Gene expression and post-gene expression influence of Spondias mombin (Hog plum) and Nigella sativum (Black seed) on diabetogenic Wistar rats.

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Abstract

Background: Diabetes Mellitus (DM) is a metabolic disorder characterised by chronic hyperglycaemia due to the body's inability to appropriately metabolism and utilize carbohydrates (sugars), further affecting protein and lipid metabolism, as a result of inadequate insulin release due to damaged pancreatic beta-cells (type 1 diabetes) or reduced tissue insulin sensitivity and elevated blood insulin (type 2 diabetes) – endocrine disorder. The possible anti-DM properties of Spondias mombin and Nigella sativum where investigated.

Materials and methods: Four (4) groups ten (10) adult Wistar rats were induced with streptozotocin (STZ) to cause a pathologic DM condition after which they were treated with metformin (positive control drug, PC), Spondias mombin methanolic extract (T1) and Nigella sativum oil (T4) for twenty-eight days. The normal control (NC) and diabetic control (DC) received distilled water and STZ. Gene expression studies and molecular docking (post-gene expression) analyses were carried out on key factors involved in DM; lipid and glucose homeostasis (peroxisome proliferator-activated receptors, PPAR-γ), transport proteins (GLUT 1 to 5), proteins of inflammatory reactions (interleukin-1-beta, IL-1β; tumour necrosis factor-alpha-1), enzymes involved in glycolysis (phosphofructokinase-1, PFK-1).

Results: It was observed that Spondias mombin, Nigella sativum, and metformin positively influenced the gene expression and post-gene expression phases to ameliorate the DM conditions.

Conclusion: Spondias mombin performed better than Nigella sativum and metformin as a potential anti-DM plant.

Introduction

According to the World Health Organisation, Diabetes Mellitus (DM) is a metabolic disorder characterised by chronic hyperglycaemia due to the body's inability to appropriately metabolism and utilize carbohydrates (sugars), further affecting protein and lipid metabolism, as a result of inadequate insulin release due to damaged pancreatic beta-cells (type 1 diabetes) or reduced tissue insulin sensitivity and elevated blood insulin (type 2 diabetes) – endocrine disorder – (Agu, 2018). The hyperglycaemia surpasses the renal glucose thresh-hold leading to large amounts of unabsorbed glomerular filtrate glucose that is passed in the urine, causing osmotic imbalance with more water is excreted with it, also, causing an increased urinary volume and frequency of urination as well as thirst. The other symptoms of DM could include itching, hunger, weight loss, and body weakness. Certain plants, due to their phytochemical compositions, have been reported to possibly improve these diabetic conditions, thus, improving glucose utilization in the test models. However the mechanisms by which these plants do these are still in their rudimentary research stages. Some of the reported plants include, Spondias mombin and Nigella sativum.

Spondias mombin (Anarcadiaceae) is known commonly as 'Hog plum', 'Iyeye' and 'Olosan' (Yoruba, South West Nigeria) (Gbile, 1984), has been reported to act via insulin-like mechanism increasing adiponectin expression (Ajiboye et al., 2018); inhibit alpha-amylase and alpha-glucosidase (Ojo et al., 2013; *Eluehike and Onoagbe, 2018*) due to the presence of alpha-sitosterol (Fred-Jaiyesimi et al., 2009); reduction of glycated haemoglobin (Saronee et al., 2019); protection against lipid peroxidation, hypolipidemic properties, (Emeka et al., 2011); etc. Also, Nigella sativum has been reported to decrease glycated haemoglobin (Hamdan et al., 2019); modulate hyperglycemia and lipid profile dysfunction through the regulation of antioxidant balance, insulin secretion, glucose absorption, gluconeogenesis and gene expression (Heshmati and Namazi, 2015; Daryabeygi-Khotbehsara et al., 2017; Kaatabi et al., 2015); increase in B-cell functions (Bamosa et al., 2010); inhibition of platelet aggregation, regulation of eNOS, VCAM-1 and LOX-1 genes expression that are involve in the endothelial dysfunction, reduction of the expression and secretion of cytokines, including, MCP-1, interleukin-1β, TNF-α, NF-κB, and Cox-2, that are involved in anti-inflammatory properties (Mohebbati and Abbasnezhad, 2020); etc. Some literatures have attributed these properties of Nigella sativum to the presence of thymoquinone.

Thus, to further unravel the mechanisms involved in the possible anti-diabetic potentials of Spondias mombin and Nigella sativum, the experiment was designed to investigate the potentials of these plants to sensitize insulin and enhances glucose metabolism (peroxisome proliferator- activated receptor *gamma, PPAR-γ); to transport glucose intracellularly (GLUT-2); to metabolise glucose through the glycolytic pathway (phosphofructokinase-1, PFK-1); to modulate cellular stress and improve metabolism (tumor necrosis factor-alpha, TNF-alpha, and interleukin-1, IL-1)*.

Materials And Methods

Experimental rats: 30 adult albino male Wistar rats with weights between 150 g – 210 g were used for this experiment. The rats were supplied by Mr. Silvanus Innih of the Anatomy Department, University of Benin, Benin City, housed in the Department of Biochemistry animal house, and were acclimatized for one week before the study. They were fed standard rat chow and water ad libitum. A written approval for the study was obtained from the Research Ethics Committee Guideline Principles on Handling of Animals of the College of Medicine, University of Benin (CMR/REC/2014/57), and was strictly adhered to.

Preparation of Spondias mombin methanol extract: A large quantity of fresh parts of the plant was collected from trees in house-hold gardens in Benin City and around University of Benin vicinity, Edo state, Nigeria. The plant was identified by Dr Bamidele of the Department of Plant Biology and Biotechnology, University of Benin and authenticated by Professor Idu of the same Department. A voucher specimen number UBHs 345 was deposited at the Herbarium of Department of Plant Biology and Biotechnology, University of Benin. The samples (leaves, roots, stem bark and pulp) were washed and pulverized separately after drying at room temperature (about 25 ^{o}C) for 4 weeks. Each pulverized plant sample was macerated in ethanol for 48 hours after which it was filtered through cheese cloth. The obtained extracts were then concentrated in vacuo using rotary evaporator to obtain viscous gels which were air-dried to gel-like solids. The gel-like crude methanolic extracts gotten from the various parts of the plant were reconstituted to obtain a stock solution using distilled-deionized water as solvent. The reconstituted crude extract (T1) was stored in small-capped plastic containers in a refrigerator at -4 ^{o}C until used.

Preparation of Nigella sativum oil extract: Nigella sativa seeds were collected from the open market. The plant seeds were cleaned, desiccated and crushed to fine powder using mortar and pestle. The Nigella sativa seed powder (100 g) was extracted in petroleum ether (400 mL) for 4-6h using Soxhlet apparatus. The solvent was evaporated from the extract using rotary apparatus, resulting in Nigella sativa seed oil (T4).

Administration of extracts: The extracts were administered with the aid of a gavage, acting as an oro-gastric tube. Utmost care was taken to not inflict oral or oesophageal injuries on the rats.

Experimental protocol for the studies: Induction of diabetic condition was done using streptozotocin, 60 mg/kg (b.w.) dissolved in cold citrate buffer (pH of 4.50). Only rats with fasting blood glucose greater than 200 mg/kg (b.w.) were considered as the diabetic rats and were used for the study. The normal control group was given 2 ml of distilled water; diabetic control group received the streptozotocin treatment only; the positive control group were diabetic rats treated with the metformin standard (50 mg/kg b.w.) only; while T1 group were the diabetic rat treated with Spondias mombin (200 mg/kg, b.w.), and T4 were the diabetic rat treated with Nigella sativum (2.50ml/kg b.w.).

Animal Sacrifice: Prior to sacrifice (after 28 days of treatment), the rats were fasted overnight, weighed and sacrificed after they were anaesthetized with chloroform. The liver samples were harvested and collected into the eppendoff tube for RNA isolation. The pancreas were also collected for histopathological evaluation.

Histological assay: Pancreas tissues taken from the animals were cut into sections and stained with hematoxylin and eosin and examined microscopically.

Biochemical analysis: Total RNA isolation: Total RNA was isolated from whole tissues following a method described by Omotuyi et al. (2018). Briefly, the liver tissues were homogenized in cold (4 °C) TRI reagent (Zymo Research, USA, Cat:R2050-1-50, Lot: ZRC186885). Total RNA was partitioned in chloroform (BDH Analytical Chemicals, Poole, England Cat: 10076-6B) following centrifugation at 15,000 rpm/15 min (Abbott Laboratories, Model: 3531, Lake Bluff, Illinois, United States). RNA from the clear supernatant was precipitated using equal volume of isopropanol (Burgoyne Urbidges & Co, India, Cat: 67-63-0). RNA pellet was rinsed twice in 70% ethanol (70 ml absolute ethanol (BDH Analytical Chemicals, Poole, England Cat: 10107-7Y) in 30 ml of nuclease-free water (Inqaba Biotec, West Africa, Lot no: 0596C320, code: E476-500ML)). The pellets were air-dried for 5 min and dissolved in RNA buffer (1 mM sodium citrate, p^H 6.4).

Complimentary DNA (cDNA) conversion: Prior to cDNA conversion, total RNA quantity (concentration (µg/ml) = 40 * A_{260}) and quality (≥ 1.8) was assessed using the ratio of A_{260}/A_{280} (A=absorbance) read using spectrophotometer (Jen-way UV-VIS spectrophotometer model 6305, UK). DNA contamination was removed from RNA was removed following DNAse I treatment (NEB, Cat: M0303S) as specified by the manufacturer. 2 µl solution containing 100 ng DNA-free RNA was converted to cDNA using M-MuLV Reverse transcriptase Kit (NEB, Cat: M0253S) in 20 µl final volume (2 µl, N^9 random primer mix; 2 µl, 10X M-MuLV buffer; 1 µl, M-MuLV RT (200 U/µl); 2 µl, 10 mM dNTP; 0.2 µl, RNase Inhibitor (40 U/µl) and 10.8 µl nuclease-free water). The reaction proceeded at room temperature O/N. Inactivation of M-MuLV Reverse transcriptase was performed at 65°C/20 min.

PCR amplification and agarose gel electrophoresis: PCR amplification for the determination of genes whose primers (Primer3 software) (Ajiboye et al., 2018) are listed below (table 1.0) were done using the following protocol: PCR amplification was performed in a total of 25 µl volume reaction mixture containing 2 µl cDNA (10 ng), 2 µl primer (100 pmol) 12.5 µl Ready Mix Taq PCR master mix (One Taq Quick-Load 2x, master mix, NEB, Cat: M0486S) and 8.5 µl nuclease-free water. Initial denaturation at 95 °C for 5 minutes was followed by 20 cycles of amplification (denaturation at 95 °C for 30 seconds, annealing (see TM values for each primer pair on table 1.0) for 30 seconds and extension at 72 °C for 60 seconds) and ending with final extension at 72 °C for 10 minutes. In all experiments, negative controls were included where reaction mixture has no cDNA. The amplicons were resolved on 1.5% agarose gel (Cleaver Scientific Limited: Lot: 14170811) in Tris (RGT reagent, china, Lot: 20170605)-Borate (JHD chemicals, China, Lot 20141117)-EDTA buffer (pH 8.4).

Amplicon image processing: In-gel amplicon bands images captured on camera were processed on Keynote platform as previously reported (Omotuyi et al., 2020) and quantified using image-J software. All graphs were plotted as mean +/- SEM using graph-pad prism.

Table 1: Primer sequences the genes used for real - time RT-PCR

Genes	Primer sequence
TNF α primers	Forward 5'- AAGTAGTGGCCTGGATTGCG-3'
	Reverse 5'- ACTCAGGCATCGACATTCCG-3'
GLUT -2	Forward 5'- TCCTCGCCTGGTTCTACTGA-3'
	Reverse 5'- TTTGAGTGTGAGCCTTGCCA-3'
Phosphofructokinase, Liver type (PFK-L)	Forward 5'- TCTGGGACTCCTCCCTTAGC-3'
	Reverse 5'- GGAGTGGTGATGTGGGTCTG-3'
Actin, beta (ACT-β)	Forward 5'- CCACCAGTTCGCCATGGAT-3'
	Reverse 5'- CCCACCATCACACCCTGG-3'
IL-1β primers	Forward 5'-CCTTCCAGGATGAGGACAA -3'
	Reverse 5'-TGAGTCACAGAGGATGGGC -3'

Molecular docking experiments

The GLUT-2 protein model prediction: After ascertaining that crystal structure of GLUT-2 and GLUT-4 for human are not available to date, the three-dimensional structure was predicted (Duddela et al., 2010). The structural model for the GLUT-2 inward-facing conformation was predicted based on the crystal structure of GLUT-1 (PDB ID: 6THA) with the Molecular Operating Environment (MOE) software (https://www.chemcomp.com/). Interestingly, GLUT-1 and GLUT-2 share 52% and 68% protein amino acids sequence identity and similarity, respectively, as determined with Align function in MOE (Schmidl, et al.,2021).

Preparation of protein: The starting crystallography structure (protein data bank (PDB) ID of 6THA for glucose transporter isoform – 1 (Glut – 1), 1T4Q for interleukin – 1 – beta (IL-1beta), 2AZ5 for tumor necrosis factor – alpha – 1 (TNF-α), 4XYK for phosphofructokinase – 1 (PFK – 1), 3PRG for peroxisome proliferator activated receptor-gamma (PPAR - γ), required for docking was retrieved from the protein data bank repository (http: //www.rcsb.org). Prior to docking, water and ligand coordinates were deleted, while the appropriate charges where added. The proteins where viewed with Discovery studio to study their 2-D amino acid residues interactions.

Preparation of ligands: The 3D structure of elucidated compounds were built using ChemDraw and converted to PDB format for docking studies using GaussView. The ligand molecules (compounds) were appropriately optimized.

Alpha-sitosterol served as the test molecule present in the Spondias mombin extract – plant sterols have been proven to exert antidiabetic activities and inhibits the absorption of dietary cholesterol (Grundy et al., 1969; Lees et al., 1977); while thymoquinone served as the test molecule present in Nigella sativum oil – Thymoquinone has been reported as the major mechanistic compound present in Nigella sativum that ameliorates the clinical conditions associated with DM (Kanter, 2008; Abdelrazek et al., 2018). Adenosine diphosphate, nonyl beta-D-glucopyranoside served as internal ligands with which the amino acid residues at the binding sites were predicted; nitrooctadecenoic acid, rosiglitazone, rilonacept, *TNF-α-inbitor-2*, *TNF-α-inbitor*-SPD-304*, rac-benpyrine, palmitoyl-coA, and S*-hexadecyl-CoA served as the standard inhibitors (some of which were obtained from, https://www.medchemexpress.com), and metformin served as the positive control ligand.

Molecular docking: Molecular Docking calculations were performed using Autodock Vina (Trott and Olson, 2010). The modeled structures of Glut – 1, IL – 1β, TNF – α1, PFK – 1 and PPAR - γ, and elucidated compounds were loaded the Autodock/Vina and all the water molecules were removed prior to the upload. The various ligand conformations were viewed using the Discovery Studio software and protein coordinates snapshots were taken using snipping tools.

Statistical analysis: Data were entered into the Microsoft Excel spread sheet (v.10) prior to descriptive analysis. The data are presented as mean ± SEM, and were analyzed using the Duncan's multiple range analyses of variance, ANOVA. Correlation analyses were done using Pearson's correlation (p=0.05) of the Statistical Package for Social Sciences, SPSS®, Version 21.0, IBM Corp., Armonk, NY, USA. Histograms and line plots were done using Graph Pad software®, Prism 5, Version 5.01, La Jolla, CA, USA. Values of p ≤ 0.05 were considered significant.

Results

Table 1. Body weight measurement of diabetic rats treated with extract of Spondias mombin and Nigella sativum leaf

Treatment	Body weight measurement(grams)				
	Day 0	Day 7(after STZ administration)	Day 14	Day 28	Weight difference (WD)
Normal control	158.7±4.30	156.5±2.50	169.6±9.60*	210.3±6.81*	+51.60
Diabetic control	165.4±4.30	145.0±0.02*	134.7±2.76*	109.0±6.18*	-56.40
Positive control	173.1±3.35	147.2±0.01*	146.0±3.20*	147.3±4.50*	-25.80
Spondias mombin treated diabetic rats (T1)	188.4±0.09	145.8±2.55*	157.5±5.05*	165.3±2.51*	-23.10
Nigella sativum treated diabetic rats (T4)	214.1±6.70	185.5±0.50*	175.6±3.92*	204.0±1.54*	-10.10

Data are body weights (grams) of rats treated with extracts for 28 days and are expressed as means ±SEM (n=6). *p ≤0.05 when compared with the normal control values at day 0. The weight difference (WD) was calculated as the difference between day 28 (final weight) and day 0 (initial weight).

Table 2. Organ weight of diabetic rats treated with extract of Spondias mombin leaf

Treatment	Organ weight(grams)	
	Liver	Pancreas
Normal control	6.30±0.17	0.37±0.05
Diabetic control	7.43±1.70*	0.50±0.14*
Positive control	6.03±0.30	0.20±0.01
Spondias mombin treated diabetic rats	6.90±0.10	0.23±0.05
Nigella sativum treated diabetic rats	7.67±0.45*	0.33±0.05

Data are organ weights of diabetic rats treated with extract for 28 days and are expressed as means ±SEM (n=6). *p ≤0.05 when compared with the normal control.

Table 3. Concentration of fasting blood glucose level (mg/dl) in diabetic rats treated with extract of Spondias mombin leaf

Treatment	Fasting blood glucose concentration (mg/dl)				
	Day 0	Day 7 (after STZ administration)	Day 14 (after commencement of treatment)	Day 28 (after commencement of treatment)	Difference in blood sugar (DBS)
Normal control	102.7± 1.80	104.0±2.50	97.5±0.01*	102.0±2.00	-0.70
Diabetic control	97.8±3.20	296.0±1.80*	302.0±1.65*	322.5±4.20*	+224.70
Positive control	106.2±1.50	289.3±0.55*	270.3±1.40*	270.0±2.50*	+163.80
Spondias mombin treated diabetic rats	104.3±2.00	292.3±1.55*	233.7±2.25*	212.7±1.60*	+108.40
Nigella sativum treated diabetic rats	104.8±1.68	310.33±0.20*	235.3±3.00*	116.30±2.50*	+11.50

Data are fasting blood glucose concentration of diabetic rats treated with extract for 28 days and are expressed as means ±SEM (n=6). *p ≤0.05 when compared with the values at day 0. The difference in blood sugar (DBS) was calculated as the difference between day 28 (final weight) and day 0 (initial weight).

Table 4. Binding affinities of the ligands against the proteins

Protein	Ligand	PBD ID	Affinity (Kcal/mole)	Dist. From rmsd l.b.	Best mode rmsd u.b.
PPAR-γ	Metformin	3PRG	-04.00	27.225	29.223
	Thymoquinone		NR	NR	NR
	Alpha-sitosterol		-08.10	01.519	02.215
	Rosiglitazone		-07.70	01.419	01.941
	Nitrooctadecenoic acid		-05.60	03.797	07.168
	Fluro-thymoquinone		-05.60	02.559	03.049
	Di-fluoro-alpha-sitosterol		-07.50	03.458	05.244
GLUT-2	Metformin	6THA	-04.50	01.623	02.423
	Thymoquinone		-06.30	20.003	21.240
	Alpha-sitosterol		-10.60	04.473	06.536
	Glupin analogue		-08.90	04.326	07.153
	Nonyl beta-D-glucopyranoside		-06.80	03.796	06.444
	Fluro-thymoquinone		-06.70	19.272	20.659
	Di-fluoro-alpha-sitosterol		-10.60	01.993	03.001
IL-1β	Metformin	1T4Q	-04.50	01.623	02.443
	Thymoquinone		-04.30	28.850	30.399
	Alpha-sitosterol		-06.60	14.973	18.568
	Rilonacept		-06.80	00.000	00.000
	Fluro-thymoquinone		-04.60	02.056	04.064
	Di-fluoro-alpha-sitosterol		-06.20	16.152	18.053
TNF-alpha1	Metformin	2AZ5	-08.50	03.278	07.452
	Thymoquinone		-05.90	01.107	04.617
	Alpha-sitosterol		-09.10	01.432	02.724
	TNF-α-inbitor-2		-08.80	02.479	03.956
	TNF-α-inbitor-SPD-304		-08.80	02.966	05.919
	Rac-benpyrine		-07.80	02.806	05.564
	Floro-thymoquinone		-05.80	02.163	04.704
	Di-floro-alpha-sitosterol		-08.70	03.321	07.437
PFK-1	Metformin	4XYK	-03.90	35.333	35.814
	Thymoquinone		NR	NR	NR
	Alpha-sitosterol		-07.50	01.459	02.426
	Palmitoyl-CoA		NA	NA	NA
	S-hexadecyl-CoA		NA	NA	NA
	ADP		-08.20	05.990	09.137
	Floro-thymoquinone		-05.00	55.096	56.638
	Di-floro-alpha-sitosterol		-07.10	01.956	02.683

Binding affinities above -07.00 Kcal/mole indicated an appropriately bonded ligand on the surface of the proteins (RMSD = root mean square distance). NR=no results (did not dock); NA=not available.

Table 5. A general summary of the performances of Spondias mombin (T1) and Nigella sativum (T4) as anti-DM plants

	PPAR-γ		GLUT-2		IL-1β		TNF-alpha1		PFK-1	
	Gene expression	Post-gene expression	Gene expression	Post-gene expression	Gene expression	Post-gene expression	Gene expression	Post-gene expression	Gene expression	Post-gene expression
Treatments										
Spondias mombin	↑	↑	↑	↑	↑	↑	↑	↓	↑	↑
Nigella sativum	↑	↓	↑	↓	↓	↓	↓	↑	↑	↓
Metformin	↓	↓	↑	↓	↓	↔	↑	↔	↔	↓

↑=significant high influence compared to other treatments; ↓=low influence compared to other treatments; ↔=average influence compared to other treatments.

Discussion

Glucose is an essential cellular source of energy metabolism, but abnormalities in its digestion, assimilation, transport and utilization can portend serious life threatening conditions, which could include the various forms of diabetes, cardiovascular diseases, diseases associated with body lipid storage and utilization, and even various forms of cancers. Various hormones, metabolic enzymes, transport and storage proteins, membrane receptors, etc. play key roles in the metabolism of sugar, starting with the buccal digestion of carbohydrates, down to its assimilation into blood, through the maintenance of blood glucose levels and the aided intracellular mobilization, down-stream to glycolysis for energy and storage as glycogen. Any biochemical or pathological misnomer at any of these mentioned levels can lead to serious glucose utilization conditions, including diabetes mellitus (DM). In DM, the implicated culprit is insulin; in which, in one case, the beta-cells of the pancreas which produces insulin are unable to release into the blood due the absence of morphologically functional beta-cells – type 1-DM, common with autoimmune disorders; the beta-cells may be producing insulin, but the amount released into the blood to stimulate the membrane-bound insulin receptors (IR) are either not enough cause a maximal stimulation (insulin resistance) or that certain other agents decreased the IR receptiveness to the pancreatic hormone – type 2-DM, common with body fat storage disorders; in pregnancy, one of the metabolic modifications to provide the growing foetus with energy is to decrease the IR-kinase activities, decreasing maternal glucose utilization, thus, increasing blood insulin and glucose levels – gestational DM. However which of the conditions as mentioned, any medical aids that will improve glucose metabolism with very minimal side effects and highly affordable, especially in type 1 and 2 DM, is highly beneficial. Various epidemiological studies have indicated that hyperglycaemia is the main cause of complication related with coronary artery disease, renal failure, ulcerations of parts of the body, blindness, limb amputation, neurological complications and pre-mature death (Lopez-Candales, 2001). Plant remedies have offered very beneficial health improvements with regards to glucose utilization of DM patients over the years due to their rich phytochemical compositions. The potentials of Spondias mombin (Hog plum) and Nigella sativum (Black seed) to ameliorate DM conditions have been reported, thus, the design of this research to further elucidate the mechanistic bases of their anti-diabetic properties. We investigated the responses of key proteins to treatments by these plants, viz, glucose transporter isoform – 1 (Glut – 1), interleukin – 1 – beta (IL-1beta), tumor necrosis factor – alpha – 1 (TNF-α), phosphofructokinase – 1 (PFK – 1), and peroxisome proliferator activated receptor-gamma (PPAR - γ); by carrying studies including, genomics, proteomic, and molecular docking and computer-aided compound screening. Spondias mombin (T1) significantly decreased blood glucose in oral glucose tolerance test (Adediwura and Kio, 2009); Nigella sativa (T4) has been reported to modulate hyperglycaemia and dyslipidaemia through various possible mechanisms which could include its antioxidant properties and effects on insulin secretion, glucose absorption, gluconeogenesis and gene expression, thus, improving glucose homeostasis (Heshmati and Namazi, 2015).

Wistar rat models whose pancreas have been challenged by streptozocin administration after seven days of treatment were used for these studies. Changes in the body weights (table 1) of indicated that the diabetic control (-56.40g), positive control (-25.80g), T1 (-23.10g) and T4 (-10.10g) groups, demonstrated a progressively decreased body weight (especially the diabetic control), compared to the normal control (+51.60g), in which understandably, there was a significant weight gain. This thus suggested on a macroscopic view that the group treated with Nigella sativum better ameliorated the deleterious effect associated with weight loss in the DM rats better than Spondias mombin, and even the group treated with the standard metformin drug. These observations were corroborated by the observed significantly enlarged average pancreatic weight of the diabetic control group (0.50g), compared to the other groups – positive control (0.20g), T1 (0.23g), and T4 (0.33g) – as well as the normal control (0.37g). The enlarged diabetic control pancreas is suggestive of developing lesions due to tissue damage arising from the streptozotocin treatment, and a possible organ compensatory effects to meet up with the body demand of insulin. It is also important to state that the mechanism by which the beta-cells releases its preformed insulin via vesicular mobilization to the apical extracellular environment, involves the inward movement of glucose into the beta-cells for increased metabolism to generate increased intracellular ATP which shuts the ATP-sensitive potassium channel for onward membrane depolarization to open up the calcium ion channel for intracellular flow of calcium ion for the eventual release of insulin molecules extracellularly. This scenario occurring in the diabetic control rats can be imagined as a situation involving the constant recruitment of glucose and other metabolites to initiate depolarization, but no release of insulin. This is quite a superficial explanation, but on an extensive mechanistic scale, Szkudelski (2001) reported that streptozotocin enters the pancreatic B-cell through the GLUT-2 to initiate DNA alkylation (damage), inducing the activation of poly ADP-ribosylation, which causes the depletion of intracellular NAD+ and ATP. Further depletion and dephosphorylation of ATP after streptozotocin treatment releases the nucleotides that are acted on by xanthine oxidase producing other deleterious end

products, reactive oxygen species (superoxide, hydroxyl and peroxyl free radicals), and nitric oxide (inhibits aconitase activity). All these events lead to beta-cells necrosis, resulting in the accumulation of necrotic tissues, which also attract immune components to the pancreatic tissue.

As would be expected (table 3), there was a significantly progressive increase in blood glucose concentration of the diabetic control (+224.70mg/dl), compared to the normal control (-0.70mg/dl) which demonstrated a rather slightly hypoglycaemic condition. Again, the T4 group treated with the Nigella sativum oil was able to better reduce the hyperglycaemia (DM) (+11.50mg/dl), compared to the T1 group treated with Spondias mombin extract (+108.40mg/dl) and the group treated with the standard metformin drug (+163.80mg/dl). These remarkable observations of these plants, especially the Nigella sativum, further served as prelude to study the mechanisms that may be involved in the high blood glucose lowering in DM.

The relative expression analysis of peroxisome proliferator-activated receptors (PPAR-γ) (figure 1), indicated that Spondias mombin (T1) and Nigella sativum (T4) significantly induced the PPAR/actin-β gene transcription, compared with the positive and normal control groups. However, T1 performed better than T4. The results of the ligand binding energies (table 4) corroborates this observation as the representative compound of Spondias mombin, alpha-sitosterol (-08.10 Kcal/mole) and its analogue, Di-fluoro-alpha-sitosterol (-07.50 Kcal/mole), demonstrated stronger bonding energies to PPAR-γ, compared to the positive control and other ligands.

The peroxisome proliferator-activated receptors (PPAR-γ) have been reported as members of the nuclear receptor supergene family which are considered as key sensors of both lipid and glucose homeostasis. The role of the PPAR-γ isoform in glucose metabolism and homeostasis is illustrated by the fact that anti-diabetic thiazolidinediones have been shown to be bona fide PPAR-γ ligands. Given the central role of PPAR-γ as a mediator in glucose regulation, the structure should be an important tool in the development of improved anti-diabetic agents. (Uppenberg et al., 1998). The relevance of PPAR-γ as an important therapeutic target for the treatment of DM is due to its hypoglycemic effects in DM patients, through the improvement of insulin sensitivity, and also from its critical role in the regulation of cardiovascular functions, especially, through the decrease in TNF-α (Villacorta et al., 2009).

From figure 2, the positive control drug, metformin, induced the expression of GLUT-2 better, compared to the Spondias mombin (T1) and Nigella sativum (T4). However, the T1 treated group expressed GLUT-2 better than the T4-treated group; the expression of GLUT-2 in the T1 treated group did not show any significant decrease compared to the metformin-treated group, whereas, the expression of GLUT-2 in the T4-treated group was significantly lower compared to the metformin-treated group. Streptozotocin treatment significantly decreased GLUT-2 expression, compared to the T1 and T4 groups.

The results of the ligand binding energies (table 4) however suggested that alpha-sitosterol (-10.60 Kcal/mole) and its analogue, di-fluoro-alpha-sitosterol (-10.60) of Spondias mombin demonstrated the highest binding dynamics with the GLUT-2 protein, even better than the standard molecule, glupin analogue (-08.90 Kcal/mole), thus, corroborating the gene expression results (figure 2). There might be other pharmacodynamics and biochemical explanations as to why the positive control, metformin, induced the GLUT-2 expression better than Spondias mombin (and even Nigella sativum).

The human glucose transporters GLUT-1 and GLUT-3 (and GLUT-2) all perform central roles in glucose uptake and homeostasis as canonical members of the Sugar Porter (SP) family. GLUT transporters share a fully conserved substrate-binding site with identical substrate coordination, but differ significantly in transport affinity in line with their physiological function (Custodio et al., 2021) – the sole aim is intracellular glucose transport and normoglycemia homeostasis; once this is achieved, DM is ameliorated. Glupin (a derivative of indomorphane) has been identified as a potent inhibitor of glucose uptake by selectively targeting and upregulating glucose transporters GLUT-1 and GLUT-3. Glupin suppresses glycolysis, reduces the levels of glucose-derived metabolites, and attenuates the growth of various cancer cell lines (Ceballos et al., 2019).

Interestingly, Nigella sativum (T4) did not demonstrate any significant influence in the inactivation cum suppression of IL-1β production in response to the underlying DM conditions, compared to the positive control group (figure 3). This thus suggests that the mechanism underlying the anti-DM that we had earlier observed with the activation of the GLUT and PPAR proteins to enhance glucose homeostasis do not include ameliorating the inflammatory influence of IL-1β, such that, it may involve the detoxification of the proinflammatory agents by the rich antioxidant compounds of the plant. In contrast, Spondias mombin (T1) greatly inhibited the expression of IL-1β compared to the positive control, metformin (and Nigella sativum). This property of Spondias mombin further supports its reported potencies to wade off the wide-spread systemic inflammatory reactions associated with the activities of glucose (DM) in the red blood cells (RBC) – glycated haemoglobin, the blood vessels – vasculopathy, the nephrons – nephropathy, the neurons – neuropathy, the retina – retinopathy, etc. This anti-inflammatory properties of Spondias mombin can be associated with its alpha-sitosterol content which expressed a far superior binding energy of approximately -07.00 Kcal/mole to the IL-1β protein, compared to the standard rilonacept which also had an approximately -07.00 Kcal/mole binding energy, also to the IL-1β protein (table 4). Rilonacept, which served as the standard ligand against IL-1β, blocks IL-1β signaling by acting as a soluble decoy receptor that binds the pro-inflammatory cytokine, IL-1β, and prevents its interaction with its cell surface receptors. Rilonacept also binds IL-1α and IL-1 receptor antagonist (IL-1ra) with reduced affinity. The synthesis and release of IL-1β requires two distinct signals which are normally initiated by damage-associated molecular patterns, either pathogen-associated molecular patterns, e.g. bacterial RNA or lipopolysaccharides, or endogenous irritants, e.g. uric acid or heat shock proteins that are associated with the DM condition like the C-reactive proteins (Agu, 2018). One signal leads to synthesis of pro-IL-1β and components of the protein complex inflammasome NLRP3/cryopyrin. A second signal leads to the assembly of the inflammasome and activation of caspase-1. Active IL-1β is released from pro-IL-1β by caspase-1. In order to exert its pro-inflammatory actions, IL-1β must bind two receptors, the interleukin-1 receptor (IL-1-R1) and an accessory protein (IL-1-RAcP). These receptors form a complex at the membrane necessary for signal transduction. Rilonacept works by trapping IL-1β before it binds its receptor complex, preventing the pro-inflammatory effects that cause some of the symptoms associated with DM (Dubois et al., 2011)

Spondias mombin (T1) and Nigella sativum (T4) were able to inhibit the expression of TNF-α (figure 4), compared to the streptozotocin-treated diabetic control, but could not completely restore the level of the TNF-α protein to the normal control (NC), due to the underlying DM condition. However, the Nigella sativum-treated group performed better compared to the Spondias mombin-treated and metformin-treated groups ($p<0.05$). In contrast, the Nigella sativum thymoquinone ligand did not demonstrate a significant binding energy with the TNF-α protein (table 1). Alpha-sitosterol demonstrated a significantly higher

binding energy (-09.10 Kcal/mole) compared to the metformin positive control (-08.50 Kcal/mole) and the standard ligands; *TNF-α-inbitor-2 (-08.80 Kcal/mole), TNF-α-inbitor*-SPD-304 (-08.80 Kcal/mole), and *rac-benpyrine (-07.80 Kcal/mole). This again suggests that the anti*-TNF-α properties of Nigella sativum observed (figure 4) could be associated with its abilities to detoxify pro-oxidant reactions, as with IL-1β - Spondias mombin performed better than Nigella sativum as an anti-inflammatory agent. It is also very important at this juncture of the discussion to identify that the observed variance of the activities of these two plants and their representative ligands to express anti-inflammatory potentials can be viewed from two phases, viz, phase one; their influence on the gene expression cascade (transcription-translation) to suppress/inhibit or induce/activate the expression of the genes for these inflammatory proteins (and other proteins so discussed), and phase two; their influence (enhancement or distortion of activities/functions) on the proteomic-metabolomic cascade (protein-receptor-effect) to form complexes with the translated proteins which induces a conformational change, prevent them from binding to their receptors and thus, preventing the inflammatory reactions from occurring – the higher the binding energies, the stronger the complex between the ligand and the protein. From the observation of this experiment, Spondias mombin (T1) performed better in the phase one and two against the IL-1β but only did well in the phase two against TNF-α. Nigella sativum (T4) only performed better than Spondias mombin (as an anti-inflammatory plant) in the phase one against TNF-α. A summary of the interactions between Spondias mombin (T1) – Nigella sativum (T4) and the phase one – phase two events are presented in table 5.

As has been previously reported, the inhibition of TNF-α protects the ocular retina against diabetic-like changes in rodent models. The mechanism by which TNF-α induces deleterious retinal changes have not been reported in details, but it has been shown in literatures that TNF-α was able to inhibit the normal insulin signal transduction, leading to increased apoptosis in both retinal endothelial cells (REC) and Müller cells. The drug, etanercept significantly reduced TNF-α levels in β2KO mice, leading to increased insulin receptor phosphorylation on tyrosine 1150/1151. SOCS3 levels were increased in β2KO mice, which were reduced after etanercept treatment. Pro-apoptotic proteins were reduced in etanercept-treated β2KO mice suggesting that the inhibition of TNF-α in DM could ameliorate tissue and organ damage associated with chronic hyperglycaemia (Jiang et al., 2014). TNF-α, a pro-inflammatory cytokine with a major pathogenic role in DM, may promote IR by inducing Ser312 phosphorylation (P-Ser312) of insulin receptor substrate (IRS)-1, thus, downregulating the activities of the phosphorylated (P-)AKT, resulting in IR (Stagakis et al., 2012). Inflammation and insulin resistance are closely linked and inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-8 may inhibit the insulin signalling cascade by multiple mechanisms. TNF-α induces phosphorylation of IRS-1 at serine instead of tyrosine residues and promotes IR. Both IL-6 and TNF-α may inhibit the transcription of IRS-1 and glucose transporter (GLUT-4) genes, thus, reducing glucose transport and enhancing IR in obese patients (Wellen and Hotamisligil (1994); Hotamisligil et al., 1996; Rotter et al., 2003). IRS-1 mediates its metabolic effects through the PIP-3 kinase pathway which results in phosphorylation of AKT and other downstream molecules. TNF-α-induced IR involves inhibition of AKT through a ceramide-activated protein-phosphatase (PP) 2A. Individuals with IR display reduced insulin stimulation of the PIP-3 kinase pathway, and insulin-stimulated AKT kinase activity is reduced in patients with type-2 DM (Stagakis et al., 2012). Treatment with anti- TNF-α has been shown to restore the phosphorylation status of Ser^{312}-IRS-1 and AKT, which are important mediators in the insulin-receptor signalling cascade.

TNF-α-inhitor-2 is a potent and orally active inhibitor of *TNF*-α, with a fifty percent inhibitory concentration (IC_{50}) of 25 nmole/dm^3. *TNF*-α-inhitor-2 causes a conformational change that distorts the *TNF*-α trimer protein upon binding, leading to distorted signalling when the trimer binds to TNF-receptor-1. Also, *TNF*-α-inhitor-SPD-304 is a selective TNF-α inhibitor, which promotes the dissociation of TNF trimers and therefore blocks the interaction between the *TNF*-α and its receptor. *TNF*-α-inhitor-SPD-304 has an IC_{50} of 22 μmole/dm^3 for inhibiting in vitro TNF receptor-1 (TNFR1) binding to TNF-α. Rac-benpyrine, a racemic analogue of benpyrine, is a potent and orally active TNF-α inhibitor. Rac-benpyrine has been reported with the potential inhibit TNF-α mediated inflammatory and autoimmune diseases.

Phosphofructo-1-kinase (PFK-1) is one of the rate-limiting enzyme in glycolysis. Skeletal muscle PFK-1 (PFK1SM) deficiency has been reported to cause impaired β-cells insulin secretion in response to increased blood glucose, predisposing the patients to type-2 DM – the pancreas is functional, but there is peripheral IR (Ristow et al., 1997). PFK-1 is a subject of multiple regulatory mechanisms that include, allosteric regulation by metabolites and control by hormones. It has also been reported that PFK-1 is downregulated in the cardiac muscle (PFK1CM) of DM patients, so that it plays a central role in the decreased cardiac muscle (CM) glucose utilization of DM patients, reducing CM fibre contraction tone, thus, leading to co-morbidity that includes congestive heart disease (CHD) (Da Silva et al., 2012).

From our experimental observations (figure 5), metformin (PC), Spondias mombin (T1) and Nigella sativum (T4) all induced a significant expression of PFK-1, compared to the streptozotocin-treated diabetic control (DC) with a significantly induced PFK-1 expression (as is expected in DM). However, Spondias mombin (T1) performed better that the metformin-treated group, and even far better than the Nigella sativum-treated group. Binding energy studies revealed that alpha-sitosterol (-07.50 Kcal/mole) and its analogue, di-floro-alpha-sitosterol (-07.10Kcal/mole) had the highest binding energies.

From literatures, metformin (PC) nullifies the downregulation of PFK-1 in the heart muscle (HM) of streptozotocin-induced diabetic mice. It was has been claimed despite the observed increase in PFK-1 activity in DM, this effect of metformin has no link with PFK-1 transcription and expression, suggesting that metformin possibly alters the intracellular localization of PFK-1 augmenting glucose utilization (Da Silva et al., 2012). The very low binding energy obtained for metformin (-03.90 Kcal/mole) supports this claim, that the mechanism of metformin make have nothing to do with phase one and two in its process of ameliorating DM. In some reported claims, skeletal muscle PFK-1 was inhibited by *S*-hexadecyl-CoA, a non-hydrolyzable palmitoyl-CoA analogue, demonstrating that covalent acylation of PFK-1 may not be required for inhibition, but an induced conformational in the protein structure; fatty acyl-CoA modulates phosphofructokinase activity through both covalent and noncovalent interactions (Jenkins et al., 2011; Nicolau et al., 2006).

Conclusion

Diabetes mellitus (DM) is a metabolic disease that involves chronic high blood glucose concentration with other precipitated pathologic and comorbid conditions. Thus, any remedial intervention that can maintain healthy glucose homeostasis would be beneficial to sufferers. Apart from distortions of insulin availability and sensitivity, a hosts of other intracellular and extracellular factors are perturbed leading to body unable to efficiently utilize glucose, including,

sensors of both lipid and glucose homeostasis (peroxisome proliferator-activated receptors, PPAR-γ), transport proteins (GLUT 1 to 5), proteins of inflammatory reactions (interleukin-1-beta, IL-1β; tumour necrosis factor-alpha-1), enzymes involved in glycolysis (phosphofructokinase-1, PFK-1), etc. Invariably, if these factors can be restored to their normal physiologic functions and activities, DM can be corrected.

From this experiment, the positive control drug, metformin and Nigella sativum were able restore the observed metabolic distortions due streptozotocin treatment, but however, Spondias mombin performed excellently better, both as a regulatory influencer of gene expression and modulator of the protein macromolecules of these genes

Declarations

Compliance with Ethical Standards:

All applicable international, national, and/or institutional guidelines for the care and use of animals were strictly followed.

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In-text Graphics

In-text graphics 1-4 are available in the Supplementary Files section.

Figures

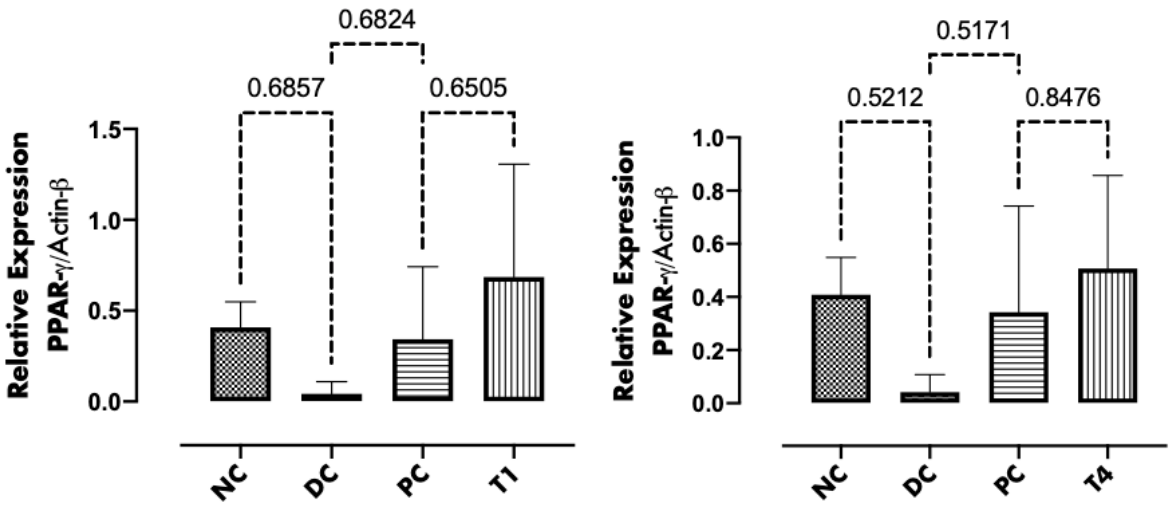

Figure 1

Relative expression of PPAR-γ following the various treatments compared to Spondias mombin (T1) and Nigella sativum (T4)

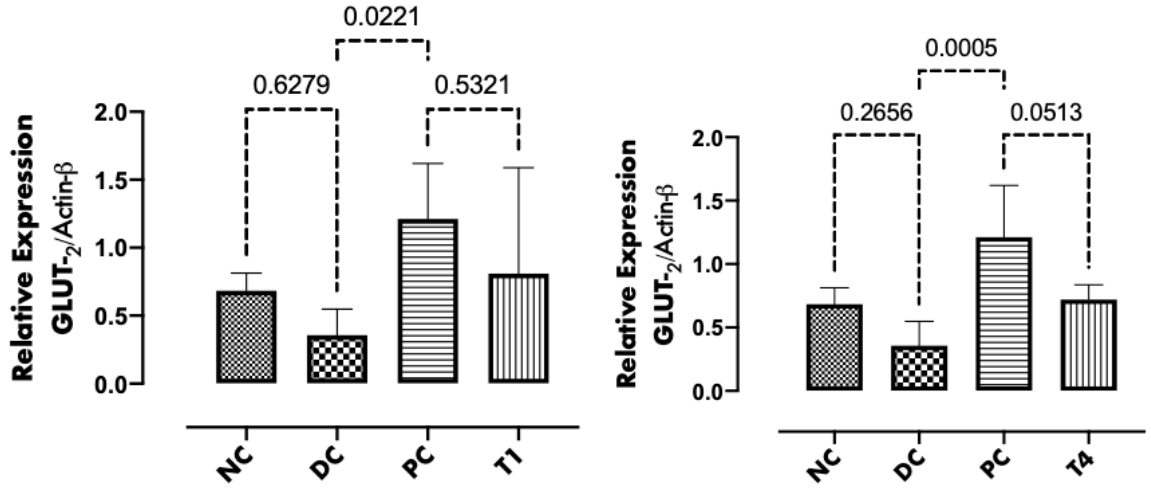

Figure 2

Relative expression of GLUT-2 following the various treatments compared to Spondias mombin (T1) and Nigella sativum (T4)

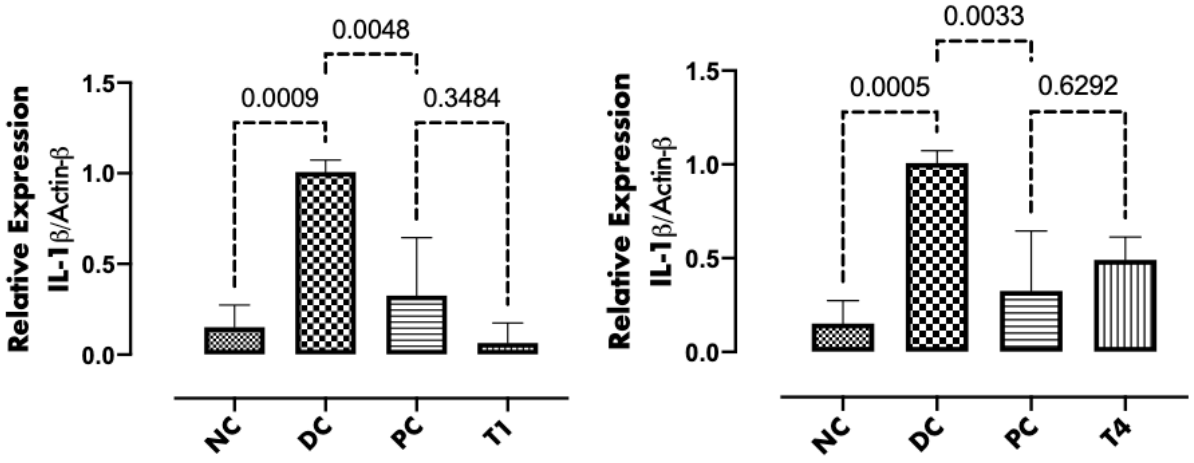

Figure 3

Relative expression of IL-1β following the various treatments compared to Spondias mombin (T1) and Nigella sativum (T4)

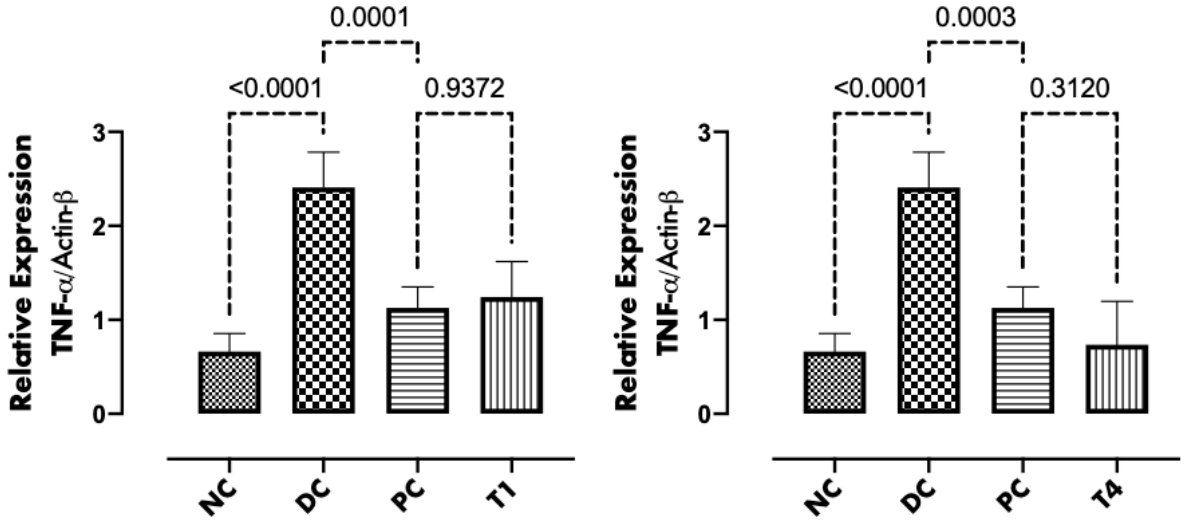

Figure 4

Relative expression of TNF-α following the various treatments compared to Spondias mombin (T1) and Nigella sativum (T4)

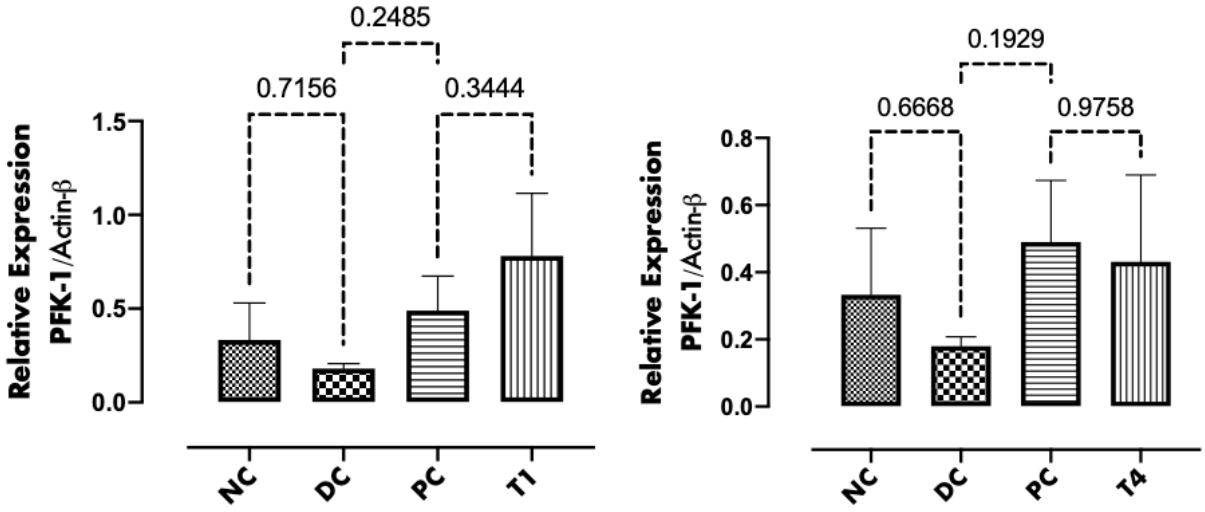

Figure 5

Relative expression of PFK-1 following the various treatments compared to Spondias mombin (T1) and Nigella sativum (T4)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphic1.png
- Graphic2.png
- Graphic3.png
- Graphic4.png