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Essential Oil of Cymbopogon Citratus Against Diabetes: Validation by *In vivo* Experiments and Computational Studies

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Abstract

This study evaluates the antidiabetic activities of essential oil obtained by steam distillation of the leaf sheath of Cymbopogon citratus (CCEO) in poloxamer-407 induced type 2 diabetic (T2D) Wistar rats. The sample was then analyzed by gas chromatography-mass spectroscopy (GCMS) identifying 23 compounds representing 96.9% of the oil. The major compounds of essential oil were geranial (42.4%), neral (29.8%), myrcene (8.9%) and geraniol (8.5%). When compared to diabetic control rats, the CCEO treated diabetic rats presented significant amelioration of glycaemia, insulinamia and lipid dysmetabolism, accompanied by increased GLP-1 content in cecum and remarkable reduction of oxidative markers. Histopatholgical analysis of pancreas showed increase in β-cell mass, islet number and quality of insulitis. HYBRID and FRED docking were performed for 48 documented CCEO phytoconstituents for putative action mechanism concerning three proteins namely PTP-1B, PPAR-γ and DPP-IV having diabetic therapeutic properties. Phytoconstituents like myrcenol, linalool, α-elemol and β-Eudesmol showed significant interaction with PPAR-γ and DPP-IV while only pimelyl dihydrazide showed interaction with PTP-1B. The results provided a pharmacological evidence of CCEO as antidiabetic mediated by interaction of various phytoconstituents with multiple targets operating in diabetes mellitus.

Keywords: Cymbopogon citratus; Antidiabetic; GCMS; PPAR-γ; PTP-1B; DPP-IV; Docking

Introduction

Type 2 diabetes (T2D) is a chronic metabolic disorder characterized by abnormalities of glucose homeostasis by various organs. It accounts for about 85-95% of all diagnosed cases of diabetes and is associated with considerable morbidity and mortality [1].

Several treatment strategies are being adopted to ameliorate T2D by therapeutic intervention. Synthetic drugs, despite having excellent potencies, had offered unwanted therapeutic profiles marked by fluid retention, drug-induced hypoglycemia, liver malfunctioning, weight gain and cardiac dysfunction [2]. Thus, there is a lot of scope for herbal medicines which have emerged as safe and relatively economical alternative therapy found to be effective as add on therapy, in the long-term management of type 2 diabetes [3].

Numerous research has been carried out world wide on molecular targets towards the development of newer antidiabetic agents like nuclear receptor PPAR-γ (peroxisome proliferator-activated receptor gamma) [4,5], human incretin-degrading enzyme DPP-IV (dipeptidyl peptidase IV) [5], protein-tyrosine phosphatase 1B (PTP1B) [6], etc. These therapeutic targets may improve the efficacy-to-safety ratio, resulting in durable maintenance of glycaemic control in the majority of people with diabetes. Several structurally unrelated natural products, including alkaloids, flavonoids or organic acids had also been described to interact with these diabetic proteins in micromolar concentrations [7].

The PPAR-γ is encoded by the PPARG gene in human. It is the key regulator of gene expression of glucose metabolism, inflammation, and other pathways like fatty acid storage [8], hence PPAR-γ agonists have been used in the treatment of hyperglycemia [4]. The DPP-IV, encoded by the DPP-IV gene in human is a membrane-bound, serine protease ectoenzyme. It is responsible for the degradation and inactivation of a number of glucose regulating incretin hormones like glucagon like peptide-1 (GLP-1) that causes an increase in the amount of insulin released from the β-cells. GLP-1 is found in numerous sites, including the kidney, intestine, and capillary [9]. DPP-IV has clinical significance for development of new class of oral antihyperglycemics called DPP-IV inhibitors [10]. PTP-1B, encoded by the PTPN1 gene in human and is a negative regulator of the insulin signaling pathway and regulates the lipogenesis and hypertriglyceridemia associated with T2D [6]. It has also emerged as a promising potential therapeutic target for T2D [6].

It has been documented in various literatures that essential oils of plant origin exhibit a wide spectrum of biological activities [11]. Cymbopogon citratus (DC) Stapf. (Lemon grass) (Graminaceae) is a source of essential oil widely used as a component of ethnopharmaceuticals in tropical and subtropical countries [12]. Among a range of essential oils isolated from different plant sources, lemongrass oil exhibit highest antioxidant activity and protect lipids peroxidation [13]. Essential oils from the aerial parts of different species of Cymbopogon have folkloric medicinal use in fever, digestive disorders, diabetes, inflammation and nerve disorders [12]. The biological activity of the essential oil obtained from C. citratus is due to the presence of citronellal and citral [12], which plays

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important detoxification and anti-cancer roles in the body by inducing glutathione-S-transferase (GST) activity [14,15].

Despite its folkloric use, there are insufficient supporting scientific data forming the basis of the present investigation which evaluates systematically, the beneficial effects of essential oil from leaf sheath of C. citratuus (CCEO) in poloxamer-407 (PX-407) induced diabetic conditions and the putative action mechanism of its phytoconstituents computationally concerning three diabetic therapeutic proteins namely PTP-1B, PPAR-γ and DPP-IV.

Poloxamer-407 (PX-407) is a non-ionic copolymer, commonly used as surfactants, emulsifying agents and solubilizing agent in pharmaceutical formulations. In rats, intraperitoneal administration of PX-407 develops hyperglycemia with low degree of toxicity (LD_{50} ≥ 1.8 g/kg body weight). It causes impaired response in the glucose tolerance test and loss of β-cell sensitivity to glucose and hence, considered as a useful experimental model to study the activity of antihyperglycemic agents [16]. Molecular docking was performed for 48 phytoconstituents from CCEO documented in literature hitherto to propose their biochemical interaction with diabetic proteins, namely PPAR-γ, DPP-IV and PTP1B using HYBRID [17] and FRED [18] docking program.

Materials and Methods

Chemicals

Poloxamer-407, heparin, glucose oxidase, peroxidase, o-dianisidine, bovine serum albumin, coomassie brilliant blue G-250, cysteine hydrochloride, thiosemicarbazide and diacetyl monoxime were purchased from Sigma Chemical Co. (St Louis, MO, USA). Blood glucose diagnostic kit was purchased from Span diagnostic Limited, Surat, India while glycosylated haemoglobin (HbA_1c) diagnostic kit from Monozyme India Limited, Secunderabad, India. The biochemical kits for measuring the lipid profiles were procured from Biosys, Bangalore and Ketamine from Neo Pharmaceuticals, Bangalore, India. The enzyme-linked immunosorbent assay (ELISA) kit for insulin assay and GLP 1 determination was purchased from Millipore Corporation (Linco Research, Inc., St. Charles, M.O.63304). Refined grade of casein was purchased from Nimesh Corporation (Mumbai, India), salt mixture from SISCO Research Lab Pvt. Ltd. (Mumbai, India) and standard pellet diet from Lipton Rat Feed Ltd. (Pune, India). All other chemicals and solvents used in this study were obtained from Merck, India and were of analytical grade.

Collection and preparation of plant materials

The leaf sheath of Lemongrass was collected from Hajipur locality in northern India, in 2010, and purchased from local folk medicinal plant dealer in Patna, Bihar, India. The plant was identified and botanically authenticated at source and a voucher specimen (CAS#106/2011) has been deposited in the post graduate department of biochemistry, Patna University, Patna for future reference. Five hundred grams of plant samples were chopped into small pieces and crushed using a mortar and pastel to increase the surface area. Then, the plant samples were dried using a freeze dryer (Beta 1-8LD, Martin Christ, German) at -55° C, and 0.070 mbar for 48 h to remove the moisture content.

Extraction of essential oil and GCMS analysis

The freeze dried sample of lemongrass (200 g) was steam-distilled for 1.5 h in a Clevenger-type apparatus, with 700 ml of water at fixed temperature and extraction time based on the optimization range to give a mixture of water/ essential oil [19]. The essential oil was cooled and separated from the water layer by using dichloromethane and dried in excess of anhydrous sodium sulphate (Na_2SO_4). It was then stored in dark at 4°C and analyzed for its major constituents by GC and GC–MS.

GC analysis of CCEO was carried out on a Hewlett Packard 5890 II GC instrument. A HP-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) was used with a HP 5972 mass selective detector and an electron ionization system with ionization energy of 70 eV. Helium was used as the carrier gas at a flow rate of 1 ml/min keeping constant inlet pressure at 200 p.s.i. Injector and MS transfer line temperatures were set at 220 and 290°C, respectively. The GC column temperature was gradually increased from 40°C to 240°C at a rate of 3°C/min with a hold time of 10 min followed by final increment up to 250°C at a rate of 10°C/min. One microlitre (µl) of diluted samples (1/100 in acetone) were injected manually and splitless. The constituents of CCEO were identified by using standards of the main components, comparing their relative retention times and matching the mass spectra fragmentation pattern with those of NBS75K library data stored in the GC–MS database.

Experimental animals and induction of diabetes

The healthy male albino Wistar rats (150-160 g) were kept under good hygienic condition and fed on standard pellet diet (SPD) (Lipton Rat Feed Ltd., Pune, India) and ad libitum water throughout the experimental period. Rats had undergone multiple administrations of freshly prepared PX-407 solution (10 mg/ kg body weight once a day for 6 weeks) followed by an hour of fasting. They were allowed to access the respective food and water ad libitum for 6 weeks. Rats with fasting blood glucose level of 200 mg/dl or higher were considered to be diabetic and were used in the study. During the experimental period, the animals were observed within cages daily for mortality or signs of toxic effects. The experimental study was approved by the Institutional Animal Ethics Committee of Department of Health, Patna, Bihar, India.

Experimental design

The rats were divided into four different groups having six rats in each group: DC group (diabetic control rats), NC group (non-diabetic control rats), DT_{400} group (diabetic rats treated with 400 mg of CCEO / kg b.w.), and DT_{800} group (diabetic rats treated with 800 mg of CCEO / kg b.w.). Body weights were recorded weekly during the experimental period. Treatment with CCEO was started from the 3rd week of PX-407 injection, which was considered as the 1st day of treatment.

Analytical parameters

Glycaemic, insulinemic and lipidic profile: Blood samples were collected from the caudal vein by means of a small incision at the end of the tail. Plasma glucose levels were measured using a Glucose oxidase commercial kit (Span diagnostic Limited, Surat, India). Taking into account the variability of serum glucose levels in rats, glycosylated hemoglobin (HbA1c) levels were used as an index of glucose control (Monozyme India Limited, Secunderabad, India). Serum insulin (SI) was estimated by radioimmunoassay method using the kit from Bhabha Atomic Research Centre, Mumbai, India. Insulin sensitivity of individual animals was evaluated using the previously validated homeostasis model assessment (HOMA) index [20] using formula: . The values used (insulin and glucose) were obtained after an overnight of food deprivation. The serum was separated and analyzed spectrophotometrically for triglyceride (TG) and total cholesterol (TC) using diagnostic reagent kit (Nicholas Piramal India Ltd., Mumbai).

Endogenous enzymatic and non-enzymatic antioxidant levels: At the end of the experimental period, rats from different groups were fasted overnight and sacrificed under anesthesia (i.p. ketamine, 150 mg/kg body weight). The whole liver was dissected out subsequent to saline water perfusion followed by preparation of their 10% liver homogenate with ice cold saline-EDTA. The protein content was measured with the Folin phenol reagent [21]. The homogenate was further subjected to the evaluation of non-enzymatic (reduced glutathione and total thiols) and enzymatic antioxidant (catalase, GST, and SOD) status using spectrophotometric methods [22]. Lipid peroxidation levels of the liver homogenates were also determined [23].

GLP-1 content in the cecum

The cecal contents from excised cecum of sacrificed rat were removed. The cecal tissue was thoroughly washed with cold phosphate buffer saline (PBS; pH 7.4) and homogenized at 4ºC in PBS containing DPP-IV inhibitor (20 µl/ml PBS). The homogenate was then centrifuged at 10,000 g for 10 min followed by the supernatant collection and GLP-1 level determination by ELISA kit, (Millipore, Corporation. Linco Research, Inc., St. Charles).

Histological studies: Pancreases of animals from different experimental groups were examined in situ, excised, and perfused with 10% neutral formalin. It is then embedded in paraffin, thinly sectioned using a microtome, stained with Hematoxylin-Eosin (HE), mounted in neutral DPX medium and examined under a light microscope. Histopathological studies were performed to estimate the beta cell mass, increase in the islet number and to check the quality of insulitis.

Molecular docking studies

Data collection and ligand preparation: To gain further insight in the interaction of fruit extracts with diabetic proteins, 48 active phytoconstituents of CCEO, reported in various literatures were selected for docking studies (Table 3). Two dimensional structures of these phytoconstituents were either retrieved from PubChem [24] or drawn using tool msketch available from ChemAxon (http://www.chemaxon.com). The correct protonation states were enumerated by using tautomer enumeration program (http://www.eyesopen.com). The partial charges were assigned by using MMFF94 [25] force-field available in molcharge (http://www.eyesopen.com) programs. Further, stereocenters were enumerated using flipper (http://www.eyesopen.com) in those molecules where a stereocenter does not have a specified stereochemistry. The 3D conformers (maximum 200 per molecule and 10 Kcal/mol energy cut-off) have been generated using OMEGA (http://www.eyesopen.com) and used for docking to three diabetic proteins namely DPP-IV, PPAR-γ and PTP1B.

Protein preparation and self-docking: The receptors for three selected proteins namely DPP-IV, PPAR-γ and PTP1B were prepared using fred-receptor preparation tool (http://www.eyesopen.com). There are 72, 113 and 120 structures available in PDB database for DPP-IV, PPAR-γ and PTP1B respectively. Out of these, PDB IDs 1X70 (2.10 Å) [26], 2HWQ (1.97 Å of resolution) [27] and 1T49 (1.90 Å) [28] were selected respectively for DPP-IV, PPAR-γ and PTP1B. Protein constraints were set-on based on interaction described in PDB complexes of above mentioned proteins. These hydrogen bonding constraints are set on residues E205, E206, Y631, Y547 in DPP-IV, H449, Y473 in PPAR- γ and N193, F196, E276, F280 in PTP1B allosteric sites. The phytoconstituents satisfying at-least one constraints are successfully docked using docking program. The correctness of protein preparation step was checked by self-docking process in which the co-crystallized ligand was re-docked in the receptor. For acceptable self-docking the root mean square deviation (RMSD) should be less than 2.00Å compared to the reference structure (http://www.ccdc.cam.ac.uk/products/life_sciences/gold/case_studies/flexible_sidechains/). The docking was performed using FRED [18] and HYBRID [17] docking program. To pose and score new ligands, HYBRID uses the structure of both the target protein and the shape and chemical features of the bound ligand to the active sites [18], while FRED uses only the structure information of the target protein and can be found in online supplementary data. Forty eight phytoconstituents documented hitherto from C. citratus have been docked to three proteins-1T49 (PTPB1), 2HWQ (PPAR-γ), 1X70 (DPP4) involved in diabetes and molecular interactions have been plotted using PoseView [29].

Statistical analysis

Data were expressed as the mean ± S.E.M, group means were compared by one-way ANOVA and the Tukey–Karmer Post Hoc test was applied to identify significance (p-value<0.001) among groups. Graphs are plotted using MATLAB version 7.8.0 R2009a, Natick, Massachusetts: The Mathworks Inc. 2009.

Results

Composition of the essential oil of CCEO

The essential oil obtained from the steam distillation of lemongrass sample was analyzed by GC MS identifying 23 compound representing 96.9% of the oil (Table 1). The major compounds of essential oil were geranial (44.5%), neral (31.2%), myrcene (9.6%) and geraniol (7.9%) (Table 1).

Serial No.	Components	KI [a]	Percentage (%)
	Myrcene	981	8.9
	(Z)-β-ocimene	1035	0.2
	(E)-β-ocimene	1048	0.3
	6,7-epoxymyrcene	1091	0.1
	Linalool	1101	0.3
	2,2-octa-3,4-dienal	1105	0.1
	menth-3-en-9-ol	1149	0.1
	Citronellal	1154	1.9
	cis-chrysanthenol	1162	0.5
	epoxy rose furane	1171	0.1
	Nerol	1231	0.2
	Neral	1245	29.8
	Geraniol	1255	8.5
	Geranial	1277	42.4
	geranyl formate	1298	0.1
	géranyl acetate	1377	3.1
	β-caryophyllene	1418	-
	trans-α-bergamotene	1435	-
	Caryophyllene oxide	1586	0.3
	Hydrogenated monoterpens		10.7
	Oxygenated monoterpens		80.8
	Hydrogenated Sesquiterpens		3.1
	Oxygenated Sesquiterpens		0.5
	Total		96.9

[a]KI was calculated using a series of n-alkanes

Table 1: Chemical composition of essential oil of Cymbopogon citratus (CCEO).

Effect of CCEO on body weight and lipid profile

The diabetic control (DC) rats demonstrated higher body weight (+107%; p<0.001) when compared with the normal control (NC) rats (Table 2). Compared to the DC rats, administration of CCEO to diabetic rats at two different concentrations (DT_{400} and DT_{800}) did not reduce the body weight significantly (Table 2). The DC rats also presented higher total cholsterol (TC) (+173%; p<0.001) and TGs (+589%; p<0.001) values compared to NC rats. Compared to the DC rats, the diabetic rats treated with lower dose of CCEO (DT_{400}) showed significantly lower values of serum TC (+23.5%; p<0.001) and TGs (+34.25%; p<0.001). In contrast, the DT_{800} treatment had no diminishing effects, neither on TC nor on TGs levels (Table 2).

Effect of CCEO on glycaemia, HbA1c, insulinaemia, and insulin Resistance

As expected, the DC rats showed significantly (p<0.001) higher level of glucose (+359%), HbA1c (+274%), and insulin (+268%), together with higher insulin resistance (HOMA-IR), when compared with their treated counterparts (Table 2). Diabetic rats of both of the groups (DT_{400} and DT_{800}) showed a reduction in glucose levels, when compared to the DC ones; nevertheless, the reduction was particularly evident in the DT_{800} rats (−47.3%; p<0.001). When compared, the glucose levels of the DT_{800} versus the DT_{400} group rats, a significant lower value in the first was also found (−25.0%; p<0.001) (Table 2). The 12-week treatment program also significantly diminished the HbA1c levels (−20.73%; p<0.01) in the diabetic rats, although inconsequential changes were detected in HbA1c levels of the DT_{800} group rats. In agreement with the above results, CCEO treatments in diabetic rats (DT_{400} and DT_{800}) significantly reduced (p<0.001) the insulin levels as well as insulin resistance evaluated by HOMA-IR index (Table 2). Nevertheless, this drop in insulin resistance was more evident in the DT_{800} rats (−65.84%) than in the DT_{400} rats (-19.75%).

Effect of CCEO on endogenous enzymatic and non-enzymatic antioxidant levels

Reduced glutathione (GSH): NC rats showed basal GSH levels of about 10.86 ± 0.17 nmoles/mg of protein. PX-407 induction for six weeks led to significant reduction (p<0.001) in GSH levels (3.19±0.37 nmoles/mg of protein). Treatment with higher doses of CCEO to diabetic rats (DT_{400} and DT_{800}) showed significant (p<0.001) improvement in GSH levels (Figure 1A).

Total thiol: Basal total thiol levels in NC rats were found to be 8.78 ± 0.96 μmoles/mg of protein. Diabetic rats showed significantly decreased (p<0.001) levels of total thiols (3.21 ± 0. 39 μmoles/mg of protein). Moreover, treatment of diabetic rats with CCEO (DT_{400} and DT_{800}) showed significantly (p<0.01) increased levels (Figure 1B).

Lipid peroxidation: NC rats showed basal TBARS (thiobarbituric acid reactive substances) levels of about 11.83 ± 1.79 nmoles/g of liver tissue. Diabetic rats showed significantly increased (p<0.001) TBARS levels (104.19 ± 3.24 nmoles/g of tissue). Treatment with CCEO (DT_{400} and DT_{800}) significantly (p<0.001) abolished the increase in TBARS levels induced by PX-407 (Figure 1C).

Glutathione-S-transferase (GST)

Normal basal GST activity in NC rats was found to be 0.059 ± 0.007 U/mg of protein. Diabetic rats exhibited significantly reduced (p<0.001) levels of GST (0.0063 ± 0.0004 U/mg of protein). The two tested doses (DT_{400} and DT_{800}) of CCEO significantly (p<0.001) increased the levels of GST (Figure 1D).

Catalase: Normal basal level of catalase activity in NC rats was found to be 172.86 ± 6.79 U/ mg of protein. Diabetic rats showed significantly decreased (p<0.001) levels of catalase by about more than 3-folds (i.e. 49.56 ± 3.26). However, treatment with CCEO (DT_{400} and DT_{800}) showed significantly (p<0.001) increased levels of catalase to the near normal values (Figure 1E).

Superoxide dismutase (SOD)

NC rats showed basal SOD levels of about 30.87 ± 2.37 U/mg of protein. Diabetic rats exhibited significantly reduced (p<0.001) levels of SOD by about one fourth (7.38 ± 0.76 U/mg of protein). Treatment with CCEO (DT_{400} and DT_{800}) showed significantly (p<0.001) increased levels of SOD to the near normal values (Figure 1F).

Effect of CCEO on pancreas weight: The pancreatic weight of diabetic rats was found lower than those of the NC rats (p<0.001). However, CCEO-treatment to diabetic rats (DT_{400} and DT_{800}) revealed significant increase (p<0.001) in pancreas weight compared to DC rats (NC: 2.46 ± 0.08 g, DC: 1.32 ± 0.04 g, DT_{400}: 1.76 ± 0.39 g, DT_{800}: 2.14 ± 0.41 g) (Figure 2A). Even so, when other parameters were normalized, CCEO treated diabetic rats showed an increase in pancreatic weight compared to NC rats.

Effect of CCEO treatment on GLP-1 levels in cecum tissue: To examine whether CCEO treatment influenced GLP-1production, the incretin level in intestinal tissue was determined. In cecum, diabetes (DC group) resulted in 76% decrease in GLP-1 concentration expressed in picomoles per total cecum compared to NC rats. The CCEO treatment significantly increased GLP-1 concentration in a dose dependent manner. Due to the significant (p<0.001) cecal tissue enlargement in DT rats in comparison to DC rats, GLP-1 content was 1.9-fold higher in DT_{400} and 2.1-fold higher in DT_{800} (Figure 2B).

	Control rats			Treated rats			
	NC	DC	Effect	DT400	Effect	DT800	Effect
Body weight (g)	157.13 ± 2.30	325.50 ± 2.73**	⇑	336.00 ± 3.76	⇔	322.40 ± 3.85	⇔
Glucose (mg/dl)	133.72 ± 2.08	614.85 ± 3.84**	⇑	432.55 ± 15.49*	⇓	323.96 ± 4.89*	⇓
HbA1c (%)	3.38 ± 0.12	12.59 ± 0.12**	⇑	09.98 ± 0.79	⇓	11.58 ± 0.67	⇔
Insulin (pmol/l)	50.31 ± 0.99	184.85 ± 1.28**	⇑	162.66 ± 0.83*	⇓	158.98 ± 1.76*	⇓
HOMA-IR	2.34 ± 0.98	40.35 ± 2.02**	⇑	32.38 ± 1.65*	⇓	13.78 ± 1.39*	⇓⇓
TC (mg/dl)	75.95 ± 1.17	208.87 ± 1.37**	⇑	159.85 ± 2.65*	⇓	202.29 ± 2.48	⇔
TGs (mg/dl)	60.25 ± 0.71	418.06 ± 3.19**	⇑	274.87 ± 2.59*	⇓	398.95 ± 3.73	⇔

Results are mean±SEM of 6 rats per each group. Statistically significant differences are indicated by the symbols: * versus normal control rat group and ** versus diabetic control rat group. p< 0.001

Table 2: Effect of CCEO essential oil on body weight, glucose, HbA1c, insulin, HOMA-IR and lipid profiles (TC and TGs) on different rat groups.

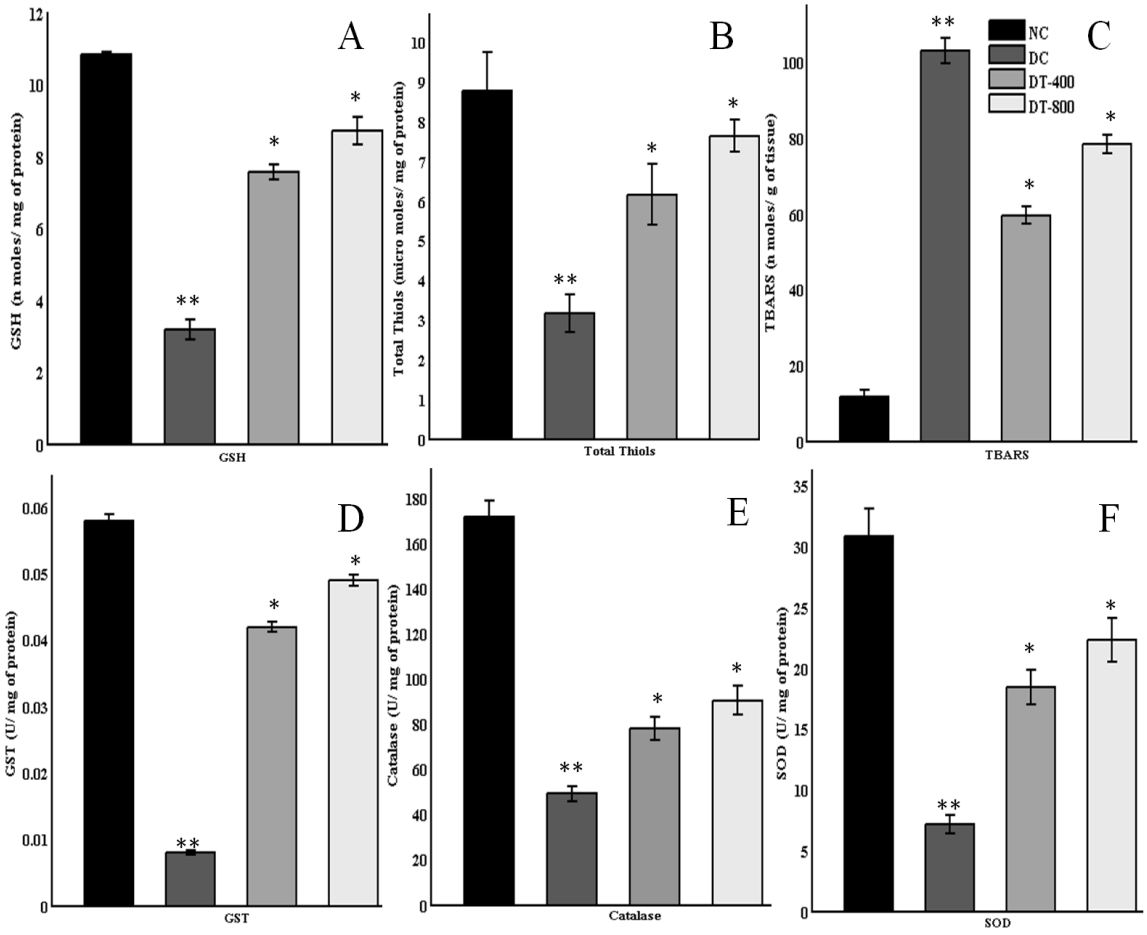

Figure 1: Effect of treatment with CCEO on non-enzymatic antioxidants GSH (A) and total thiols (B) as well as TBARS (C) and enzymatic antioxidants GST (D), Catalase (E) and SOD (F) in the liver homogenates of different rat groups. NC, untreated normal control animals; DC, diabetic control animals; DT_{400}, diabetic animals treated with a low dose of CCEO (400 mg /kg b.w.); DT_{800}, diabetic animals treated with a high dose of CCEO (800 mg /kg b.w.). Each bar represent the mean ± SE (n=6). **p<0.001, compared to the normal control rats. *p<0.001, compared to the diabetic control rats.

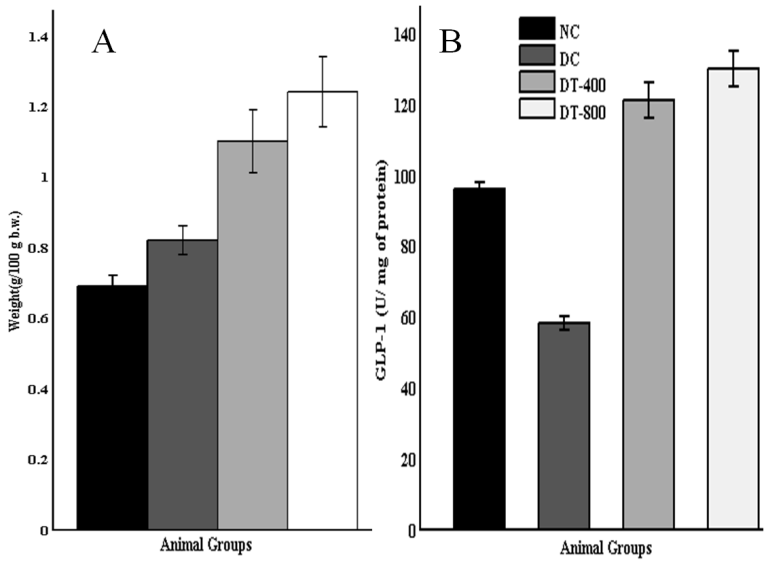

Figure 2: Effect of CCEO treatment on pancreas weight and GLP-1 contents. Each bar represent the mean ± SE (n=6). NC, untreated normal control animals; DC, diabetic control animals; DT_{400}, diabetic animals treated with a low CCEO (400 g /kg b.w.); DT_{800}, diabetic animals treated with a high CCEO dose (800 g /kg b.w.). Each bar represent the mean ± SE (n=6). **p<0.001, compared to the normal control rats. *p<0.001, compared to the diabetic control rats.

Histopathological analysis

The effect of CCEO on the number of pancreatic islets, its size and the grade of insulitis on different rat groups was shown in Table S1; Supplementary information. In the DC group, the number and size of islets decreases significantly ($p<0.001$) and the grade of insulitis is also very high, when compared to NC rats (Figure 3). In DC_{400} and DC_{800} rats there was a significant improvement in the number of islets as well as the grade of insulitis.

LD_{50}

The behavior of the treated rats appeared normal. No toxic effect was reported up to the effective dose of the CCEO, as there was no death in any of these groups.

Molecular docking studies

Self-docking: In case of PTP1B and DPP-IV the self-docking RMSD was observed around 1 Å indicating acceptable quality of docking preparation while for PPAR-γ self docking observed RMSD was 1.45 Å.

CCEO phytoconstituents docked to PTP1B, PPAR-γ and DPP-IV: Out of 48 phytoconstituents, a total of 14 and 13 phytoconstituents respectively were successfully docked to PPAR-γ and DPP-IV while only pimelyl dihydrazide (Figure 4) was capable to dock to PTP1B (Tables 4 and 5).

Discussion

The studies assessing the physiological and biochemical effects of bioactivities of natural or synthetic products in humans are logistically and methodologically complex. Therefore we should rely on laboratory animal models of treatment and computational docking protocols that mimic the treatment response in humans [3]. Now a day, the PX-407 induced diabetic rats are commonly used as an animal model of human T2D to study pathophysiological characteristics, in addition to therapeutic influences. PX-407 induction to neonatal rats at the dose of 10 mg/kg b.w. intraperitoneally, increases body weight, manifest hyperglycemia, an impaired response to the glucose tolerance test and loss of β-cell sensitivity to glucose [30]. These alterations in biochemical parameters are inevitable, when the unrestrained diabetic status advances, considerable modifications in these biochemical indices are expected.

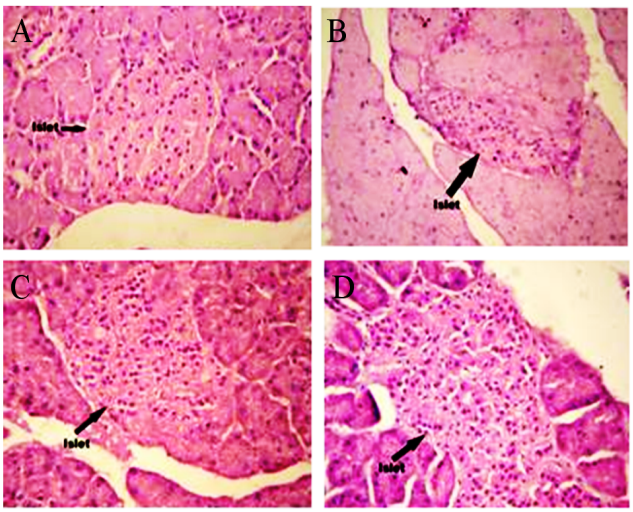

Figure 3: Histopathology of pancreas showing variation in islet number, size and grade of *insulitis* in each rat groups. (A) NC, untreated normal control animals; (B) DC, diabetic control animals; (C) DT_{400}, diabetic animals treated with a low dose of CCEO (400 mg /kg b.w.); (D) DT_{800}, diabetic animals treated with a high dose of CCEO (800 mg /kg b.w.).

Compound No	Name	Pub-chem CID
1	β-Myrcene	31253
2	β-Ocimene	5281553
3	α-Pinene oxide	91508
4	τ -Muurolol	3084331
5	1-Octyn-3-ol	13166
6	Allo-ocimene	5368821
7	Myrcenol	10975
8	Linalool	6549
9	Trans-chrysanthemal	579152
10	Citronellal	7794
11	(-)-Isopinocampheol	10524983
12	5-Cyclopropylidene-1-pentanol	551450
13	3-undecyne	143689
14	3-carvomenthenone	6987
15	Neral	643779
16	Geranial	638011
17	Citronellol	8842
18	Dextro-carvone	16724
19	Cycloisolongifolene	563197
20	Trans-(-)-Carveol	94221
21	Nerol	643820
22	Methyl-n-nonyl-ketone	8163
23	Geranic-acid	5275520
24	Geranyl-acetate	1549026
25	Isolongifolene-4-5-9-10-dehydro	588771
26	Levo-β-elemene	10583
27	α-Gurjunene	15560276
28	α-Bergamotene	86608
29	γ-Muurolene	12313020
30	α-Muurolene	12306047
31	α-Amorphene	12306052
32	β-Sesquiphellandrene	519764
33	α-Farnesene	5281516
34	δ-Cadinene	441005
35	δ-Cadinene	441005
36	Valencene	9855795
37	α-Selinene	10123
38	α-Guaiene	107152
39	Isocaryophyllene	5281522
40	α-Elemol	92138
41	Germacrene-D	5373727
42	Viridiflorol	94174
43	Humulene	5281520
44	τ-Cadinol	519662
45	β-Eudesmol	91457
46	(E,E)-Farnesal	5280598
47	Pimelyl dihydrazide	284646
48	Di-n-octyl phthalate	8346

Table 3: Phytoconstituents documented hitherto for docking from C. citratus plant.

The effects of medicinal plants in diabetic organism are of great interest due to their possible synergistic effects on metabolic profile. In the present study, the higher dose of CCEO (in DT_{800})

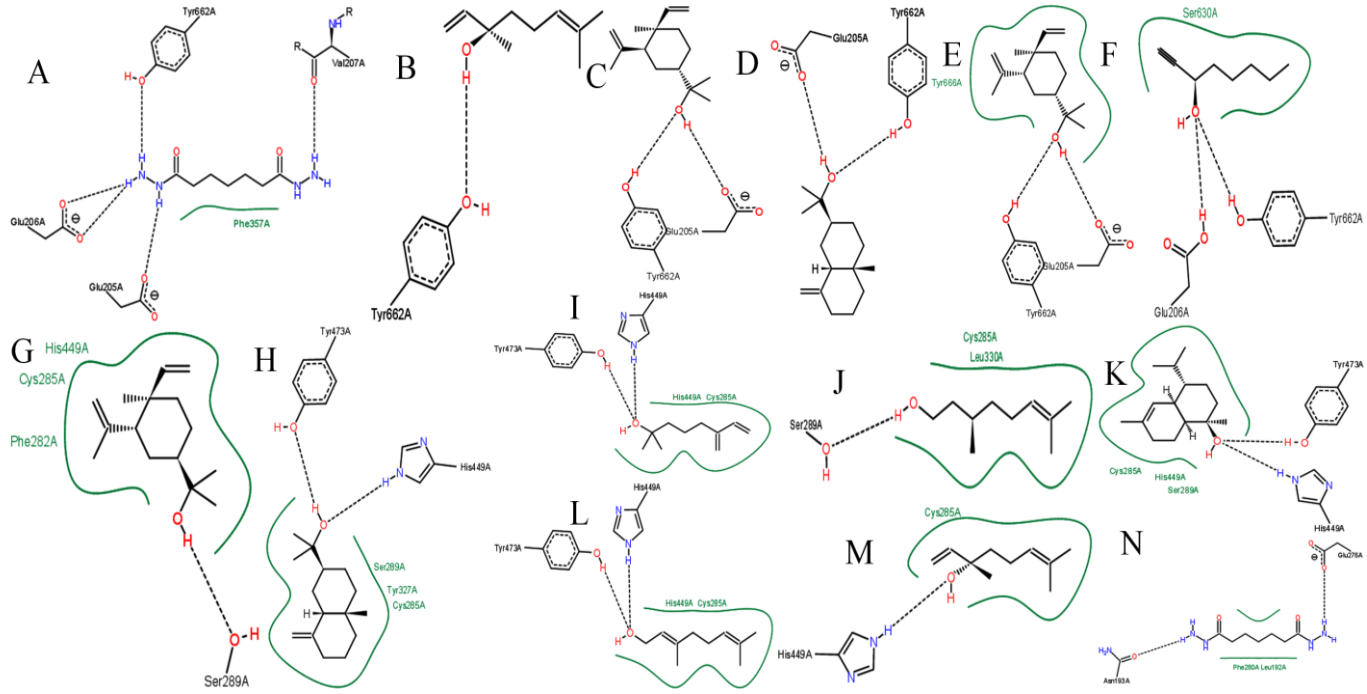

Figure 4: 2D docked plots for compounds shown to have maximum interactions with active sites of DPP-IV: Pimelyl dihydrazide (A), Linalool (B), α-Elemol (C), τ-Cadinol (D), Trans-(-)-Carveol (E), 1-Octyn-3-ol (F); PPAR-gamma: α-Elemol (G), β-Eudesmol (H), Myrcenol (I), Nerol (J), Citronellol (K), Viridiflorol (L), Linalool (M), and PTP1B: Pimelyl dihydrazide (N).

PDB ID	Name	Ligand ID	RMSD (Å)	Interacting Residues in PDB	Interacting Residues in self-docking	Type of Interaction
1X70	Dipeptidyl peptidase IV (DPP-IV)	715	0.71	Phe357A; Tyr662A	Tyr662A	Pi-Pi
				Tyr662A; Glu206A; Glu205A	Tyr662A; Glu206A; Glu205A	Hydrogen Bond
				Ser630A; Trp629A; Gly628A; Tyr547A	Ser630A;Trp629A; Trp627A	Hydrobhobic
2HWQ	Peroxisome Proliferator- Activated receptor Gamma (PPAR-γ)	DRY	1.45	His449A; Tyr473A	Tyr473A; Ser342A	Hydrogen Bond
				Glu276A; Asn193A	Glu276A; Asn193A	
1T49	Protein tyrosine phosphatase 1B (PTP1B)	892	1.11	Phe280A; Phe196A	Phe280A; Phe196A	Pi-Pi
				Glu276A; Asn193A	Glu276A; Asn193A	Hydrogen Bond
				Phe280A; Phe196A; Leu192A	Phe280A; Phe196A; Leu192A	Hydrobhobic

Table 4: Protein Structures studied.

significantly lowers plasma glucose as well as insulin level in diabetic rats accompanied by a decline in insulin resistance, calculated by HOMA-IR. Therefore, we hypothesize that CCEO administration at higher dose was capable of improving peripheral insulin resistance, albeit insignificant effect on hepatic resistance, proposing that hyperinsulinaemia might be the spontaneous effect of insulin resistance in the liver. These favorable effects of CCEO may be attributed to synergistic action of their phytoconstituents particularly the essential oil fraction on multiple targets including PPAR-γ activation and DPP-IV inhibition which may therefore regulate the hyperglycemia, lipogenesis and hypertriglyceridemia associated with T2D.

Despite amelioration in glucose metabolism by CCEO treatment, it was unable to induce beneficial modifications in the lipid profile (TGs and TC). This was supported by our docking results in which the CCEO phytoconstituents showed poor interaction with PPAR-γ ligand. It has also been demonstrated that CCEO treatment improved the lipidic profile of diabetic rats by reducing the total cholesterol and TGs contents [31].

Hyperglycemia and hypertriglyceridemia are well known to increase the generation of reactive oxygen species (ROS) and subsequent lipid peroxidation which are known to accelerate the pathogenesis of T2D, as evidenced by the glucose and lipid toxicity theories [1]. The catalytic actions of antioxidant enzymes are important for the effective removal of oxygen radicals. Increased generation of free radicals by PX-407 induction to rats reduces the activities of antioxidant enzymes such as catalase, GST and SOD as observed in DC group rats. The CCEO treatment presented a significant normalizing effect on these antioxidant enzymes in accordance with earlier findings [32].

Our study showed that CCEO treatment significantly increased GLP-1 content in the cecum of diabetic rats accompanied by an important cecal tissue enlargement. This result was also supported by our docking results in which some of the phytoconstituents showed strong inhibition of DPP-IV. Some important documented biological effects of GLP1 include insulin secretion, glucagon and somatostatin inhibition over and above pancreatic beta-cell mass development, maintenance,

Compd No.	Pubchem Cid	HYBRID_score	FRED Score	Interacting Residues	Major Interactions
Phyroconstituents docked to DPP-IV; Bioactive ligand has HYBRID Score (--14.51)					
47	284646	-7.689908	-7.55946	Tyr662A, Glu206A, Glu205A	Hydrogen bond
				Phe357A	Hydrophobic
8	6549	-6.635406	-6.814846	Tyr662A	Hydrogen bond
40	92138	-6.566278	-6.800921	Tyr662A, Glu205A	Hydrogen bond
45	91457	-6.539947	-6.594956	Tyr662A, Glu205A	Hydrogen bond
20	94221	-6.328463	-6.694277	Tyr662A, Glu205A	Hydrogen bond
				Tyr666A	Hydrophobic
5	13166	-6.253001	-7.696756	Tyr662A, Glu206A	Hydrogen bond
				Ser630A	Hydrophobic
21	643820	-5.999903	-5.764823	Tyr662A	Hydrogen bond
				Phe357A	Hydrophobic
17	8842	-5.863444	-6.1567	Tyr662A, Glu205A	Hydrogen bond
				Phe357A	Hydrophobic
7	10975	-5.70859	-6.477645	Glu205A	Hydrogen bond
4	3084331	-5.659113	-5.829168	Glu205A, Glu206A	Hydrogen bond
				Phe357A	Hydrophobic
44	519662	-5.659113	-5.829168	Glu206A, Glu205A	Hydrogen bond
				Phe357A	Hydrophobic
42	94174	-5.616273	-5.616273	Tyr662A, Glu206A, Glu205A	Hydrogen bond
12	551450	-5.354326	-6.937231	Tyr662A, Glu206A, Glu205A	Hydrogen bond
11	10524983	-4.870677	-4.896286	Tyr662A, Glu206A	Hydrogen bond
Phytoconstituents docked to PPAR-gamma; Bioactive ligand has HYBRID Score (-18.02)					
40	92138	-10.236771	-10.405219	Ser289A	Hydrogen bond
				His449A, Cys285A, Phe282A	Hydrophobic
45	91457	-9.405942	-8.870893	Tyr473A, His449A	Hydrogen bond
				Ser289A, Tyr327A, Cys285A	Hydrophobic
7	10975	-7.995707	-7.924927	Tyr473A, His449A	Hydrogen bond
				His449A, Cys285A	Hydrophobic
21	643820	-7.462873	-7.680222	Tyr473A, His449A	Hydrogen bond
				His449A, Cys285A	Hydrophobic
17	8842	-7.419712	-8.056846	Ser289A	Hydrogen bond
				Cys285A, Leu330A	Hydrophobic
42	94174	-7.40029	-7.656871		
8	6549	-7.087233	-7.087233	His449A	Hydrogen bond
				Cys285A	Hydrophobic
44	519662	-7.001183	-7.001183	Tyr473A, His449A	Hydrogen bond
				His449A, Cys285A, Ser289A	Hydrophobic
4	3084331	-7.001183	-7.001183	Tyr473A, His449A	Hydrogen bond
				His449A, Cys285A, Ser289A	Hydrophobic
12	551450	-6.80504	-6.795523	His449A	Hydrogen bond
				His449A, Cys285A	Hydrophobic
5	13166	-6.175961	-6.407844	Ser289A	Hydrogen bond
47	284646	-5.996939	-6.861071	Ser289A, Tyr473A	
				Cys285A	Hydrophobic
20	94221	-5.449536	-5.992487	Ser289A	Hydrogen bond
				Cys285A	Hydrophobic
Phytoconstituents docked to PTP1B; Bioactive ligand has HYBRID Score (-18.02)					
47	284646	-5.941802	-6.128138	Asn193A, Glu276A	Hydrogen bond
				Phe280A, Leu192A	Hydrophobic

Table 5: Docked phytoconstituents from CCEO and their interaction with DPP-IV, PPAR-gamma and PTP1B.

and expansion [33]. Our results confirmed that after CCEO treatment the insulin-positive cell mass was enlarged which may well elucidate the significant rise in fasting plasma insulin levels, even if this rise was not adequate to control fasting glycemia. Most likely, these effects are proportional to the length of the treatment and are potentially beneficial in the perspective of long-term treatment.

PX-407 partially damages the pancreatic islets and so decreases the number of β-cells and insulin globules [30]. This damage leads to an inflammation in the pancreas which is known as insulitis. Our result showed that, in CCEO treated diabetic rats there was a significant ($p<0.001$) improvement in the number of islets as well as the grade of insulitis compared to DC groups. The increased β-cell mass would increase the secretion of insulin, which may increase the peripheral utilization of glucose [34]. Hence, one of the reasons behind the observed antihyperglycemic activity of CCEO was the increase in the islet size and activity to near normal values.

The time of harvest is one of the key factors influencing the chemical composition, quality and quantity of the plant essential oil. Based on literature data, it appears that citral, geraniol, linalool, limonene and β-myrcene have been found as major compounds in many other Cymbopogon species [35]. With the possibility that these phytoconstituents may interact with its constituents to potentiate their antidiabetic effect synergistically; further investigation regarding their interactions would be rewarding [36].

Further, in order to understand the mechanism of action, docking studies were performed by HYBRID and FRED program for documented 48 CCEO phytoconstituents with respect to three antidiabetic proteins namely PPAR-γ, PTP-1B, DPP-IV. The 3D conformers of these phytoconstituents were used for docking to three proteins. In the case of PPAR-γ, it has been reported that the direct interaction between a ligand and the C-terminal helix in the ligand-binding domain (LBD), forming the activation function 2 (AF-2) has a decisive role in the ligand-induced receptor activation [8]. Based on this conservation of interactions with important residues in the active site of PPAR-γ, the modulating role of phytoconstituents can be proposed. Full agonist of PPAR-γ forms hydrogen bond with Y473 on the AF-2 helix H12 [37], whereas partial agonists, did not always interact with this helix to activate PPAR-γ [38]. For DPP-IV inhibition H-bonding with Glu205 and Glu206 as well as extension of hydrophobic part in S1 sub-pocket has been reported to be important. DPP-IV contains two sub-pockets S1 and S2.The hydrophobic S1 pocket is composed of residues V656, Y631, Y662, W659, Y666, and V711 while hydrophobic S2 pocket is determined by the side chains of residues of R125, F357, Y547, P550, Y631, and Y666. Based of docking results, synergistic effect of these phytoconstituents on PPAR-γ and DPP-IV can also be proposed.

Thus, the in vivo experiment, fully supported by molecular docking proves that *C. citratus* essential oil is antidiabetic in nature. Consequently, this study safely submits that application of *C. citratus* essential oil is a supplement to the existing oral anti diabetic drugs and it minimizes the transformation of prediabetics into diabetics.

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