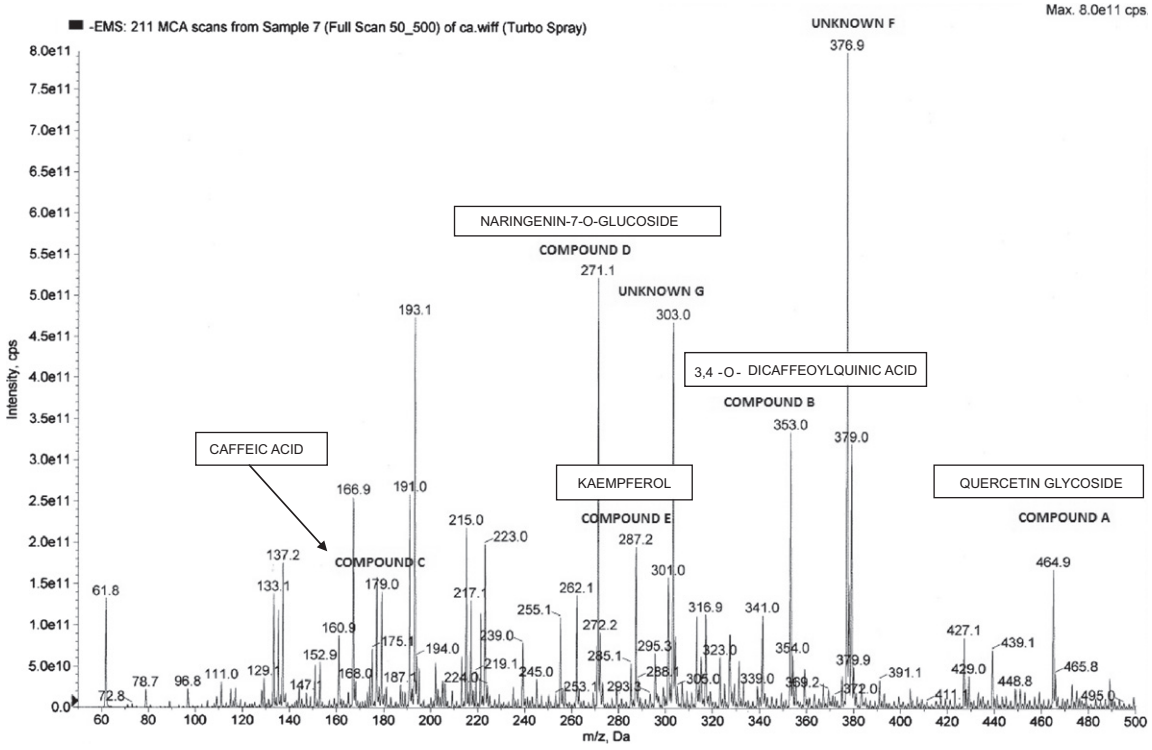

Fig. 1. The mass spectrometric main (parent) chromatogram of CAMFs indicates the presence of characterized compounds on the basis of their mass fragmentation pattern as (compound A) quercetin glycoside, (compound B) 3,4-*O*-dicaffeoylquinic acid, (compound C) caffeic acid, (compound D) naringenin-7-*O* glucoside, and (compound E) kaempferol with other unknown compounds.

3. Results

3.1. Fraction yield and LCMS-MS analysis

The final yield of the obtained CAMFs fractions, was 12.6% w/w. Phytochemical analysis using LCMS-MS demonstrated the presence of quercetin glycoside, 3,4-*O*-dicaffeoylquinic acid, caffeic acid, naringenin-7-*O* glucoside, and kaempferol, as the major compounds in CAMFs based on the main peaks selected, as well as other unknown compounds (Fig. 1). The fragmentation patterns of known compounds were in agreement with those documented in the literatures. The mass spectrometric characterization of Compound A indicates the presence of quercetin glycosides (Lin and Harnly, 2007). 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 (Lin and Harnly, 2008; Gouveia and Castilho, 2011). Compound C exhibits fragment ions at *m/z* 179 and *m/z* 135, the characteristic ions of caffeic acid (Luo et al., 2003; Lin and Harnly, 2008; Plazonic et al., 2011). 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 (Charrouf, et al., 2007). (E) Compound E was identified as kaempferol based on mass fragments at *m/z* 287 and *m/z* 151(Sun et al., 2007).

3.2. GC–MS analysis

The possible chemical composition in CAMFs analyzed by GC/MS is presented in Table 1. Major peaks were selected and identified on the basis of similarity index. The most abundant components determined are decanoic acid, dodecanamide, pentadecanoic acid, 14-methyl-methyl ester, tetradecanamide, hexadecanoic acid, ethyl ester, octadecanoic acid, and ethyl ester. Compounds with lower percentage (less than 80 similarity index, SI) were considered as minor constituents and these compounds were excluded from the list.

3.3. Effect of CAMFs on β-TC6 cell proliferation

To evaluate whether CAMFs is cytotoxic towards β-TC6 cells, we apply various concentrations of CAMFs and cell viability was analyzed using MTT assays 24 h after treatment. Fig. 2a illustrates the dose–response curves in the end-point cytotoxicity assay. CAMFs concentrations of up to100 μg/mL did not exert cytotoxic effects on β-TC6 cell viability. Next we monitored treated β-TC6 cells for 3 days using Xcelligence Real-time Cell Proliferation (RTCA) assays. RTCA data showed that β-TC6 cells treated with 50, 25, 12.5, 6.25 or 3.125 μg/mL of CAMFs proliferated in a similar manner compared to control cells throughout the 72-h treatment, as shown by an increase in the CI values, (Fig. 2b). However, high-concentration treatment (100 μg/mL) decreased the CI values after 72 h, suggesting that high concentration of CAMFs may inhibit β-TC6 cell proliferation after prolonged treatment. The IC_{50} values of CAMFs on β-TC6 cells at 24, 48, and 72 h were 518, 416, and 94 μg/mL, respectively.

3.4. Effect of CAMFs on 2-NBDG uptake

Fig. 3 illustrates the endocytosis of the fluorescent glucose analog 2-NBDG by mouse β-TC6 cells and increased intracellular 2-NBDG fluorescence is visible in distinct groups of cells. Fluorescence appeared to be restricted to the cytoplasm. As the duration defined for 2-NBDG uptake would have been sufficient for the stabilization of 2-NBDG uptake and metabolism,

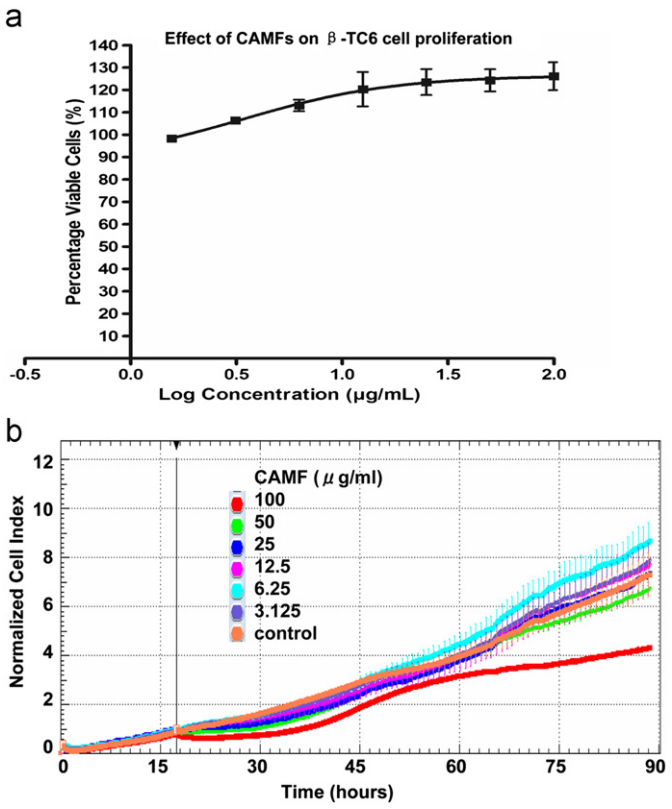

Fig. 2. (a) MTT assay growth dose–response curves of CAMFs tested on mouse β-TC6 cells. (b) Dynamic monitoring of cell proliferation using RTCA. β-TC6 cells were seeded in a 16X E-plate device and continuously monitored up to 90 h after treatment with various concentrations of CAMFs. CI values were normalized to the time point of CAMFs addition, indicated by the vertical black line.

Table 1
Compounds tentatively identified in CAMFs.

Peak No.	RT[a]	Compounds[b]	SI[c]	MW	Molecular Formula
1	9.40	Decanoic acid, ethyl ester	95	200	$C_{12}H_{24}O_2$
2	11.57	Tetradecanamide	86	227	$C_{14}H_{29}NO$
3	12.41	Octadecanamide	81	281	$C_{18}H_{35}NO$
4	13.10	Dodecanamide	93	287	$C_{16}H_{33}NO_3$
5	16.30	Pentadecanoic acid, 14-methyl-methyl ester	91	270	$C_{17}H_{34}O_2$
6	17.70	Hexadecanoic acid, ethyl ester	86	284	$C_{18}H_{36}O_2$
7	20.80	Octadecanoic acid, ethyl ester	81	312	$C_{20}H_{40}O_2$

[a] RT, Retention time (min).
[b] Compounds listed according to their relative area percentage (peak area relative to the total peak area percentage.
[c] SI, Similarity index.

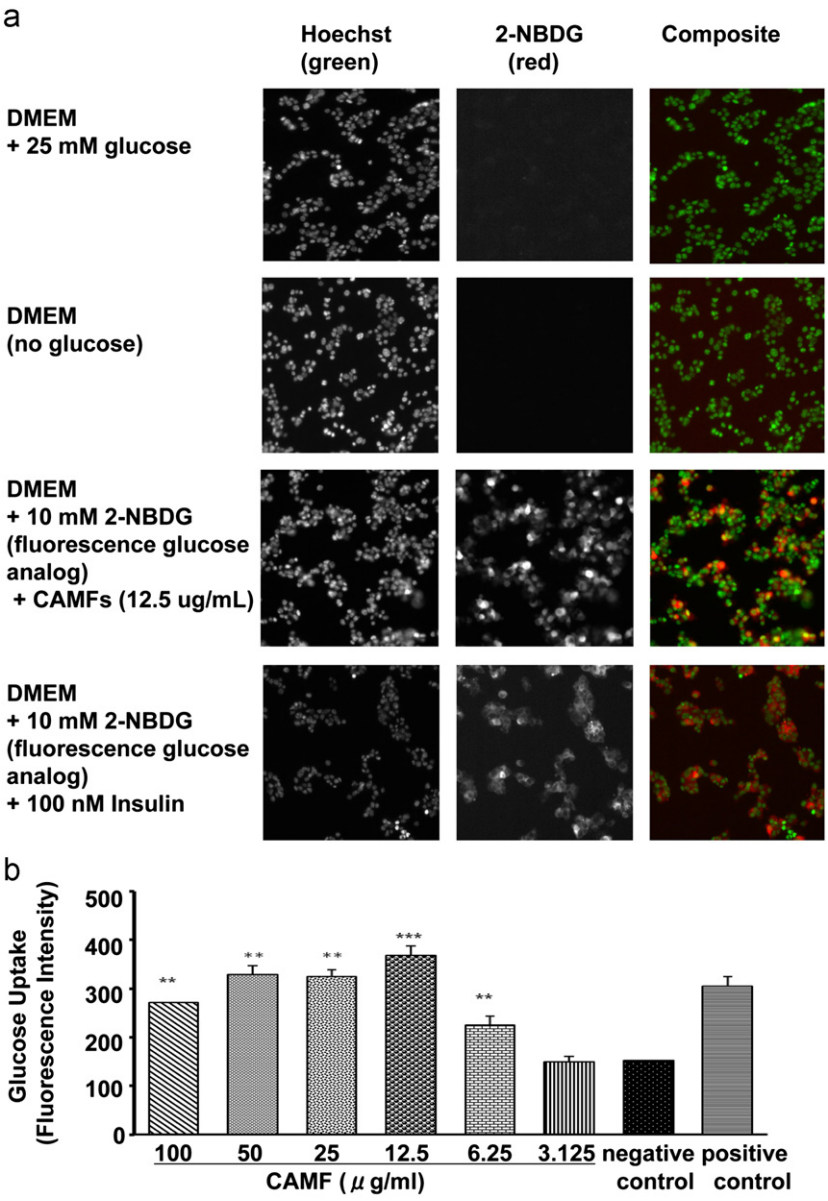

Fig. 3. (a) Fluorescence images of β-TC6 cells exposed to 10 mM 2-NBDG for 30 min revealing heterogeneous 2-NBDG uptake and metabolic activity. The cells were imaged after a 10-min washout of 2-NBDG. (b) Glucose uptake in β-TC6 cells after 60-min exposure to 10 mM 2-NBDG and CAMFs. Significant difference compared to negative control ($^{**}P < 0.01$ or $^{***}P < 0.001$).

this heterogeneous intracellular fluorescence suggests that β-TC6 cells retain the heterogeneous glucose uptake activity of native β-cells.

3.5. Effect of CAMFs on insulin secretion

Fig. 4 displayed the result of insulin secretion. In the absence of glucose, insulin secretion was low in the β-TC6 cells. However, CAMFs increased insulin secretion in a dose-dependent manner at glucose concentrations of 6.25, 12.5, 25, and 50 mM). In particular, at 12.5 μg/mL CAMFs elicited a marked increase in insulin secretion in the β-TC6 cells ($^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$).

3.6. Effect of CAMFs on glucose transporter proteins

At 24 h after CAMFs treatment, the protein level of GLUT-1, GLUT-2 and GLUT-4 in β-TC6 cells were examined by Western blotting analysis. As presented in Fig. 5a–c, CAMFs increased GLUT-2 and GLUT-4 protein levels in a dose-dependent manner compared to control ($^{*}P < 0.05$ or $^{**}P < 0.01$). In contrast, CAMFs did not alter GLUT-1 protein level (Fig. 5a).

3.7. Acute oral and intraperitoneal toxicity study

The acute oral and i.p toxicity studies revealed CAMFs non-toxic nature; no lethality or toxic reactions were observed at any of the doses tested. Based on these findings, 100 mg/kg and 50 mg/kg doses were chosen as the maximum doses for further experiments involving i.p and oral routes of administration, respectively.

3.8. Effect of CAMFs on blood glucose levels of type 1 diabetic rats

Blood glucose levels were evaluated weekly in normal, CAMFs-treated, and untreated diabetic rats, the outcomes of which are listed in Table 2. The standard drug insulin (6 U/kg) produced

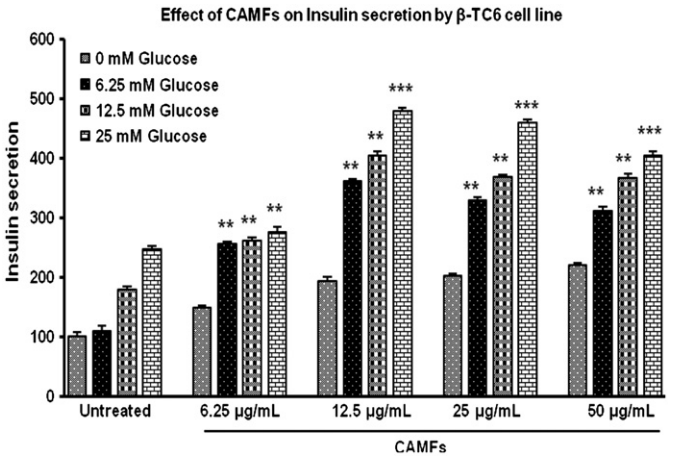

Fig. 4. Insulin release elicited by CAMFs in β-TC6 cells. The cells were incubated in Krebs/HEPES buffer (pH 7.4) containing no glucose or 6.25, 12.5, or 25 mM glucose in the absence or presence of CAMFs for 60 min at 37 °C.

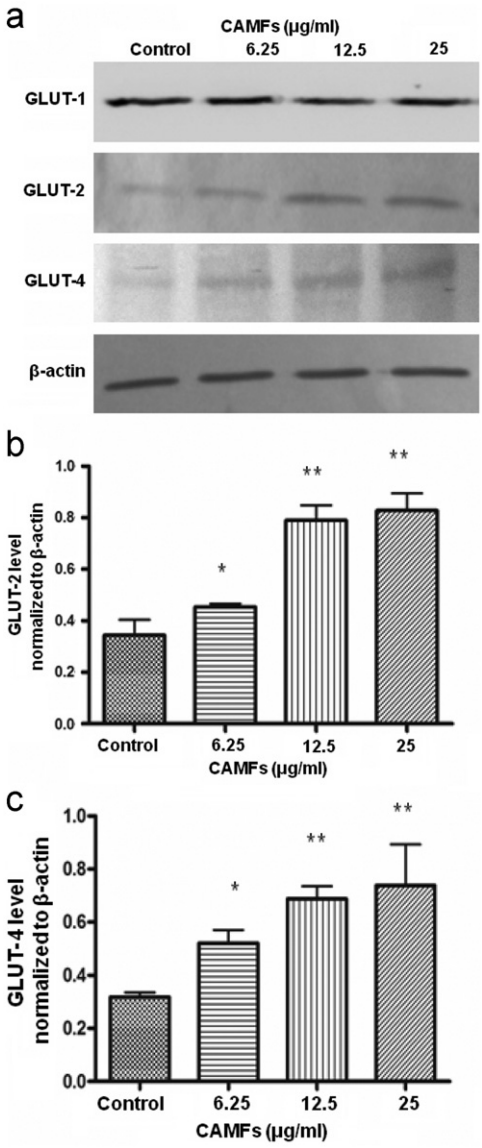

Fig. 5. (a) Western blot analysis of GLUT-1, GLUT-2 and GLUT-4 expression in control and CAMFs-treated samples. Membranes were stripped and reprobed with β-actin. Histogram (b and c) denotes band intensity ratio of GLUT-2 and GLUT-4 after normalization with β-actin in non-treated β-TC6 cells.

significant decrease in blood glucose levels, compared to untreated diabetic rats, starting from week 1 and produced maximal fall of 64.08% after end of the study period (4-week). While administration of 100 and 50 mg/kg of CAMFs demonstrated significant reduction in blood glucose levels at week-4 when compared with untreated diabetic rats, and exhibited decreases of 23.21% and 21.05% after 4-week treatment period. The blood glucose levels of normal rats were not changed, whereas in the untreated diabetic rat blood glucose levels were significantly and continuously elevated, until end of the study period.

3.9. Effect of CAMFs on blood glucose levels of type 2 diabetic rats

The results of the weekly blood glucose evaluations in normal, CAMFs-treated, and untreated diabetic rats are presented in Table 3. The blood glucose levels of normal rats were not altered until the end of the study period, while that in the untreated diabetic rats were significantly elevated. Oral administration of 100 and 50 mg/kg of CAMFs and 50 mg/kg of glibenclamide produced a significant reduction in blood glucose levels at weeks 1–4 when compared with untreated diabetic rats. In the final week of study period, 100 mg/kg of CAMFs produced the maximal decrease of blood glucose level (51.40%) as compared to untreated diabetic rats, and 50 mg/kg of CAMFs caused 46.47% decline, whereas glibenclamide group achieved 50% drop in blood glucose levels.

3.10. Effect of CAMFs on OGTT of type 2 diabetic rats

Result of OGTT is showed in Table 4. In diabetic rats, CAMFs at 100 and 50 mg/kg demonstrated a significant reduction in blood glucose levels after 60-min of glucose load, compared to untreated diabetic rats. Similarly, after 90 min the blood glucose levels were continuously reduced to 66.31% and 52.94% after CAMFs treatment, whereas the glibenclamide-treated group caused (60.96%) reduction, respectively. This was followed by a reduction as great as 67.59% and 54.74% following the respective CAMFs doses at 120 min, whereas glibenclamide showed 62.01% markdown in the blood glucose levels.

3.11. Effect of CAMFs on insulin level and body weight of type 1 and type 2 diabetic rats

Fig. 6a illustrates the insulin levels in the experimental animals. In type 1 and type 2 groups, untreated diabetic rats exhibited significant reduction of insulin in the serum, compared to the normal control rats. Upon daily treatment of standard positive and 100 or 50 mg/kg bw of CAMFs to diabetic rats for 4 weeks, demonstrated significant improvement in insulin levels of type 2 diabetic rats as compared to untreated diabetic rats, whereas type 1 diabetic rats did not produce any significant sign of improvement.

Moreover, we observed a significant loss in the body weight of untreated type 1 and type 2 diabetic rats, as compared to normal control rats after 4 weeks of study period (Fig. 6b). While daily intake of food and water was significantly increased only in type 2 diabetic rats (Fig. S1a and S1b). Standard positive and CAMFs treatment significantly increased the body weight of type 2 diabetic rats compared to untreated diabetic rats. In contrast, only 100 mg/kg of CAMFs resulted in elevation of body weight in type 1 diabetic rats.

Table 2
Effects of CAMFs on fasting blood glucose level of type 1 diabetic rats.

Group	Fasting blood glucose level (mmol/L)				
	Pretreatment week	Treatment weeks			
	Week 0	Week 1	Week 2	Week 3	Week 4
Normal control	4.2 ± 0.13	4.4 ± 0.26	3.9 ± 0.18	3.6 ± 0.22	4.1 ± 0.32
Diabetic control	23.8 ± 1.19	25.6 ± 1.41	27.5 ± 1.24	28.7 ± 1.77	32.3 ± 2.69
Insulin (6 U/kg)	24.6 ± 1.21	9.9 ± 0.67[a (61.32)]	9.1 ± 0.97[a (66.90)]	10.4 ± 1.16[a(63.76)]	11.6 ± 1.36[a(64.08)]
CAMFs (100 mg/kg)	24.9 ± 0.94	23.8 ± 1.08[(7.03)]	22.2 ± 1.37[(19.27)]	23.1 ± 1.08[(19.51)]	24.8 ± 1.27[a(23.21)]
CAMFs (50 mg/kg)	25.2 ± 0.67	23.3 ± 0.88[(8.98)]	23.7 ± 0.97[(13.81)]	24.6 ± 1.21[(14.28)]	25.5 ± 1.03[a(21.05)]

Values denote mean ± SD, $n=6$.

[a] Represents statistical significance compared to diabetic control ($P<0.05$).
Values given in parenthesis represent percent decline in the blood glucose level in comparison with diabetic control group within the same week.

Table 3
Effects of CAMFs on fasting blood glucose level of type 2 diabetic rats.

Group	Fasting blood glucose level (mmol/L)				
	Pretreatment week	Treatment weeks			
	Week 0	Week 1	Week 2	Week 3	Week 4
Normal control	4.0 ± 0.21	4.2 ± 0.19	4.4 ± 0.25	4.3 ± 0.34	4.1 ± 0.29
Diabetic control	11.8 ± 0.57	12.1 ± 0.91	12.9 ± 1.04	13.7 ± 1.17	14.2 ± 1.37
Glibenclamide (50 mg/kg)	11.9 ± 0.65	7.6 ± 0.32[a(37.19)]	7.4 ± 0.43[a(42.63)]	7.5 ± 0.66[a(45.25)]	7.1 ± 0.36[a(50.00)]
CAMFs (100 mg/kg)	12.2 ± 0.56	7.8 ± 0.44[a(35.53)]	7.7 ± 0.51[a(40.31)]	7.3 ± 0.33[a(46.71)]	6.9 ± 0.49[a(51.40)]
CAMFs (50 mg/kg)	12.7 ± 0.71	8.4 ± 0.39[a(30.57)]	8.9 ± 0.39[a(31.00)]	8.1 ± 0.46[a(40.87)]	7.6 ± 0.53[a(46.47)]

Values denote mean ± SD, $n=6$.

[a] Represents statistical significance compared to diabetic control ($P<0.05$).
Values given in parenthesis represent percent decline in the blood glucose level in comparison with diabetic control group within the same week.

Table 4
Effects of CAMFs on fasting blood glucose level of type 2 diabetic rats after glucose load.

Group	0 min	30 min	60 min	90 min	120 min
Normal control	3.9 ± 0.17	7.9 ± 0.25	7.1 ± 0.29	6.3 ± 0.32	5.1 ± 0.41
Diabetic control	15.3 ± 0.52	22.2 ± 0.64	19.2 ± 0.4	18.7 ± 0.53	17.9 ± 0.35
Glibenclamide (50 mg/kg)	7.3 ± 0.39	9.8 ± 0.23[a(55.85)]	8.1 ± 0.42[a(57.81)]	7.3 ± 0.59[a(60.96)]	6.8 ± 0.44[a(62.01)]
CAMFs (100 mg/kg)	6.8 ± 0.26	9.5 ± 0.37[a(57.20)]	7.3 ± 0.31[a(61.97)]	6.3 ± 0.28[a(66.31)]	5.8 ± 0.48[a(67.59)]
CAMFs (50 mg/kg)	8.2 ± 0.71	10.4 ± 0.43[a(53.15)]	8.8 ± 0.47[a(54.16)]	8.8 ± 0.35[a(52.94)]	8.1 ± 0.29[a(54.74)]

Values denote mean ± SD, $n=6$.

[a] Represents statistical significance compared to diabetic control ($P<0.05$).
Values given in parenthesis represent percent decline in the blood glucose level in comparison with diabetic control group in that time period.

4. Discussion

Centratherum anthelminticum seeds are commonly used in India to treat diabetes. The seed extracts are available in Ayurvedic formulation for treating diabetes and various disorders. However, the anti-diabetic potential and the mechanism of CAMFs have not been thoroughly investigated. In the present study, we demonstrated that CAMFs exhibited potential anti-diabetic effects on pancreatic β-TC6 cells. Moreover, our *in vivo* results indicate that CAMFs possess glibenclamide-like activities in type 2 diabetic rats with lesser effects seen in type 1 diabetic rats.

Both MTT assays and RTCA results showed that CAMFs is not toxic to β-TC6 cells at 50 μg/ml, which is in agreement with the *in vivo* acute toxicity studies. We further investigated the effect of CAMFs on glucose uptake and insulin secretion by β-TC6 cells. Our results revealed that CAMFs dose-dependently stimulates glucose uptake and enhances insulin secretion in β-TC6 cells. The fluorescent glucose analog 2-NBDG has been used to determine cell viability as well as to estimate glucose uptake rates in a variety of cell types (Poitout et al., 1995; Yoshioka et al., 1996; Yamada et al., 2000; Leira et al., 2002; Zou et al., 2005; Yamada et al., 2007). β-TC6 cell is a β cell derivative, which can endocytose the glucose analog 2-NBDG as shown by increasing intracellular fluorescence in the cytoplasmic regions after CAMFs treatment. In addition, we detected higher insulin secretion in these cells, corresponding with enhanced 2-NBDG glucose uptake. These observations on β-TC6 cell lines clearly establish the potent ability of CAMFs in increasing insulin release and glucose uptake.

Following characterization of CAMFs effects in promoting glucose uptake and insulin secretion, we studied the most crucial factor in the insulin-signaling cascade, glucose transporter proteins, which are essential transporters responsible for the translocation of insulin-regulated glucose into the cells. Our results showed that CAMFs treatment increased GLUT-2 and GLUT-4 protein expression without any significant changes in GLUT-1 protein level compared with the control group. GLUT-4 is classically referred as "insulin-responsive transporter" and increased GLUT-4 expression may result in higher sensitivity to insulin which subsequently potentiates the influx of glucose into the cell (Suzuki and Kono, 1980; Slot et al., 1991; Kraegen et al., 1993). Whereas GLUT-2 is a transmembrane carrier protein mainly found in pancreatic β-cells. Increased GLUT-2

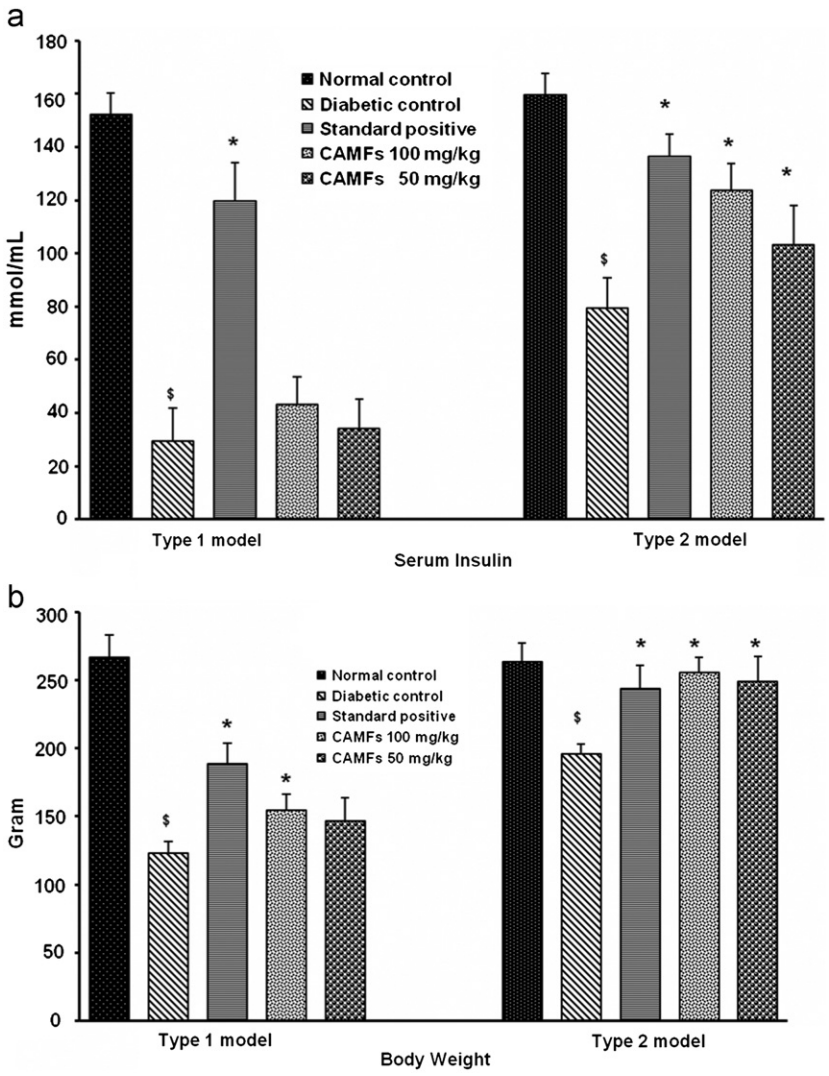

Fig. 6. Effects of CAMFs on insulin level and body weight of type 1 and type 2 diabetic rat models after 28 days (4 weeks) in comparison with diabetic and normal control rats. At the end of the treatment, rats were fasted for 12 h and blood was drawn to collect the serum for insulin measurement. Panel denotes (a) serum insulin and (b) whole body weight. The data are presented as means ± SD ($n=6$). $^{\$}$Significant difference compared to the normal control group ($P<0.05$). *Significant difference compared to the diabetic control ($P<0.05$).

expression is thought to play a constitutive role and responsible for higher glucose uptake in β-cells (Hyo-Sup et al., 2008). In contrast, loss of GLUT-2 may accompany the onset and contribute to the pathogenesis of insulin-dependent (DM1) and non-insulin-dependent diabetes mellitus (DM2) (Bjornholm and Zierath, 2005). Thus, phytochemicals in CAMFs could stimulate insulin-signaling cascade by upregulating GLUT-2 and GLUT-4 expression, leading to increased glucose uptake in the pancreatic β-TC6 cells.

Based on these positive findings, we studied the anti-diabetic potential of CAMFs in the management of type 1 or type 2 diabetic rats. Daily administration of varying concentrations of CAMFs to both groups of animals for 4 weeks produced differential reduction in the blood glucose levels. We found that only type 2 diabetic rats exhibited marked reduction of blood glucose, with less effect observed in the type 1 diabetic rats. Although type 1 diabetic rats displayed significant reduction of blood glucose levels after second week of treatment period, the overall percentage declined were less significant compared to type 2 diabetic rats. This was probably caused by the severe destruction of pancreatic β-cells by STZ in type 1 diabetic rats as these rats had very low to non-detectable serum insulin levels that were not significantly improved upon treatment with CAMFs. On the other hand, in the type 2 diabetic rats, nicotinamide administration prior to STZ injection may have partially protected β-cells by scavenging nitric oxide (NO) and rescued the β-cells from STZ-induced apoptosis (Masiello et al., 1998). Thus, the beneficial effects of CAMFs treatment in type 2 diabetic rats was likely due to improved insulin release and glucose uptake in remnant β-cells. Moreover, elevated insulin secretion by CAMFs can promote conversion of inactive glycogen synthetase to the active form, which increases the conversion of blood glucose into glycogen by enhancing the glycolytic and glycogenic processes with concomitant decrease in glycogenolysis and gluconeogenesis (Mahomed and Ojewole, 2003; Andrade-Cetto and Wiedenfeld, 2004).

In the present study, we observed significant loss in the body weight of type 1 and type 2 diabetic rats, a symptom synonymous with diabetes mellitus (Pupim et al., 2005). The characteristic loss of body weight associated with STZ-induced diabetes could be due to dehydration and catabolism of fats or breakdown of tissue proteins, which leads to wasting of muscle. CAMFs treated type 2 diabetic rats exhibited comparable increases in body weight. This could be the result of increased glucose uptake, insulin secretion and decreased fasting blood glucose levels, indicating improved glycemic control in the rats. Such findings of improvement in body weight amongst diabetic animals are consistent with treatment of other medicinal

plants reported to have potential anti-diabetic effects (Pari and Saravanan, 2004; Nagarajan et al., 2005).

In this study, we have scientifically verified the traditional basis for the use of *C. anthelminticum* seeds in diabetes mellitus. The anti-diabetic potential of CAMFs may be attributed to the polyphenolic constituents as identified by LCMS-MS including quercetin glycoside, 3,4-*O*-dicaffeoylquinic acid, caffeic acid, naringenin-7-*O*-glucoside and kaempferol. These dietary polyphenols possess wide therapeutic benefits and many researchers have demonstrated the anti-diabetic, anti-oxidant, and anti-inflammatory activities with these compounds (Jung et al., 2006; Ortiz-Andrade et al., 2007; Shih et al., 2012). In addition, decanoic and pentadecanoic acid which are characterized by GC–MS with high similarity index in CAMFs have previously been shown as potential modulating ligands for peroxisome proliferator-activated receptors (PPARs) (Malapaka et al., 2012). Thus, combination of these compounds in CAMFs may exert synergistic anti-diabetic effects in the type 2 diabetic rats.

5. Conclusions

In summary, the present findings suggest that the anti-diabetic effects of CAMFs may be due to the enhancement of 2-NBDG glucose uptake, stimulation of insulin secretion and inducing higher GLUT-2 and GLUT-4 transporter protein expression in β-TC6 cells. These observations were corroborated by the beneficial effects of CAMFs in attenuating hyperglycemia and augmenting insulin secretion in type 2 diabetic rat model. These findings supports the use of CAMFs as a potential adjunct dietary treatment of insulin-resistant type 2 diabetes and a potential source for the discovery of new orally active agent(s) for future therapy of diabetes.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2012.08.014.

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