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Moringa concanensis Nimmo ameliorates hyperglycemia in 3T3-L1 adipocytes by upregulating PPAR-γ, C/EBP-α via Akt signaling pathway and STZ-induced diabetic rats

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ABSTRACT

Moringa concanensis Nimmo is a medicinal plant for treating various human illnesses including menstrual pain, high blood pressure, jaundice, inflammation, pain, fever, sore eyes, and cholesterol in Indian folk medicine. Despite its versatility, its antihyperglycemic mechanism of action (*in vitro* and *in vivo*) remains unclear. Therefore, in this study we developed the possible antihyperglycemic mechanism of action in 3T3-L1 cells by evaluating mRNA and protein expression, which are associated with adipogenesis and lipogenesis (insulin sensitizer). Also, the antihyperglycemic activity of the ethanolic extract of *M. concanensis* Nimmo leaves (EEMCN) was evaluated on glucose, insulin, biochemical, and lipid profile in experimental diabetic rat models induced with streptozotocin (STZ). Results showed that EEMCN leaves enhanced lipid accumulation in 3T3-L1 cells, as assessed by Oil Red O staining, and upregulated gene expression level of PPAR-γ, C/EBP-α, t-SREBP, FAS, Glut-4, adipogenin, DAG, and LPL through Akt signaling in 3T3-L1 cells. Also, EEMCN treatment increased body weight and insulin level and lowered blood glucose, HbA1c, amylase, and lipid profile level in STZ-induced diabetic rats. In conclusion, EEMCN possesses *in vivo* antidiabetic potential, having such efficacy through a mechanism of action that involves antihyperglycemic, hypoglycemic, and potential insulin sensitizer (PPAR-γ, C/EBP-α/Akt over expression) action.

1. Introduction

Diabetes mellitus, commonly known as a group of chronic metabolic disorders, is a public health issue whose prevalence worldwide has been on the rise. It is characterized by hyperglycemia with unusual carbohydrate, fat, and protein metabolism resulting from defective pancreatic β-cells or insulin deficiency/action. This chronic hyperglycemic circumstance of diabetes is associated with long-term damage, dysfunction, and organ failure, particularly the nerves, eyes, blood vessels, heart, and kidney [1]. The total type 2 diabetes population in developed and developing countries has increased from 285 million to 387 million in 2010–2014, because of their lifestyle, age, obesity, and food habits [2]. Diabetes is also associated with an increase in lifestyle diseases including stroke, ischemic heart and hypertensive disease, kidney failure, and blindness. Several approaches are available to control diabetes mellitus, including dietary intervention, aerobic exercise, insulin injection, and a wide array of hypoglycemic drugs (troglitazone (TRZ), metformin, and sulphonylureas). However, long-term conventional synthetic drug treatment is expensive, hardly accessible to people, and may also cause severe side effects; therefore, herbal pharmacotherapy has expanded globally, becoming more readily available affordable to people [3]. Recently, researchers have been paying more attention on the development of antidiabetic drugs from plant sources used in traditional medicine systems. Because of conventional medicine, natural products can prove to be a better treatment than currently used molecules responsible for the severe ambiguous side effects of diabetes [4].

Moringa concanensis (*M. concanensis*) Nimmo is an Indian medicinal plant belonging to the Moringaceae family, growing in various locations including India as well as Asian and Arab countries. Tribal

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communities in the Western Ghats region of Tamil Nadu use it to treat antifertility [5]. The *M. concanensis* Nimmo root and root bark are used for various human illnesses including paralysis, rheumatism, abscess, and epilepsy, and their fruits are used to treat liver and kidney disease as well as joint pain [6]. *M. concanensis* Nimmo leaves can also minimize menstrual pain, high blood pressure, jaundice, constipation, skin tumors, and cholesterol, and its gum can control dental problem and headaches, and flowers have been used to treat thyroid storms (hyperthyroidism) and leucorrhea [7]. The Ayurveda and Unani systems of medicine also use it to treat inflammation, pain, fever, and sore eyes (sensations) [8]. Seeds of this plant can treat goiter, glycosuria, and lipid disorders [9]. To the best of our knowledge, *M. concanensis* Nimmo leaves have not been investigated for its hyperglycemic activity mechanism of action in 3T3-L1 cells and against STZ-induced diabetic rats. Therefore, in this study, we establish the efficacy of *M. concanensis* Nimmo on hyperglycemic activity in *in vitro* and *in vivo* models. Overall, this finding provides a scientific foundation for developing and applying *M. concanensis* Nimmo as a functional food with ameliorative efficacy in hyperglycemia.

2. Materials and methods

2.1. Preparation of experimental samples

Fresh leaves of *M. concanensis* Nimmo were collected from Perambalur, Tamil Nadu, India. The specimen sample was authenticated by the Botanical Survey of India (BSI), Tamilnadu Agricultural University, Coimbatore, Tamil Nadu, India. The voucher specimen (No.BSI/SRC/5/23/2016/Tech-151) was deposited in the herbarium cabinet. The leaves were rinsed with normal saline, dried in a shaded area to remove water, and then turned into powder using a blender. Of the powdered leaves, 10 g was extracted with 100 ml of ethanol using a Soxhlet apparatus and filtered by using Whatman No. 1 filter paper. The filtrate was then dried under reduced pressure (4.579 mm of Hg) and controlled temperature (-40 ℃). The concentrated ethanolic extract of *M. concanensis* Nimmo leaves (EEMCNL) was stored at -20 ℃ until further analysis.

2.2. Cell culture and chemicals

T3-L1 preadipocytes were procured from the American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Gaithersburg, MD, USA). The mRNA extraction and RT-PCR kits were purchased from Invitrogen (Carlsbad, CA, USA). Oil Red O staining, dimethyl sulfoxide (DMSO), dexamethasone, and 3-isobutyl-1-methylxanthine were obtained from Sigma-Aldrich (St. Louis, MO, USA). STZ was also purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents used in the study were of analytical grade.

2.3. Hypoglycemic activity in 3T3-L1 preadipocyte

2.3.1. Cytotoxicity assay

WST (water-soluble tetrazolium) was used to determine cytotoxicity assay. Cells were seeded into 96 wells at a density of 1×10^4 cells/well, including the DMEM medium with 10% FBS, and the plates were incubated for 24 h at 37 ℃ with 5% CO_2. After incubation, cells were then exposed to various concentrations (10–100 μg/ml) of EEMCNL. After 24 h and 48 h incubation of cells, WST was added and incubated for another 2 h. The absorbance of each well was noted at a wavelength of 450 nm using an ELISA microplate reader (Packard Instrument Co., Downers Grove, IL, USA).

2.3.2. Adipocyte differentiation

Adipocyte differentiation was made following the previously published protocol of Choi et al. [10], with few modifications. Briefly, 3T3-L1 preadipocytes were seeded into 6-well plates containing DMEM medium with 10% FBS at a density of 3×10^4 cells/well, and then the plates were incubated at 37 ℃ with 5% CO_2. After 24 h of 100% confluence of cells, growth media were changed with differentiation media (DMEM containing 10% FBS, 0.5 mM of DMI (3-isobutyl-1-methylxanthine), 1μM dexamethasone, 1.7 μM insulin). For the experiment, cells were differentiated in DMI with the presence of the selected concentration of EEMCNL for two days. After the incubation period, 1.7 μM insulin (postdifferentiation media) was added with differentiation media in the presence of EEMCNL for the next two days. Afterward, the cell differentiation media were substituted with cell culture growth media containing EEMCNL every two days for eight days.

2.3.3. Lipid quantification (Oil Red O staining)

At the end of the experiment, cells were washed thrice using PBS, and cells were fixed in formaldehyde at room temperature for 1 h. After washing, 3 ml of freshly prepared Oil Red O staining solution was added to each well, and plates were incubated at 37 ℃ for 15 min. After incubation, excess Oil Red O was washed out with PBS and distilled water. Cells were photographed using an inverted microscope (CKX41, Olympus Corporation, Tokyo, Japan). Also, Oil Red O stain was extracted from the wells using 100% isopropanol, and absorbance was noted at a wavelength of 490 nm [10].

2.3.4. Adipogenic gene expression quantification by quantitative RT-PCR

At the end of the experiment, cellular RNA was extracted from 3T3-L1 adipocytes using an RNeasy lipid tissue kit (Qiagen, Valencia, CA, USA) based on manufacturer protocol. Extracted RNA was quantified with UVS-99 μvol UV/Vis spectrometer-ACT gene, and 500 ng of cellular RNA was used to synthesize cDNA using oligo (dT) primers and reverse transcriptase provided by Superscript III first-strand synthesis system for RT-PCR (Invitrogen). SYBR green-based real-time PCR was carried out on an ABI 7500 PCR system (Applied Biosystems, Foster City, CA, USA). The target gene expression levels were normalized against housekeeping gene β-actin [10].

2.3.5. Protein extraction and immunoblotting

At the end of the experiment, cells were washed thrice with ice-cold PBS, added with 300 μl of RIPA lysis buffer/well included protease (1X) and phosphate inhibitors cocktail, and incubated for 5 min at 4 ℃ (Roche, Switzerland and Sigma-Aldrich, USA). The cells were then centrifuged for 10 min at 4 ℃ to remove unwanted material. The protein content was quantified using a Bio-Rad protein assay kit (Bio-Rad, Seoul, Korea). A Western blot was performed based on the Western Breeze chemiluminescence protocol (Invitrogen, Seoul, Korea) using monoclonal antibody against the targeted gene. Band signal were analyzed with an enhanced chemiluminescence kit (Bio-Rad, Seoul, Korea) on a chemiluminescence imaging system (Davinch Chemiluminescence, Seoul, Korea).

2.4. Hypoglycemic activity against STZ-induced diabetic rats

2.4.1. Animals and diets

Male Wistar albino rats (weighting 120–150 g) were purchased from Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Tamil Nadu, India, and were housed at 25 ± 5 ℃ in a well-ventilated animal house under a 12 h light–dark cycle for 1 week to adapt to their new environmental conditions. The animals were fed with commercial rat pellets and water *ad libitum*. The study was approved and carried out according to the guidelines of the Institutional Animal Care Ethics Committee (IAEC no: 07/2016/IAEC/KASC), Kongunadu Arts and Science College, Coimbatore, Tamil Nadu, India.

2.4.2. Experimental design

A total of 30 male Wistar albino rats weighing 120–150 g were