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

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ABSTRACT

This study aimed to ascertain the potential of *Centratherum 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 H_2O_2-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.

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, *Centratherum 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; HbAlc, 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.

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promotes low-grade inflammation via activation of nuclear factor-κB (NF-κB) (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-1β, 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-1β (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 cumin) 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 H_2O_2-induced NF-κB 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 spectometry 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 μL. Separation of the compounds was performed using a Luna 3-μm RP C18 column (100 × 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 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 μL was transferred to 96-well mircoplate (TPP, USA). To this, 80 μL of Folin–Ciocalteu reagent (1:10) were added and mixed thoroughly. After 5 min, 160 μL of sodium bicarbonate solution ($NaHCO_3$ 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% CO_2 in a humidified incubator. The growth medium was changed every 3 days.

2.2.2. NF-κB 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 μg/mL CAMFs, or were left untreated. The cells were then stimulated for NF-κB translocation with 50 μM of H_2O_2 for 30 min. NF-κB staining was performed according to the manufacturer's instructions with an NF-κB 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-κB–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 ± 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