



Antidiabetic Effects of Olive Oil Against Streptozotocin Induced Diabetes In Rats

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ABSTRACT

Diabetes mellitus, a prevalent health issue throughout the world, is characterized by abnormal insulin secretion caused by the overproduction of reactive oxygen species (ROS) which affects the integrity of the lysosomal membrane affecting lysosomal enzymes. It is diagnosed by the presence of hyperglycemia. Natural products as antioxidants can constrict the effect of these ROS. Therefore, treatment of diabetic patients with antioxidant, such as extra virgin olive oil (EVOO) may be of advantage in attenuating certain complications. This study aimed to evaluate the antidiabetic role of olive oil against hyperglycemia in streptozotocin (STZ) induced diabetic rats. Thirty-six healthy male Sprague-Dawley rats were divided into six groups (6 rats per group). Diabetes was induced by single intraperitoneal injection of STZ. Group 1 served as non-diabetic control (NC) and group 2 as diabetic control (DC). Animals in groups 3, 4 and 5 were treated with metformin, olive oil and combination of olive oil with metformin respectively. Rats in group 6 were given olive oil orally for 2 weeks daily before induction of diabetes and then throughout the study. All treatments were gavaged orally for six weeks. The effect of olive oil was assessed by measuring the changes in body weight of rats, determination of glycemic control and estimation of oxidative stress markers. The results showed the significant increase in blood glucose level, glycated haemoglobin (HbA1c) and malondialdehyde (MDA) levels. While as the body weight of rats and insulin were significantly reduced in DC rats. Moreover, MDA level remained higher in diabetic rats treated with metformin and/or olive oil. In contrast, pretreatment with olive oil significantly decreased blood glucose and HbA1c levels. Furthermore, treatment of olive oil with metformin decreased blood glucose and HbA1c levels but it increased total antioxidant capacity (TAC) significantly. In conclusion, pretreatment with olive oil to protect against diabetes and the combined treatment of olive oil with metformin to animals might offer additional antidiabetic and antioxidant effect to metformin. Therefore, it could be a promising strategy for diabetes therapy. We recommend more investigations on humans to study the complementary effect of combination of olive oil and metformin on body tissues.

Keywords: Antidiabetic, Antioxidant, Olive oil, Oxidative stress, Rats, Streptozotocin.

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INTRODUCTION

Diabetes mellitus is a group of metabolic diseases caused by the insufficient secretion of insulin or defects in insulin action or by both. It is described by chronic hyperglycemia with metabolic disorders of carbohydrate, fat and protein.^{1,2} Diabetes mellitus is a common health issue around the world with increasing prevalence. The number of adults affected by diabetes in the world is estimated to rise from 382 million in 2013 to 592 million by 2035.³ The main causes for this worldwide ascent in diabetes mellitus are increase in aged population, expanding patterns towards obesity, unhealthy diet and stationary way of life.^{4,5} Diabetes mellitus has two main types: Insulin-dependent diabetes mellitus (IDDM) commonly known as Type 1 diabetes mellitus (T1DM) and Non-insulin dependent diabetes mellitus (NIDDM) commonly known as Type 2 diabetes mellitus (T2DM).⁶ Type 1 diabetes mellitus is an auto-immune condition characterized by β -cells destruction that eventually causes diabetes mellitus in which insulin is required to inhibit the progress of ketoacidosis, coma and death. T1DM is usually diagnosed during childhood or early adolescence and it accounts for only about 5-10% of all cases of diabetes.¹ Type 2 diabetes mellitus is a progressive and complex metabolic illness described by hyperglycemia and insulin resistance with insufficient compensatory insulin-secretory reaction.⁷ T2DM is generally adult-onset diabetes and accounts about 90–95% of all the diabetic cases.⁸ The symptoms of diabetes mellitus include increased thirst, polyuria, obscuring of vision, increased hunger, weight loss, hypertension and irregularities of lipoprotein metabolism. Acute complications of diabetes mellitus like ketoacidosis or non-ketotic hyperosmolar state might develop and cause stupor, coma and death.⁸ The long-term impacts of diabetes mellitus incorporate gradual advancement of the particular complexities of retinopathy with potential visual impairment, nephropathy that may prompt renal impairment, and/or neuropathy with danger of foot ulcers, charcot joints, and features of autonomic dysfunction.⁹ People with diabetes are at raised risk of cardiovascular, peripheral vascular and cerebrovascular disease.¹ Prevention and control of diabetes mellitus is a great challenge and requires change in way of life towards more physical movement and low calorie admission maintaining a strategic distance from inactive propensities. However, numerous individuals think that it is hard to change their way of life and look for simple options. So some conventional constituents of nourishment might end up being for prevention and control of diabetes mellitus.¹⁰ There is convincing experimental and clinical evidence that diabetes mellitus is associated with increased production of free radicals that further lead to oxidative stress and damage of cell components.⁶ Therefore, free

radical damage effectively contributes to the etiology of several chronic health problems such as atherosclerosis, cancer, neurodegenerative diseases, diabetes mellitus and inflammatory diseases.¹¹ Expanded oxidative stress seems to be a pernicious component prompting insulin resistance, dyslipidemia, β -cell dysfunction, hindered glucose tolerance and ultimately causing T2DM.¹² Several research studies established the relationship of oxidative stress and the pathogenesis of insulin resistance through insulin signals inhibition and adipocytokines dysregulation.^{13,14} T2DM and insulin resistance are definitely connected with a pro-oxidant environment in both rat and human studies, which recommends that oxidative stress might essentially change both the pathogenesis and progression of these diseases *in-vivo*.¹⁵

Antioxidants are substances that neutralize free radicals or their actions and keeps up the body's delicate oxidant/antioxidant equilibrium. Antioxidants have been observed to protect β -cells from damage caused by free radicals by repressing the peroxidation chain reaction, thus might prevent the progress of diabetes.^{16,17} Intraperitoneal injection of α -lipoic acid, a known antioxidant to streptozotocin (STZ) diabetic Wistar rats standardizes thiobarbituric acid reactive substances (TBARS) levels in plasma, pancreas, liver, and the retina.¹⁸ Streptozotocin [(STZ), (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose)] is a naturally occurring chemical synthesized by *Streptomyces achromogenes*. STZ is particularly toxic to the insulin-producing beta cells of the pancreas in mammals. It is used in medicine for treating certain cancers of the Islets of Langerhans and used in medical research to induce both IDDM and NIDDM.¹⁹ Consequent study including STZ induced diabetic rats demonstrated that diabetes-induced increase in lipid peroxidation could be avoided by antioxidant therapy.²⁰ Therefore, antioxidant rich diet may enhance antioxidant defense mechanism and protect against oxidative harm caused by free radicals.²¹ Various regular antioxidants, for example, vitamin E and phenolic compounds are known to have hypoglycemic, hypolipidemic or both actions.²² People who have T2DM need diabetes medications or insulin therapy. There are numerous kinds of glucose-depressing medications which treat diabetes by enhancing insulin sensibility, expanding insulin creation and/or diminishing the measure of glucose in blood.⁴ Chemical medications have numerous reactions; along these lines, screening for new antidiabetic sources from normal antioxidants is still appealing because they are harmless and great option for treatment of diabetes mellitus. Metformin hydrochloride [(MFH), (N,N-dimethyl biguanide)] is an oral anti-diabetic medicine from the biguanide group.²³ Metformin brings down blood glucose concentrations in T2DM without creating obvious hypoglycemia in patients with T2DM and it doesn't bring about hyperinsulinemia. Metformin is contraindicated in patients with renal

or hepatic deficiency, in very elderly patients, and in patients with conditions of circulatory dysfunction such as congestive heart inability, because of expanded danger of lactic acidosis.²⁴ Recently, researchers have shown an increased interest to assess the efficiency and safety of specific herbal formulation that have been used for treating diabetes in traditional medicine presenting possibilities for detection of new antidiabetic medicines.^{25,26} It has turned out clear that utilization of Mediterranean diet with high intake of cereals, fruits, fish, legumes, vegetables and olive oil, is connected to decreased danger of hardening of arteries (atherosclerosis), cardiovascular diseases and specific forms of cancer and general mortality.²⁷ This lower incidence has been partly ascribed to the dietary utilization of virgin olive oil (VOO) by Mediterranean populations. Some of these impacts have been credited to the antioxidant compounds and huge amount of monounsaturated fatty acids (MUFA) in the olive oil.²⁸ Olive oil is the most representative food of the traditional Mediterranean diet.²⁹ Olive oil, a product of *Olea europaea* L, is made out of a glycerol portion (90–99 %) and non-glycerol or unsaponifiable portion (0.4–5%).³⁰ It is a source of not less than 30 phenolic compounds, a significant number of which add to the resistance of olive oil to oxidative rancidity.³¹ The three most focused components of olive oil are glycoside oleuropein, hydroxytyrosol (3,4-dihydroxyphenyl ethanol) and tyrosol which give extra virgin olive oil (EVOO) its severe taste and strong antioxidant action in both *in-vivo* and *in-vitro*.³⁰ Normally, hydroxytyrosol is a better antioxidant and radical scavenger than oleuropein and tyrosol.³¹ Olive oil phenolic compounds have indicated antioxidant and anti-inflammatory properties and have favorable impacts on particular physiological parameters, e.g., plasma lipoproteins, oxidative harm, inflammatory markers, platelet and cell capacity, antimicrobial action and bone validity.^{29,31,32} Currently, there has been an expanding interest to assess the EVOO activity against metabolic disorders like hypercholesterolemia, hypertriglyceridemia, hypertension, obesity, fatty liver and insulin resistance, all firmly connected to diabetes and coronary illness.^{27,33} Olive oil-rich diet ameliorate diabetes, as it improves insulin sensitivity and its possible good consequences by raising level of high density lipoproteins (HDL), bringing down low density lipoproteins (LDL) and triacylglycerol (TAG) level, and guaranteeing better glucose level control.^{34,35} Several studies have shown that olive oil enhances insulin secretion in human and rats.³⁶ It has been observed that olive leaf extracts inhibit hyperglycemia, hyperlipidemia and oxidative stress induced by diabetes, while as it reduces lipid peroxides.^{37,38}

Therefore, the aim of this study was to evaluate the antidiabetic effect of olive oil in reducing oxidative stress and hyperglycemia in STZ-induced diabetic rats by measuring the body weight

and serum biochemical parameters.

MATERIALS AND METHOD

Materials

Virgin olive oil (VOO) was obtained from Aladhara agricultural project, Al-Jouf, Kingdom of Saudi Arabia (KSA). STZ for induction of diabetes mellitus in experimental animals was purchased from Sigma-Aldrich, USA. Metformin (Glucophage 1000 mg tablet) was procured from local pharmacy and was dissolved in distilled water containing 0.9% (wt/vol) sodium chloride for oral administration.

Subjects

Thirty-six healthy male Sprague-Dawley rats (200-250 g) were purchased from the Experimental Animal Unit of King Fahd Medical Research Center (KFMRC), King Abdul Aziz University (KAU), Jeddah, Kingdom of Saudi Arabia (KSA) and used for experiment. The rats were housed in controlled environment of temperature 22 ± 3 °C, relative moistness of 50 - 55% and 12 hours/12 hours light/dark cycle for two weeks of acclimatization. Animals were fed nutritionally balanced diet and supplied with *ad libitum* drinking water. The study was conducted for 6 weeks. This study was carried out according to King Abdul Aziz University's policy and international ethical guidelines on the care and use of laboratory animals (The National Research Council of The National Academy of Sciences 2011). These guidelines follow the national and international laws and policies (National Institutes of Health Guiding Principles on the Care and Use of Laboratory Animals, USA). The ethical approval was obtained from the Unit of Biomedical Ethics, Faculty of Medicine, KAU.

Methods

Induction of diabetes (T2DM)

The rats were fasted overnight, but supplied with sufficient supply of water before the induction of diabetes. The diabetes was induced through a single intraperitoneal (IP) injection of STZ (40 mg/kg bw) by dissolving in freshly prepared 1 ml of 0.05 M citrate buffer (pH 4.5).³⁹ After STZ injection, rats were given 10% (wt/vol) *ad libitum* fructose solution for 3 days. After three days, the blood glucose levels of rats were measured. Animal with blood glucose level above 250 mg/dl was considered as diabetic and used in the experiment. While as those animals with blood glucose level less than 250 mg/dl were given 10% (wt/vol) *ad libitum* fructose solution for two weeks and then their blood glucose level was measured again. If the blood glucose level of these rats had raised above 250 mg/dl, then these were used in the experiment as diabetic animals.

After induction of diabetes, the body weight and fasting blood glucose levels were measured every week during the experiment. Rats were divided into six groups:

Group 1: Non-diabetic control (NC); rats were given normal saline daily by oral gavage.

Group 2: Diabetic control (DC); rats were given normal saline daily by oral gavage.

Group 3: Diabetic with metformin (DM); rats were administered daily with metformin (300 mg/kg bw/day) by oral gavage for 2 weeks and then the dose of metformin was increased to 500 mg/kg bw/day for 4 weeks.^{40,41}

Group 4: Diabetic with olive oil (DO); diabetic rats were given olive oil (1ml /100g bw/day) orally.

Group 5: Diabetic with olive oil and metformin (DOM); diabetic rats were given olive oil (1ml/100g bw/day) orally for six weeks and then the rats were treated daily with metformin (300 mg/kg bw/day) by oral gavage for two weeks and then the dose of metformin was increased to 500 mg/kg bw/day for four weeks.^{40,41}

Group 6: Diabetic with olive oil pretreatment (DOP); rats were given olive oil (1ml /100g bw/day) orally for 2 weeks before induction of diabetes by STZ injection and then throughout the study.

Oral glucose tolerance test (OGTT)

At the 30th day of the test, the rats were fasted overnight and given D-glucose orally at a dose of 2g/kg bw (200 mg/ml).⁴² Blood was collected from the tip of tail at the specific time patterns. The blood glucose concentration was measured by the Contour blood glucose monitoring system at the periods of 0, 30, 60, 90, and 120 minutes after the administration of the glucose.

Blood collection

At the end of the experiment, rats were fasted overnight with sufficient supply of water before blood collection. Animals were anesthetized by ether and blood was collected via intraorbital sinus of the rats using 75 mm heparinized micro-hematocrit capillary tubes (Clay-Adams, Parsippany, New Jersey, USA). The blood was collected in plain tubes for serum preparation and in lithium-heparin tubes for plasma preparation followed by centrifugation using tabletop centrifuge (Sigma Aldrich, USA) at 4000 rpm for 10 minutes. The serum and plasma were kept at -80 °C for biochemical analysis.

Biochemical Analysis

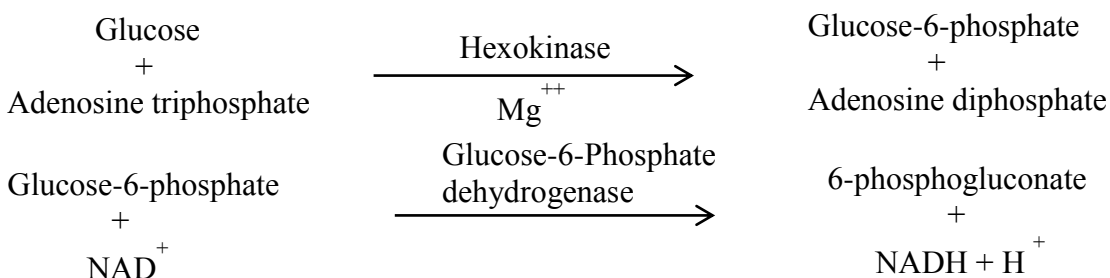
Biochemical parameters in serum like glucose, glycated haemoglobin (HbA1c), insulin, total antioxidant capacity (TAC) and malondialdehyde (MDA) were determined by using the standard

commercial kits (Dimension Vista 1500 Intelligent Lab System, Siemens Healthcare, USA) at King Abdulaziz University Hospital, Jeddah, KSA.

Determination of glycemic control

Measurement of glucose

Fasting serum glucose was measured in serum using Glucose Flex® Reagent Cartridge, (Siemens Healthcare Diagnostic Inc, Newark, USA). This kit measures glucose on the following principle:



The absorbance due to NADH and thus glucose concentration is determined using a bichromatic (340 and 383 nm) endpoint technique.

Measurement of glycated haemoglobin (HbA1c)

The HbA1c was estimated in serum using Rat HbA1c (Glycosylated Hemoglobin /Hemoglobin A1c) ELISA Kit (My BioSource, San Diego, California, USA). This assay follows the principle of Sandwich ELISA. The micro ELISA plate of this kit has been pre-coated with HbA1c specific antibody. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then, biotinylated detection antibodies specific for HbA1c and avidin-horseradish peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away and the substrate solution is added to each well. Only those wells that contain HbA1c, biotinylated detection antibody and HRP will appear in blue color. The enzyme-substrate reaction is stopped by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm +/- 2 nm. The OD value is proportional to the concentration of HbA1c. Concentration of HbA1c in the samples was calculated by comparing the OD of samples to the standard curve.

Measurement of insulin

The level of insulin in the serum was measured using Rat/Mouse Insulin ELISA Kit (Merck Millipore, Billerica, USA). This assay is a Sandwich ELISA based on: (a) capture of insulin particles from samples or standards to monoclonal mouse anti-rat insulin antibodies coated on

the wells of the microtiter plate and the binding of biotinylated polyclonal antibodies to the captured insulin, (b) wash away of unbound materials from samples, (c) binding of HRP to the immobilized biotinylated antibodies, (d) wash away of free enzyme conjugates, and (e) determination of immobilized antibody-enzyme conjugates by monitoring HRP activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme-substrate reaction is ended by the addition of stop solution. The enzyme activity is measured spectrophotometrically by the increased absorbance at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. As the increase in absorbency is directly proportional to the amount of captured insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat insulin.

Estimation of Oxidative stress markers

Measurement of total antioxidant capacity (TAC)

The TAC was measured in plasma by using OxiSelect™ Total Antioxidant Capacity Assay Kit (Cell Biolabs, Inc, San Diego, USA). This kit measures the TAC inside a sample. The principle of this assay is comparing the samples to a known concentration of uric acid standard within a 96-well microtiter plate format. Samples and standards are diluted with a reaction reagent and, upon the addition of copper, the reaction continues for a couple of minutes. The reaction is blocked and read with a standard 96-well spectrophotometric microplate reader at 490 nm. Antioxidant capacity is determined by comparison with the uric acid standards.

Measurement of malondialdehyde (MDA)

The concentration of MDA was estimated in serum using thiobarbituric acid reactive substances (TBARS) assay kit (Cayman Chemical, Ann Arbor, Michigan, USA). This kit determines MDA, a reactive compound formed from lipid peroxides that are generated under oxidative stress conditions. MDA forms an adduct with thiobarbituric acid (TBA) at high temperature of 90-100 °C and acidic conditions and is measured colorimetrically at 530-540 nm. Results are calculated from a standard curve constructed with authentic MDA.

Statistical analysis

All data were analyzed by using SPSS (Statistical Package for the Social Sciences) version 20.0 software (SPSS Inc., Chicago, IL, USA). The data were presented as mean ± standard deviation (SD). Significances among different group were determined by using one-way analysis of variance (ANOVA) test. A P-value less than 0.05 was considered as criterion for a statistically significant difference.

RESULTS AND DISCUSSION

In vivo antidiabetic activity

Effect of olive oil on the body weight of STZ induced diabetic rats

The results showed that in 1st week of experiment the body weight of the DM group of rats was significantly higher than NC group (P=0.020). In DC group, the body weight was significantly lower than NC group in the 4th and 6th weeks (P=0.017 and P=0.023 respectively). In the final week, body weight in DC and DO groups was significantly lower than NC group (P=0.024 and P=0.044 respectively) but there were no significant changes in DM, DOM and DOP groups when compared to NC and DC groups (Table 1).

Table 1: Body weight (grams) of different groups of rats at different weeks during the experiment after the induction of diabetes by streptozotocin. Data was expressed as mean \pm standard deviation (SD). Significances among different groups were determined by using one-way analysis of variance (ANOVA) test. ¹P: Significant versus control, ²P: Significant versus diabetic control.

Rat Groups	1 st week	2 nd week	3 rd week	4 th week	5 th week	6 th week	Final
Group 1: Non-diabetic control (NC)	251.25 \pm 10.37	263.50 \pm 10.39	264.01 \pm 10.95	294.33 \pm 11.78	283.80 \pm 8.14	306.08 \pm 5.43	306.08 \pm 5.43
Group 2: Diabetic Control (DC)	266.44 \pm 36.87 ¹ P =0.394	256.98 \pm 36.31 ¹ P =0.673	252.42 \pm 40.77 ¹ P =0.468	251.79 \pm 38.22 ¹ P = 0.017	254.90 \pm 48.13 ¹ P =0.141	257.46 \pm 49.29 ¹ P = 0.023	261.54 \pm 46.23 ¹ P = 0.024
Group 3: Diabetic and metformin (DM)	292.17 \pm 34.33 ¹ P = 0.020 , ² P=0.153	284.50 \pm 35.29 ¹ P =0.159, ² P=0.081	270.67 \pm 36.17 ¹ P =0.661, ² P=0.255	286.17 \pm 39.58 ¹ P =0.619, ² P=0.051	267.57 \pm 41.45 ¹ P =0.382, ² P=0.514	290.41 \pm 49.92 ¹ P =0.427, ² P=0.116	283.52 \pm 43.80 ¹ P =0.221, ² P=0.255
Group 4: Diabetic and olive oil (DO)	254.83 \pm 24.62 ¹ P =0.832, ² P=0.514	256.50 \pm 20.66 ¹ P =0.635, ² P=0.975	255.40 \pm 21.42 ¹ P =0.571, ² P=0.852	267.33 \pm 23.96 ¹ P =0.105, ² P=0.368	257.65 \pm 28.94 ¹ P =0.162, ² P=0.887	268.26 \pm 31.00 ¹ P =0.060, ² P=0.601	268.26 \pm 31.00 ¹ P = 0.044 , ² P=0.726
Group 5: Diabetic, olive oil and metformin (DOM)	277.50 \pm 48.50 ¹ P =0.126, ² P=0.534	262.00 \pm 24.26 ¹ P =0.919, ² P=0.745	260.00 \pm 21.14 ¹ P =0.792, ² P=0.634	274.18 \pm 24.13 ¹ P =0.223, ² P=0.197	267.11 \pm 27.69 ¹ P =0.368, ² P=0.529	278.73 \pm 23.75 ¹ P =0.169, ² P=0.305	276.78 \pm 25.49 ¹ P =0.114, ² P=0.428
Group 6: Diabetic and olive oil pretreatment (DOP)	274.10 \pm 22.61 ¹ P =0.182, ² P=0.666	265.50 \pm 27.47 ¹ P =0.892, ² P=0.582	265.99 \pm 28.64 ¹ P =0.896, ² P=0.396	276.56 \pm 37.17 ¹ P =0.282, ² P=0.155	272.72 \pm 34.05 ¹ P =0.549, ² P=0.360	278.98 \pm 42.21 ¹ P =0.173, ² P=0.300	278.10 \pm 36.26 ¹ P =0.131, ² P=0.389

Table 2: Blood glucose, glycated haemoglobin (HbA1c) and insulin levels in different groups of rats at different weeks during the experiment after the induction of diabetes by streptozotocin. Data was expressed as mean \pm standard deviation (SD). Significances among different groups were determined by using one-way analysis of variance (ANOVA) test. ¹P: Significant versus control, ²P: Significant versus diabetic control.

Rat Groups	Blood glucose (mmol/L)							Glycated haemoglobin (ng/ml)	Insulin (ng/mL)
	1 st week	2 nd week	3 rd week	4 th week	5 th week	6 th week	Final		
Group 1: Non-diabetic control (NC)	3.54 \pm 0.57	3.41 \pm 0.63	3.53 \pm 1.00	3.96 \pm 0.55	2.76 \pm 0.52	3.47 \pm 0.50	5.76 \pm 1.62	5.71 \pm 1.76	0.74 \pm 0.15
Group 2: Diabetic Control (DC)	31.65 \pm 1.71 ¹ P = 0.0001	26.34 \pm 10.28 ¹ P = 0.0001	10.22 \pm 3.19 ¹ P =0.136	23.92 \pm 7.07 ¹ P = 0.0001	19.54 \pm 5.42 ¹ P = 0.0001	21.32 \pm 9.55 ¹ P = 0.0001	26.45 \pm 1.04 ¹ P = 0.0001	167.17 \pm 37.01 ¹ P = 0.0001	0.48 \pm 0.07 ¹ P = 0.001
Group 3: Diabetic and metformin (DM)	33.33 \pm 0.00 ¹ P = 0.0001 ,	9.01 \pm 6.08 ¹ P =0.179,	25.35 \pm 10.42 ¹ P = 0.0001 ,	18.72 \pm 12.03 ¹ P = 0.004 ,	5.08 \pm 2.79 ¹ P =0.459,	21.04 \pm 10.82 ¹ P = 0.0001	22.96 \pm 5.97 ¹ P	67.95 \pm 11.27 ¹ P = 0.002 ,	0.56 \pm 0.12 ¹ P = 0.011 ,

	² P=0.619	² P =0.0001	² P =0.001	² P =0.308	² P =0.0001	² P =0.949	=0.0001, ² P =0.214	² P =0.0001	² P =0.256
Group 4: Diabetic and olive oil (DO)	26.59±4.91 ¹ P=0.0001 ² P=0.139	21.09±10.99 ¹ P=0.0001, ² P =0.228	24.95±10.28 ¹ P =0.0001, ² P =0.002	24.45±10.30 ¹ P=0.0001, ² P =0.916	17.37±9.60 ¹ P=0.0001, ² P =0.509	24.80±10.14 ¹ P=0.0001, ² P =0.440	18.87±9.42 ¹ P =0.0001, ² P =0.009	52.34±18.87 ¹ P=0.016, ² P =0.0001	0.55±0.08 ¹ P=0.006, ² P =0.354
Group 5: Diabetic, olive oil and metformin (DOM)	33.33±0.00 ¹ P =0.0001, ² P =0.619	18.61±8.32 ¹ P=0.001, ² P =0.079	18.57±7.27 ¹ P =0.001, ² P =0.065	19.67±10.96 ¹ P=0.002, ² P =0.404	7.40±4.36 ¹ P=0.143, ² P =0.001	7.80±3.95 ¹ P=0.316; ² P =0.004	8.47±3.25 ¹ P =0.311, ² P =0.0001	63.45±5.03 ¹ P=0.003, ² P =0.0001	0.49±0.06 ¹ P=0.001, ² P =0.848
Group 6: Diabetic and olive oil pretreatment (DOP)	16.17±11.15 ¹ P =0.0001, ² P =0.0001	20.15±7.41 ¹ P=0.0001, ² P =0.157	15.60±11.06 ¹ P =0.007, ² P =0.229	15.30±10.38 ¹ P=0.023, ² P =0.095	10.96±8.63 ¹ P=0.012, ² P =0.012	13.60±9.05 ¹ P=0.022, ² P =0.092	20.77±3.85 ¹ P =0.0001, ² P =0.047	127.15±4.28 ¹ P=0.0001, ² P =0.045	0.60±0.15 ¹ P=0.045, ² P =0.092

Table 3: Diabetic tolerance curve in different groups of rats at different periods during the experiment after the induction of diabetes by streptozotocin. Data was expressed as mean ± standard deviation (SD). Significances among different groups were determined by using one-way analysis of variance (ANOVA) test. ¹P: Significant versus control, ²P: Significant versus diabetic control.

Rat groups	0 minute	30 minutes	60 minutes	90 minutes	120 minutes
Group 1: Non-diabetic control (NC)	71.33±10.01	104.60±26.78	106.00±15.67	87.50±6.72	85.50±8.07
Group 2: Diabetic Control (DC)	275.00±172.96 ¹ P= 0.017	472.40±157.67 ¹ P= 0.0001	513.40±106.59 ¹ P= 0.0001	501.80±91.26 ¹ P= 0.0001	468.80±131.45 ¹ P= 0.0001
Group 3: Diabetic and metformin (DM)	371.33±188.11 ¹ P=0.0001, ² P=0.244	556.67±76.20 ¹ P=0.0001, ² P= 0.310	506.50±142.14 ¹ P=0.0001, ² P=0.928	521.83±191.47 ¹ P=0.0001, ² P=0.820	460.50±191.24 ¹ P=0.0001, ² P=0.916
Group 4: Diabetic and olive oil (DO)	378.50±215.34 ¹ P=0.0001, ² P=0.211	492.67±176.38 ¹ P=0.0001, ² P= 0.806	575.33±38.47 ¹ P=0.0001, ² P=0.418	480.50±127.14 ¹ P=0.0001, ² P=0.809	507.17±148.05 ¹ P=0.0001, ² P= 0.628
Group 5: Diabetic, olive oil and metformin (DOM)	133.17±78.47 ¹ P=0.430, ² P=0.089	314.50±118.75 ¹ P=0.015, ² P= 0.062	435.33±130.54 ¹ P=0.0001, ² P=0.309	325.67±198.33 ¹ P=0.007, ² P=0.051	314.17±126.22 ¹ P=0.004, ² P= 0.057
Group 6: Diabetic and olive oil pretreatment (DOP)	197.33±155.41 ¹ P=0.113, ² P=0.346	287.33±242.33 ¹ P=0.032, ² P= 0.030	279.17±247.66 ¹ P=0.021, ² P=0.004	287.83±228.34 ¹ P=0.021, ² P=0.019	248.50±190.37 ¹ P=0.036, ² P=0.008

Table 4: Serum levels of oxidative stress markers (total antioxidant capacity and malondialdehyde) in different groups of rats after the induction of diabetes by streptozotocin. Data was expressed as mean \pm standard deviation (SD). Significances among different groups were determined by using one-way analysis of variance (ANOVA) test. ¹P: Significant versus control, ²P: Significant versus diabetic control.

Rat groups	Total antioxidant capacity (mM)	Malondialdehyde (μ m)
Group 1: Non-diabetic control (NC)	0.26 \pm 0.03	0.11 \pm 0.01
Group 2: Diabetic Control (DC)	0.24 \pm 0.02 ¹ P =0.401	0.12 \pm 0.01 ¹ P = 0.001
Group 3: Diabetic and metformin (DM)	0.27 \pm 0.03 ¹ P =0.574, ² P=0.173	0.12 \pm 0.01 ¹ P = 0.001 , ² P=0.823
Group 4: Diabetic and olive oil (DO)	0.28 \pm 0.03 ¹ P =0.338, ² P=0.084	0.13 \pm 0.00 ¹ P = 0.0001 , ² P=0.152
Group 5: Diabetic, olive oil and metformin (DOM)	0.29 \pm 0.03 ¹ P =0.114, ² P= 0.022	0.12 \pm 0.005 ¹ P =0.245, ² P= 0.019
Group 6: Diabetic and olive oil pretreatment (DOP)	0.28 \pm 0.04 ¹ P =0.423, ² P=0.113	0.12 \pm 0.01 ¹ P = 0.006 , ² P=0.435

Effect of olive oil on the biochemical parameters of STZ induced diabetic rats

Effect on glycemic control

The results showed that in 1st week the blood glucose level of DC, DM, DO, DOM and DOP groups of rats was significantly higher than NC group (P=0.0001 for all) but was significantly lower in DOP group than DC group (P=0.0001). In 2nd week blood glucose level of DC, DO, DOM and DOP groups was significantly higher than NC groups (P=0.0001, P=0.0001, P =0.001 and P=0.0001 respectively) but was significantly lower in DM group than DC group (P=0.0001). In 3rd week blood glucose level of DM, DO, DOM and DOP groups was significantly higher than NC group (P=0.0001, P=0.0001, P=0.001 and P=0.007 respectively) and was significantly higher in DO and DM groups than DC group (P=0.002 and P=0.001 respectively). Blood glucose level in the 4th week was significantly higher in DC, DM, DO, DOM and DOP groups than NC (P=0.0001, P=0.004, P=0.0001, P=0.0001, P=0.002 and P=0.023 respectively). In 5th week blood glucose level of DC, DO and DOP groups was significantly higher than NC group (P=0.0001, P=0.0001 and P=0.012 respectively) but was significantly lower in DM and DOP groups than DC group (P=0.0001 and P=0.012 respectively). Blood glucose level in the 6th week was significantly higher in DC, DM, DO and DOP groups than NC group (P=0.0001, P=0.0001, P=0.0001 and P=0.022 respectively) and was significantly higher in DC group than DOM group (P=0.004). Blood glucose level in the final week was significantly higher in DC, DM, DO and

DOP groups than NC group ($P=0.0001$ for all) and was significantly higher in DC group than DO, DOM and DOP groups ($P=0.009$, $P=0.0001$ and $P=0.047$ respectively). HbA1c level of DC, DM, DO, DOM and DOP groups was significantly higher than NC group ($P=0.0001$, $P=0.002$, $P=0.016$, $P=0.003$ and $P=0.0001$ respectively) and was significantly higher in DC group than DM, DO, DOM and DOP groups ($P=0.0001$, $P=0.0001$, $P=0.0001$ and $P=0.045$ respectively). Insulin serum level of DC, DM, DO, DOM and DOP groups was significantly lower than NC group ($P=0.001$, $P=0.011$, $P=0.006$, $P=0.001$ and $P=0.045$ respectively) (Table 2).

Effect on blood glucose concentration (Diabetic tolerance curve)

The analysis of the data showed that at 0 minute, the blood glucose levels of DC, DM and DO groups were significantly higher than NC group ($P=0.017$, $P=0.0001$ and $P=0.0001$ respectively). At 30 minutes, the blood glucose levels of DC, DM, DO, DOM and DOP were significantly higher than NC group ($P=0.0001$, $P=0.0001$, $P=0.0001$, $P=0.015$ and $P=0.023$ respectively), meanwhile blood glucose level at DOP was significantly lower than DC ($P=0.030$). At 60 minutes, the blood glucose levels of DC, DM, DO, DOM and DOP were significantly higher than NC group ($P=0.0001$, $P=0.0001$, $P=0.0001$, $P=0.0001$ and $P=0.021$ respectively), but blood glucose level at DOP was significantly lower than DC ($P=0.004$). At 90 minutes, the blood glucose levels were significantly higher in DC, DM, DO, DOM and DOP than NC group ($P=0.0001$, $P=0.0001$, $P=0.0001$, $P=0.007$ and $P=0.021$ respectively), however, blood glucose level of DOP was significantly lower than DC ($P=0.019$). The blood glucose levels at 120 minutes were significantly higher in DC, DM, DO, DOM and DOP than NC group ($P=0.0001$, $P=0.0001$, $P=0.0001$, $P=0.004$ and $P=0.036$ respectively), but blood glucose level at DOP was significantly lower than DC ($P=0.008$) (Table 3).

Effect on the concentration of oxidative stress markers

There was non-significant change in the serum TAC of DC rats when compared to NC rats. TAC serum level in DOM group was significantly higher than DC group ($P=0.022$) but no significant difference was found in DM, DO and DOP groups when compared to NC and DC groups. MDA serum level in DC, DM, DO and DOP groups was significantly higher than NC group ($P=0.001$, $P=0.001$, $P=0.0001$ and $P=0.006$ respectively) and was significantly higher in DOM than DC group ($P=0.019$) (Table 4).

DISCUSSION

The present study investigated the antidiabetic effects of olive oil in STZ induced diabetes mellitus rats by studying antioxidative changes, biochemical parameters like glycemic control of

diabetes and oxidative stress markers, and body weight changes. Results of the present study showed that diabetic rats exhibited a significant increase in blood glucose level after injection of STZ. This agrees with the finding of other study.⁴³ Induction of diabetes by STZ was characterized by a severe loss in body weight, which has also been reported by another researcher.⁴⁴ The body weight of DC and DO groups was significantly lower than NC group. This may be due to lack of insulin in the blood, so the sugar cannot enter inside the cell, thus increases the percentage of sugar in the blood. The body tries to get rid of excess sugar by excretion in the urine. The elevated secretion of urine will lead to the reduction of water in the body which results in weight loss.^{45,46} Many studies reported that, this loss of weight may be related to excessive catabolism of proteins to provide amino acids for gluconeogenesis during insulin deficiency resulting in muscle wasting in DC rats.^{45,46} On the other hand, in the final week, a gradual but not significant increase in the body weight was observed in DOP, DM and DOM groups except DO group. This may be due to the improved levels of glucose and insulin in DM, DOM and DOP groups.⁴⁷ However, the present study showed that olive oil did not prevent weight loss. Administration of metformin alone or in combination with olive oil, and the pretreatment with olive oil significantly improved body weight. This weight gain may be assumed due to increased utilization of glucose by the body or by decreased gluconeogenesis. The significant weight loss of DC rats observed in this study was similar to the study which has also reported no weight gain in the diabetic rats compared to controls throughout the study.⁴⁸ Moreover, these results were in agreement with the study which reported a significant weight loss in diabetic rats.⁴⁹ Also, the present observations agree with the findings of many other researchers.^{44,50,51}

In diabetes mellitus, the condition of body is assessed mainly by measuring glycemic control (fasting blood glucose, HbA1c) and serum insulin level. HbA1c is a reliable index of glycemic control in diabetes.⁵² In the present study, it has been observed that blood glucose level in the final week of study in DC, DM, DO and DOP groups was significantly higher than NC group and was also higher in DC group than DO, DOM and DOP groups. In correspondence to our results, the experimental studies concluded that monounsaturated fatty acids (MUFA) rich groundnut and olive oil significantly improved blood glucose in NC and DC rats.^{53,54} Blood glucose level of DO, DOM and DOP groups were significantly decreased after 6 weeks when compared with DC group. Interestingly, non-significant differences were found between DOM and NC groups. These findings suggest that olive oil in combination with metformin showed significant hypoglycemic effect than olive oil alone, metformin alone and pretreatment of olive

oil. Therefore, this implies that olive oil potentiates the hypoglycemic effect of metformin. Similarly, it has been found that daily consumption of olive oil had a positive effect on fasting blood glucose and lipid profiles in healthy controls. Plus, this positive effect was much more profound in the diabetic group as levels of fasting blood glucose, triacylglycerol (TAG), cholesterol (CHO) and low density lipoproteins (LDL) decreased by 16-32%.⁵⁵ Correspondingly, the combination of metformin with antioxidant (honey) improves glycemic control and provides additional metabolic benefits, not achieved with metformin alone.⁵⁶ It has also been observed that treatment of diabetic animals with oleuropein, the main component of olive oil, significantly inhibits increase of HbA1c and serum glucose in comparison with NC group.⁵⁷

As Insulin is a known hormone responsible for maintaining the serum glucose levels. The normal function of insulin releasing β -cells in pancreas and other cellular mechanisms are distorted by oxidative stress which would contribute in induction of diabetes mellitus.⁵⁸ In STZ-induced diabetic rats, insulin deficiency or hypoinsulinemia develops as a consequence of irreversible destruction of β -cells of pancreas resulting in hyperglycemia.⁵⁹ In the present study, the serum insulin level in DC, DM, DO, DOM and DOP groups was significantly lower than NC group but it increased nonsignificantly when compared with DC group. In consistency to our findings, no improvement in insulin content of pancreas of type 2 diabetic rats was reported, when olive oil diet was given for fifty days.⁶⁰ The findings of the present study are also partially consistent with the study where it has been found that olive oil suppressed hyperglycemia without exaggerating insulin secretion.⁶¹ However, the findings of the present study are in disagreement with the ones from experimental studies in human and rats, which showed that olive oil enhances insulin secretion.³⁶ Further it was found that olive oil increased insulin secretion in isolated pancreatic islet of rats.⁶² There was non-significant increase in insulin of rats even after treatment with olive oil, metformin, olive oil and metformin, and pretreatment of olive oil. This demonstrates that their effectiveness to treat diabetic condition might rely on actions other than pancreatic β -cells insulin release. From the above findings it is clear that the antihyperglycemic activity of olive oil is certainly not because of its insulin secretagogue property but there may be involvement of other possibilities like insulin mimetic action, regulation of postprandial glucose level, increase in sensitivity of target tissues or ability to increase peripheral glucose utilization.⁶³

HbA1c, an excellent marker of overall glycemic control, was found to be increased in the DC group. The increase in HbA1c level of diabetic patient is due to glycosylation of haemoglobin, and the amount of increase is directly proportional to the fasting blood glucose levels.⁶⁴ In the present study, serum level of HbA1c in DC, DM, DO, DOM and DOP groups was significantly

higher than NC group and significantly higher in DC group than DM, DO, DOM and DOP groups. In this study, treatment of STZ-induced diabetic rats with olive oil, metformin, olive oil and metformin, and pretreatment of olive oil showed a significant decrease in HbA1c level which advocates its potential to control long term diabetic condition. Administration of olive oil to type 2 diabetic rats reduced the glycosylation of haemoglobin by its free radical scavenging property, thus decreased the level of HbA1c. A decrease in blood glucose level might also have contributed to decreased level of HbA1c in olive oil-treated type 2 diabetic rats. Oral administration of olive oil showed a significant decrease in HbA1c which indicates the efficiency of olive oil in glycemic control. These findings of the present study are in concurrence with the results of studies where olive oil significantly improved HbA1c in NC and DC rats.^{53,54} In diabetic individuals treatment with olive leaf extract was associated with a significant reduction in HbA1C value.⁶⁵ The present findings are in accordance with a study which reported that significant increase in blood glucose and HbA1c, and decrease in insulin concentrations were determined in STZ induced DC group compared to NC group of rats.⁶⁶ In agreement with our results a study where rats were given EVOO supplemented diet, reported a significant improvement of fasting blood glucose, HbA1c and serum insulin levels of DC group in comparison with NC group of rats.⁴³

Oral glucose tolerance test (OGTT) is more sensitive measure to assess the early abnormalities in glucose regulation than fasting plasma glucose or HbA1c tests.⁶⁷ In the present study, after oral administration of D-glucose (2g /kg bw/day) to all rats, blood glucose levels increased to the maximum at 60 min, then decreased gradually. DC, DM and DO groups showed an impaired glucose tolerance when compared to NC group of rats. This reflects hepatic gluconeogenesis and reduced uptake of glucose from blood into skeletal muscle and adipose tissue, following a meal. Thus, serves as a marker for the state of insulin resistance and predicts both large and small vessel vascular complications.⁶⁸ Our results showed that the blood glucose level in DOM group was slightly but not significantly decreased than DC group. While as, DOP group showed decrease in blood glucose level when compared to DC group. Thus, treatment with combination of olive oil and metformin, and pretreatment of olive oil improved glucose tolerance which is indicated by reduction in peak blood glucose level at 1 and 2 hours in diabetic treated rats during OGTT. Therefore, this implies that the pretreatment of olive oil and the combination of olive oil with metformin might have brought this effect by enhancing glucose utilization by peripheral tissues and by increasing the glycogen stores in the liver and skeletal muscle.

The role of oxidative stress in the pathogenesis and complications of diabetes mellitus is well recognized.⁶⁹ Both human and experimental animal models of diabetes exhibit high oxidative stress due to persistent and chronic hyperglycemia, which depletes the activity of free radical scavenging enzymes and promotes free radicals generation.^{70,71} In the present study, we investigate and compare the effect of olive oil on markers of oxidative stress of STZ-induced diabetic rats. STZ significantly increased oxidative stress biomarkers as indicated by increase in serum MDA level and decrease in TAC of DC group of rats. Lipid peroxidation, measured as MDA, reflects the impact of oxidative stress in cells and tissues. MDA is generated from the degradation of polyunsaturated lipids by ROS.⁷² In the present study, it has been reported that MDA concentrations in DC group increased significantly when compared to NC group. This is in similarity to the report that plasma MDA concentration increased especially in T2DM than in T1DM patients and healthy controls.⁷³ In diabetes, the elevated level of MDA is being considered as a major indicator for the occurrence of lipid peroxidation.

In this study, MDA level in the serum of DM, DO and DOP groups was higher than NC rats, thus supported the occurrence of lipid peroxidation. The diabetic rats that received olive oil in combination with metformin showed significantly lower levels of MDA than those which received olive oil alone, metformin alone and pretreatment of olive oil. This suggests that combination of olive oil with metformin offer protection against lipid peroxidative damage. Therefore, this implies that combination olive oil with metformin offer better antioxidant effect than olive oil alone, metformin alone and pretreatment of olive oil, thereby protecting the pancreas from oxidative stress induced damage. This finding, increase in oxidative stress of DC group of rats is in agreement with the observations of many researchers who reported a significant elevation in MDA concentration in serum, liver and uterus of DC group of rats.⁷⁴ Two previous studies concluded that rats treated with STZ showed a significant increase in lipid peroxidation compared with NC group.^{58,75} In contrast to our results it was found that MDA levels in both poorly and well controlled T2DM patients did not differ from control subjects.⁷⁶

TAC is a measure of the antioxidant capacity of all antioxidants in a biological sample and not of a single compound.⁷⁷ Thus, TAC may be altered during oxidative stress. In the present study, it has been observed that serum TAC level in DC group was slightly but non-significantly lower than NC group. Serum TAC level in DOM group was significantly higher than DC group. TAC level was slightly higher in DM, DO and DOP groups when compared to DC group. The reduced TAC in DC rats might imply that there was an imbalance between free radical formation and antioxidant protection. This might be a consequence of increased utilization of endogenous

antioxidants in response to elevated levels of free radicals. Our present study showed that the combination of olive oil with metformin provided a better antioxidant effect than olive oil alone or metformin alone. This might demonstrate that olive oil offers additional antioxidant effect to metformin. Several studies support the fact that olive oil is rich in oleuropein, hydroxytyrosol and tyrosol which give olive oil its strong antioxidant action both *in-vivo* and *in-vitro*.^{30,78,79} Our findings are like the ones who found that metformin did not elevate TAC.⁸⁰ However, the same authors observed that combination of metformin with antioxidant (honey) significantly increased TAC, which correlates to our findings. A study found that consumption of 18 mg/day of phenols from EVOO for 3 weeks did not affect TAC.⁸¹ However, we disagree with the observations that TAC was significantly reduced in the DC group.⁸⁰ It was also found that the serum antioxidant potential presented a significant depletion in the DC group of rats when compared to NC group of rats.⁸²

In this study, the combination of olive oil and metformin in STZ-induced diabetic rats helps to achieve serum glucose levels much lower than those achieved with olive oil or metformin alone. Olive oil as an adjuvant to metformin leads to an improved glycemic control and some additional metabolic benefits not achieved with metformin alone. This study suggests that the combination of olive oil with oral hypoglycemic agents may be a valuable adjuvant therapy to achieve and/or maintain glycemic control and possibly reduce or delay the onset of diabetic complications. This data suggests that olive oil, when administered alone might not efficiently arrest oxidative stress mediated damage in diabetic rats. The present findings demonstrated the beneficial role of pretreatment with olive oil and olive oil as supplement to metformin in ameliorating oxidative stress in STZ-induced diabetic rats. The administration of olive oil in combination with metformin activated TAC. In other words, it can be concluded that olive oil provides additional antioxidant effect to metformin. Moreover, pretreatment with olive oil for two weeks did not completely prevent STZ-induced diabetic effects in rats, however, it might reduce such complication.

CONCLUSION

In conclusion, this study establishes a basis for advising pretreatment with olive oil as an adjuvant and prophylactics supplement against diabetes and antioxidant therapy in combination with hypoglycemic drugs in the management of diabetes mellitus. This may result in better and more efficient management of diabetes mellitus and its related complications. Moreover, we recommend more investigations on humans to study the complementary effect of combination of

olive oil and metformin on body tissues. Besides, more studies should be carried out to determine if the results from pretreatment with olive oil can be appropriately protective against human diabetes.

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AUTHORS CONTRIBUTIONS

KB designed the experiment, performed data analysis, revised and improved the manuscript. EA, HA, and SK collectively worked on the idea, experimental design, and manuscript preparation. EA conducted the experiments and participated in data analysis and manuscript preparation. All the authors have read and approved the final manuscript for publication.

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