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Anti-Oxidoreductive stress Effects of Iraqi Olea Europaea L. Leaves Extract against Low Double Doses of Alloxan Induced Diabetes Mellitus in Rats

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ABSTRACT

Olive leaves extract is famous for its antioxidant and protective effects. In this study, the aqueous extract of Iraqi Olea europaea L. Leaves was investigated for its anti-diabetic effects against low double doses of alloxan induced Diabetes Mellitus in rats. Low double doses (75 mg\Kg body weight) of alloxan were injected intraperitoneally at day 1&29 of the experimental period in rats, whereas an aqueous extract of Iraqi Olea europaea L. Leaves was added continuously to their drinking water. Serum malondialdehyde concentration, total oxidative stress and oxidative stress index as oxidoreductive stress biomarker, activities of certain anti-oxidoreductive stress enzymes (glutathione peroxidase, super oxide dismutase and catalase) and concentration of reduced form glutathione wit total antioxidative stress capacity and lipid profile were estimated. Furthermore, histopathological evaluation of pancreas and liver were conducted. Obviously, the double doses of Alloxan that injected intraperitoneally were enhanced oxidoreductive stress by elevation of malondialdehyde and decreased some antioxidative stress biomarkers like GSH-Px and reduced form of glutathione and ultimately by increasing fasting blood glucose. Simultaneously, The diabetic rats treated with the extract showed reduction in fasting blood glucose by enhanced insulin sensitivity, improved some antioxidative parameters, and significantly decreased the histopathologic lesions noticed in pancreas of the treated diabetic rats. Together with converting the pathology of these organs caused by diabetes to almost normal architecture. Taken together, the aqueous extract of Iraqi olive leaves demonstrated many therapeutic criteria to cope with oxidoreductive stress mediated diabetes mellitus in alloxan injected rats.

Keywords: Alloxan, Oxidoreductive stress, Diabetes mellitus, Antioxidative stress, Olive leaves extract.

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INTRODUCTION

Diabetes mellitus (DM) is a perilous metabolic disorder characterized by the incidence of serious complications mediated by the oxidative stress dependent mechanism [1]. It is subdivided into type I and type II. The former is mediated by pancreatic damage that results in hypo-insulinemia and a deficiency in the entry of glucose to the muscles and hepatocytes whilst that later is mediated by a decline in the insulin sensitivity in the periphery that compromises glucose entry into that target cells. [1]. Both types induce oxidative stress and lead to generation of excessive amounts of free radicals. Free radicals induce tissue damage through several mechanisms, viz; peroxidation of the unsaturated free fatty acids and release of aldehyde products like malondialdehydes (MDA), isoprostan and 2 nonenal [2] Furthermore, they tend to bind to crucial biomolecules like DNA or proteins to adducts and result in loss of their biological. On the other hand, they act as signaling molecules for the transduction pathways related to the stress mechanisms. For instance, the signaling cascades related to the regulation of the insulin secretion or sensitivity [3]

Human body is endowed with endogenous antioxidant mechanisms that is represented by a cluster of antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) [4]. Besides, the nutrients sanctuary also provides plenty of antioxidants that interfere with the cascade of the free radicals mediated damage, such as; vitamin A, α tocopherol acetate (vitamin E) , vitamin C and β- carotenes [5]. Supplementation of different antioxidants rich herbal products may play a role in counteracting the oxidative stress mediated damage. They remain a possible adjunct therapy to sustain better glycemic control and reduce complications of diabetes [6].

This study aimed at screening the antidiabetic effect of olive leaves extract in a model of Alloxan induced diabetes in Wister Albino rats. In this experiment, a double dosing protocol of alloxan treatment was followed instead of the conventionally used single dosing protocol. This helps in making the mechanisms of diabetes; induced by alloxan, encompasses both the pathogenesis of both type I and type II diabetes.

Alloxan is a powerful diabetogenic urea derivative that acts as a cytotoxic glucose analog that enhances damage of β-cells of Islets of Langerhans and suppression of insulin release [7]. This results in induction of hyperglycemia and progression of the cascade of the hyperglycemia mediated oxidative stress.

The herbs sanctuary is rich with effective antidiabetic products that belong to different botanical families [8], such as; Oleaceae to which olive trees Olea Europaea belongs. Olive Olea Europaea (Oleaceae) is an evergreen small tree that can live more than 1000 years under the favorable conditions. Its products are rich with lots of healthy components, such as resin, wax, chlorophyll, tannin, saponins, gallic acid, oleuropein, oleuropeoside, verbascoside, rutin, tyrosol and hydroxytyrosol [9]. Oleuropein was found to be the most abundant bio-phenol (up to 6-9 %) in the leaves of the olive tree [10]. Its hypoglycemic and antioxidant in the diabetic models was screened previously [11] and [12]. Accordingly, this study was designed to explore the pathophysiologic consequences

of injecting rats with low double dose of alloxan by using the oxidative and antioxidant parameters and deep evaluation of the histopathological changes.

MATERIAL AND METHODS

Animals

A total of 48 adult male Wistar albino rats of eight weeks' age and 150-170 g body weight were obtained from Faculty of Pharmacy, University of Baghdad. The animals were housed in clean plastic cages and allowed to acclimatize to the laboratory environment for 10 days under the same laboratory conditions of photoperiod (12-h light:12-h dark cycle), a minimum relative humidity of 40%and room temperature 23±5 ◦C. During the acclimatization and the experimental period, the animals had free access (ad libitum) to the rodent's chow and water. The rats were housed in cages and were given a standard rodents chow. The diet contained Protein (12%), sugars (5%), fat (10%), vitamin mixtures (1%), salt mixtures (4%), fiber (4%) and starch (64 %). All the experiments and procedures were done as per rules and regulations of the Ethical Committee\College of Pharmacy\University of Baghdad.

Chemicals

All the used chemicals were of analytical grade and were obtained from Sigma-Aldrich. Alloxan was purchased from Sigma Chemicals, St. Louis, USA. All the working solutions were prepared using deionized water.

Preparation of aqueous olive leaves extract

The green olive leaves were collected from different quarters of Al Kurgh and Al Rusafa in Baghdad during April and May 2018. It was authenticated by the Herbarium Center of the Iraqi National Museum/ Baghdad-Iraq. The extraction was done as previously described by Ahmad-Qasem et al .2013 [13]. Briefly, the leaves were dried at 45°C for 90 min, ground to small pieces as small as 0.1 mm and stored at – 20 °C until further analysis. The extracts were used to prepare 5% aqueous olive leaves extract solution as per (Goldsmith et al., 2014) [14]. The prepared solutions were preserved in sterile dark bottles in a cool environment (4° C) until use.

Experimental design

Diabetes was induced by twice intraperitoneal injections of alloxan at 75mg/Kg (Body weight). The doses were given on day 1 of the first and fifth weeks of the experimental period. The animals were divided into 4 main groups (n=12), viz; Group 1 (Gr 1) which was treated as the negative control and was allowed for the free access to the rodents chow and the tap water for 56 days, Group 2 [(Gr 2) or the (AOLE-Gr)] which was assigned as the herb control and was given the basal diet along with drinking water enriched with the mentioned aqueous olive leaves extract for 56 days, Group 3 [(Gr 3) or ALX-Gr)]; the alloxan-injected group which was given alloxan at 75 mg/kg (body weight) on the first day of the first and 5the week of the study. and Group 4 (Gr 4) (ALX+AOLE-Gr) which was given Alloxan-and received the standard rodents chow along with an aqueous extract of the olive leaves.

Each of the mentioned group was subdivided into two halves (n=6) that one was exsanguinated on day 28 (subgroup A) while the rest were kept till the end of the study period (56 days) (Subgroup B).

During the study, the daily fasting tail vein glucose level was determined using the glucose meter. The animals were exposed to 10 hours fasting and the saw dust was changed before the commencement of the fasting to remove any possible remains of the pellets remains within the saw dust.

At the end of the study period, the animal's blood was collected via cardiac puncture under the A.C.E anesthesia (Alcohol, chloroform, diethyl ether) at a ratio of (1:2:3, Ethanol: Chloroform: Diethyl Ether, respectively) [15]. The blood samples were processed for serum preparation and running of the biochemistry analyses. The animals were ethically euthanized via decapitation and their organs (liver and pancreas) were collected and stored in 10% formalin for the histopathologic analyses. The carcass was wrapped and disposed as per the rules and regulations of the animal's ethics committee of the Faculty of Pharmacy/ Baghdad University.

Blood glucose level

The blood glucose was measured daily using the fasting tail venous blood. The measurements were done using the glucometer and the test strips provided by Contour -Bayer Germany. Means of the daily glucose level at the end of each week were determined for all the groups to follow the impact of the treatment on glucose homeostasis.

Blood samples

The blood samples were withdrawn in the fasting state and processed within 30 minutes of collection, centrifuged at 3000 rpm for 10 min to separate the serum and transferred in Eppendorf tubes for analysis

Biochemistry analyses

The serum samples were used to obtain the total serum proteins, albumin, cholesterol triglycerides, Alanine aminotransaminase (ALT, GPT), aspartate aminotransaminase (AST, GOT) and alkaline phosphatase (ALP) activities using the commercial kits obtained from (Bio-Merieux Laboratory Reagents and Products, France).

Oxidative stress measurement

Blood samples were used to determine the oxidative stress markers like malondialdehyde, serum total oxidant and antioxidant capacities (TOC and TAC). They were determined using the automated techniques described by Erel 2005 [16]. The oxidative stress index (OSI) was calculated through dividing the total oxidant capacity (TOC) to the total antioxidant capacity (TAC). Furthermore, the serum level of the antioxidant enzymes like glutathione peroxidase, catalase and superoxide dismutase were determined as well.

Determination of the serum level of malondialdehyde

Serum malondialdehyde (MDA) level was measured using thiobarbituric acid reactive substance (TBARS) method as described by Kikugawa et al. 1992 [17] with modifications. The serum concentration of MDA was expressed in nmol/ml. Briefly, 0.2 ml of the sample was added to 0.2 mL of phosphate buffer saline (0.2 M), and the color reaction was initiated by adding 25 μL of 0.11 M TBA solution. Then the

samples were placed in a 90°C heating block for 50 min and cooled later to form the TBARS (Thiobarbituric acid releasing substance complex). Then the complex was extracted with 0.4 ml of n-butanol and the butanolic phase was separated by centrifugation at 6000×g for 10 min. Later on, aliquots of the n-butanol phase were placed in a 96 well-plate and the absorbance was measured spectrophotometrically at 535 nm (Benchmark Plus, Bio-Rad, Hercules, CA). The calibration curve was prepared with authentic MDA standards ranging from 0–20 nmol/ ml with a detection limit of 0.079 nmol/ml. the intra- and inter- assay coefficient of variability were 1.82% and 4.01%, respectively.

Determination of total oxidant, antioxidant capacities and Oxidative Stress Index.

Each of the total oxidant (TOC) and total antioxidant (TAC) capacities were measured using the automated colorimetric measurement method found by (Erel, 2005) [16] and the commercial kits obtained by (Rel Assay Diagnostics ®, Gaziantep, Turkey).

The principle of the TOC relies on ability of oxidants present in the sample to oxidize the ferrous ion (Fe+2) ferrous-o-dianisidine complex into the ferric ions (Fe+3). The results were expressed in micromoles hydrogen peroxide equivalent per liter (µmol H2O2 Eq/L). Meanwhile, the serum total antioxidant capacity (TAC) was estimated using the automated colorimetric measurement method found by (Erel 2004) [18] wherein the hydroxy radical; that is produced as a byproduct of the Fenton reaction, reacts with colorless o-dianisidine to form a bright yellowish brown colored radical of the dianisyl substrate. The results were expressed in Trolox equivalent of millimoles per liter (Trolox Eq mmol/L). The analysis of serum samples has been done in 680 Beckman Coulter (Beckman Coulter Inc.®, CA, USA) device after reaching the room temperature.

The intraassay %CV values for the TAC measurement were 4.12% for the 0.50 (0.35-0.65) mmol Trolox equivalent / L and 1.53% for the 2.0 (1.7-2.3) mmol Trolox equivalents / L. The intraassay CV% values for TOC measurements were 3.57% for 5.5 (3.0-8.0) µmol/L and 5.17% for 19.5 (16-23) µmol/L. The calculation of Oxidative Stress Index (OSI) was done according to the formula: Total Oxidant Capacity (TOC) / Total Antioxidant Status (TAC).

Histopathological Studies

After the blood collection, the animals were ethically euthanized via decapitation and their organs (liver and pancreas) were fixed for 72 hours in 10% neutral buffered formalin saline. The tissues were embedded in paraffin and sectioned at 5-µm thickness using a rotary microtome. The sections were stained with hematoxylin-eosin (H&E) according to Luna (1968) for the light microscopy examination [20].

Statistical Analysis

The results were expressed as mean ±standard error of the mean (SEM). One-way ANOVA followed by post hoc Branferroni test was used to test the difference between the groups. The test was done using the SPSS version 18 statistical package, 2010, (SPSS Inc. Chicago, IL, USA). P- value of less than 0.05 was considered as statistically significant.

RESULTS

Fasting blood Glucose Levels

During the whole period of the experiment, there hasn't been any significant difference in the Fasting Blood Glucose (FBG) levels between (CN-Gr) and (AOLE-Gr) ($P<0.05$). On the other hand, during the first 4 weeks of the study, both of the (ALX-Gr) and (ALX+AOLE-Gr) groups experienced a transient increase in the FBG level during the first week followed by a gradual decline till the end of the 4th week. The increment was higher for ALX-Gr as compared to that of ALX+AOLE Gr. On the other hand, when the second dose of alloxan was given in the 5th week of the study, the FBG level has increased progressively up to the end of the study in the ALX-Gr. The AOLE co treatment in the subgroup B of the ALX-AOLE-Gr has prevented the alloxan induced hyperglycemia during the second month of the study (the period from week 5 to week 8) (Table-1).

Liver function test

The liver function test revealed absence of any significant difference in the serum level of the hepatic enzymes between (CN-Gr) and (AOLE-Gr) ($P<0.05$) (Table 2). Meanwhile, the deterioration in the hepatic function was obvious for the alloxan treated group on day 28 and day 56. The deterioration was stronger on day 56 in comparison to that on day 28. AOLE co treatment along with the alloxan has ameliorated the hepatic dysfunction as seen in the results of the AST and ALT levels (Table 1). Nevertheless, results of the ALP showed comparable values for the AOLE treated and untreated diabetic rats indicating that the extract has ameliorated the liver dysfunction without amelioration in the serum level of ALP.

Oxidative stress markers and antioxidant enzymes

The results revealed a prominent and a significant and progressive increase in the oxidative stress markers along with a decline in the antioxidant enzymes in the rats treated with alloxan ($P<0.05$). Treatment with AOLE did not produce that much change in the AOLE-Gr as compared to that of the CN-Gr. but it hindered the alloxan induced deterioration in the oxidative stress as seen in the results of the ALX-AOLE-Gr (Tables 3 &4).

Table 1. The mean of the daily fasting blood glucose levels (FBG) (mg/dl) at the end of each week during the entire stud period for all the groups.

Groups	1st week N=12	2nd week N=12	3rd week N=12	4th week N=12	5th week N=6	6th week N=6	7th week N=6	8th week N=6
CN-Gr	113.55^{A}	110.22^{A}	112.23^{A}	108.37^{A}	112.04^{A}	115.12^{A}	111.82^{A}	112.11^{A}

AOLE-Gr	110.75^{A}	109.12^{A}	108.92^{A}	106.45^{A}	109.33^{A}	113.95^{A}	112.55^{A}	113.85^{A}
ALX-Gr	172.65^{B}	161.34^{B}	145.80^{B}	135.52^{B}	170.33^{B}	177.53^{B}	193.20^{B}	220.12^{B}
ALX+AOLE-Gr	169.64^{B}	159.23^{B}	133.22^{C}	125.65^{C}	171.45^{B}	162.73^{C}	148.32^{C}	135.11^{C}

(CN-Gr), (AOLE-Gr), (ALX-Gr) and (ALX+AOLE-Gr) represent the negative control, AOLE (Aqueous Olive Leaves Extract) treated, Alloxan treated and alloxan + AOLE treated groups respectively.

The results were expressed as mean ± SEM. Each value not sharing a common letter superscript is significantly different (P<0.05). The sample size was 12 rats during the first 4 weeks and declined to 6 rats during the rest period of the study.

Table 2. Serum level of the hepatic enzymes (Alkaline Phosphatase (ALP), Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT)) in the different groups during the entire period of the study

Groups	ALP (IU/L)		AST (IU/L)		ALT (IU/L)	
	28 Days N=12	56 Days N=6	28 Days N=6	56 Days N=6	28 Days N=6	56 Days N=6
CN-Gr	118.75 ± 4.73^{A}	117.29 ± 4.33^{A}	121.95 ± 4.16^{A}	123.55 ± 5.10^{A}	28.65 ± 1.90^{A}	29.66 ± 1.94^{A}
AOLE-Gr	115.21±3.55^{A}	116.46±2.99^{A}	119.22±3.50^{A}	121.65±2.90^{A}	26.90±1.05^{A}	27.33±1.10^{A}
ALX-Gr	190.17±5.85^{B}	320.10±8.88^{B}	146.13±2.75^{B}	190.33±5.22^{B}	45.98±1.33^{B}	110.25±2.67^{B}
AOLE+ALX Gr	188.91±4.32^{B}	315.65±5.90^{B}	129.33±3.22^{C}	143.31±3.55^{C}	36.45±2.11^{C}	62.53±2.30^{C}

(CN-Gr), (AOLE-Gr), (ALX-Gr) and (ALX+AOLE-Gr) represent the control, AOLE (Aqueous Olive Leaves Extract) treated, Alloxan treated and alloxan + AOLE treated groups respectively.

The results were expressed as mean ± SEM. Each value not sharing a common letter superscript is significantly different (*P<0.05*). The sample size was 6 animals on days 28 and 56.

OXIDATIVE STRESS MARKERS AND ANTIOXIDANT ENZYMES

The results revealed a prominent and a significant and progressive increase in the oxidative stress markers along with a decline in the antioxidant enzymes in the rats treated with alloxan (*P<0.05*). Treatment with AOLE did not produce that much change in the AOLE-Gr as compared to that of the CN-Gr. but it hindered the alloxan induced deterioration in the oxidative stress as seen in the results of the ALX-AOLE-Gr (Tables 3 &4).

Table 3:- Serum level of the oxidative stress markers (Malondialdehyde (MDA) Concentration, Total Oxidative Stress Capacity (TOC), Total Antioxidant capacity (TAC) and Oxidative Stress Index (OSI) for all the groups during the experimental period.

	At 28 day (n=6)				At 56 day (n=6)			
	MDA nmol/ml.	TOC (μmol H_2O_2 Eq/L)	TAC (TroloxEq μmol/L)	OSI (TOC\TAC)	MDA nmol/ml.	TOC (μmol H_2O_2 Eq/L)	TAC (TroloxEq μmol/L)	OSI (TOC\TAC)
CN-Gr	15.44±1.11^{A}	11.2±3.32^{A}	1.41±0.26^{A}	0.78±0.80^{A}	16.12±1.30^{A}	12.8±3.67^{A}	1.44±0.21^{A}	0.84±0.75^{A}
AOLE-Gr	14.95±1.44^{A}	10.5±2.88^{A}	1.70±0.45^{B}	0.62±0.67^{B}	15.70±1.05^{A}	13.5±2.94^{A}	1.91±0.51^{B}	0.70±0.66^{B}
ALX-Gr	25.25±2.35^{B}	17.4±3.64^{B}	1.01±0.34^{C}	1.70±0.65^{C}	35.19±2.10^{B}	21.4±3.60^{B}	0.85±0.22^{C}	2.51±0.95^{C}
AOLE+ALX Gr	20.44±1.58^{C}	11.3±3.15^{A}	1.39±0.96^{A}	0.91±0.77^{D}	22.37±1.42^{C}	16.3±3.05^{C}	1.42±0.30^{A}	1.14±0.84^{D}

(CN-Gr), (AOLE-Gr), (ALX-Gr) and (ALX+AOLE-Gr) represent the control, AOLE (Aqueous Olive Leaves Extract) treated, Alloxan treated and alloxan + AOLE treated groups respectively.

The results were expressed as mean ± SEM. Each value not sharing a common letter superscript is significantly different *(P<0.05)*. The sample size 6 animals on days 28 and 56.

Table 4:- Serum levels of the antioxidant enzymes (Glutathione peroxidase (GSH-Px), Super oxide dismutase (SOD) and Catalase (CAT)) together with the RBCs hemolysate reduced glutathione (r-GSH) concentrations on days 28 and 56 of the experimental period.

	At 28 day				At 56 day			
	r-GSH (μmol/ml)	GSH-Px (U/ml)	SOD (U/ml)	CAT (U/ml)	r-GSH (μmol/ml)	GSH-Px (U/ml)	SOD (U/ml)	CAT (U/ml)

CN-Gr	21.07±1.10^{A}	114.4±5.6^{A}	85.53±2.5 A	3.09±0.13 A	22.05±1.15^{A}	115.5±5.1 A	87.83± 2.66^{A}	2.99± 0.16^{A}
AOLE-Gr	21.20±1.13^{A}	115.7±6.2^{A}	86.22±2.8 A	3.10±0.16 A	22.25±1.18^{A}	117.4±6.5 A	88.13± 2.13^{A}	3.01 ± 0.11^{A}
ALX-Gr	19.98±1.08^{A}	110.9±4.4^{B}	67.83±1.5 B	2.22±0.18 B	11.23±1.08^{B}	74.9±5.4^{B}	44.33± 2.33^{B}	1.01± 0.18^{B}
AOLE+ALX Gr	20.25±1.75^{A}	114.8±3.3^{A}	78.66±2.4 C	2.19±0.13 B	15.74±1.01^{C}	106.9±3.3 C	69.45± 2.20^{C}	2.01 ± 0.18^{C}

(CN-Gr), (AOLE-Gr), (ALX-Gr) and (ALX+AOLE-Gr) represent the control, AOLE (Aqueous Olive Leaves Extract) treated, Alloxan treated and alloxan + AOLE treated groups respectively. r-GSH, GSH-Px, SOD and CAT refer to the blood hemolysate reduced glutathione level, serum level of the glutathione peroxidase, superoxide dismutase and catalase respectively.

The results were expressed as mean ± SEM. Each value not sharing a common letter superscript is significantly different (*P<0.05*). The sample size was 12 on day 28 and 6 on day 56)

Table 5: - Lipid profile (Triglyceride (T.G), Total cholesterol, Low density lipoprotein (LDL) and High-Density Lipoprotein (HDL)) on days 28 and 56 of the experiment periods.

	At 28 day				At 56 day			
	Triglyceride (TG) mg/dl	Total cholesterol mg/dl	LDL-cholesterol mg/dl	HDL-cholesterol mg/dl	Triglyceride (TG) mg/dl	Total cholesterol mg/dl	LDL-cholesterol mg/dl	HDL-cholesterol mg/dl
CN-Gr	80.9±1.90^{A}	70.31±2.10^{A}	20.77±0.88^{A}	41.18±1.19^{A}	79.9±1.63^{A}	73.55±2.61^{A}	22.37±0.85^{A}	42.15±1.13^{A}
AOLE-Gr	79.4±1.42^{A}	69.77±1.91^{A}	19.79±0.67^{A}	46.16±1.21^{B}	78.33±1.11^{A}	72.11±2.43^{A}	21.45±1.01^{A}	56.72±2.02^{B}
ALX-Gr	130.8±4.35^{B}	150.38±2.41 B	55.58±1.77^{B}	29.21±1.05^{C}	199.12±1.24^{B}	201.10±5.71 B	89.18±1.77^{B}	24.55±1.14^{C}
AOLE+ ALX Gr	87.93±1.66^{C}	103.51±2.86 C	38.91±1.03^{C}	35.22±1.17^{D}	98.9±2.47^{C}	145.33±3.88 C	44.71±1.63^{C}	32.12±1.24^{D}

(CN-Gr), (AOLE-Gr), (ALX-Gr) and (ALX+AOLE-Gr) represent the control, AOLE (Aqueous Olive Leaves Extract) treated, Alloxan treated and alloxan + AOLE treated groups respectively.

The results were expressed as mean ± SEM. Each value not sharing a common letter superscript is significantly different (*P<0.05*). The sample size was 12 on day 28 and 6on day 56).

HISTOPATHOLOGICAL FINDINGS

Pancreas histopathological findings

In the non-alloxan treated groups the Islets of Langerhans appeared with normal architecture. The endocrine cells in these islets are arranged as anastomosing cords around abundant capillaries. These islets display a central core of β-cells surrounded by a peripheral, largely continuous sheath of non-β-cell cells. Peripherally, the Islet cells with small, dark nuclei are (alpha-cells), however, those with light and large nuclei are (β-cells). The islet cells are seen embedded within the acinar cells and surrounded by a fine connective tissue capsule (Figure-1).

In the pancreas of the rats; injected with diabetogenic alloxan and at the end of the first 4 weeks, some Islets of Langerhans were irregular with projections into the exocrine part of the pancreas as a result of hyperplasia/hypertrophy. Occasionally, there was an infiltration of inflammatory cells together with pronounced degenerative changes like vacuolations due to hydropic degeneration (water droplets with hazy boundaries) \and or fatty steatosis (variable size well delineated boundaries of intracellular fat droplets) of the cellular mass of the Islet of Langerhans (Figure- 2).

Meanwhile, at the end of the 8 weeks, the pancreas of the remaining rats; that had been injected with 2nd dose of alloxan, showed prominence atrophic islets, extensive cellular vacuolations and β- cells depletion or reduction (Figure- 3).

Furthermore, a frank regenerative changes in the pancreas of AOLE treated and the alloxan injected group (ALX-AOLE gr) were noticed as shown by semi-characteristic appearance of most islets with few reversible degenerative features and significant regenerative changes (Figure- 4).

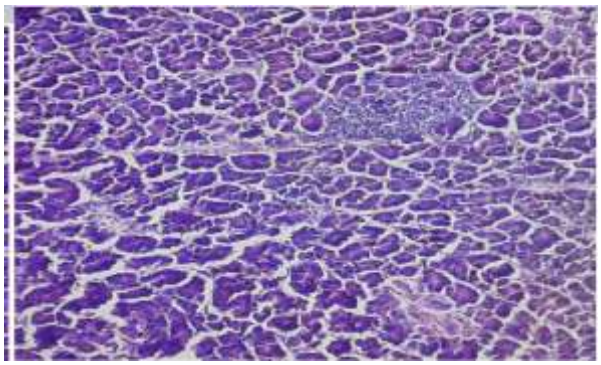

Figure-1: Histological view of a pancreas from both control and most rats from olive leaves aqueous extract groups. The islet cells are seen embedded within the acinar cells and surrounded by a fine capsule. The normal pancreatic islet architecture appearance showing a central core of β-cells enveloped by a peripheral, largely continuous mantle of non- β-cells endocrine cells. Islet

cells with small, dark nuclei on the periphery (α-cells), or **with light and large nuclei** (β-cells). (H&E stain 20X)

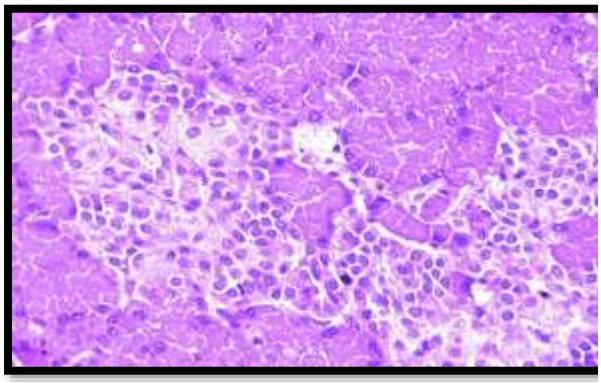

Figure-2: Pancreas of rat from alloxan treated group at the end of the first 4 weeks, a hypertrophic and hyperplastic islet appears with irregular outline and projections into the exocrine part of the pancreas together with pronounced cellular degenerative findings. (H&E stain 40X).

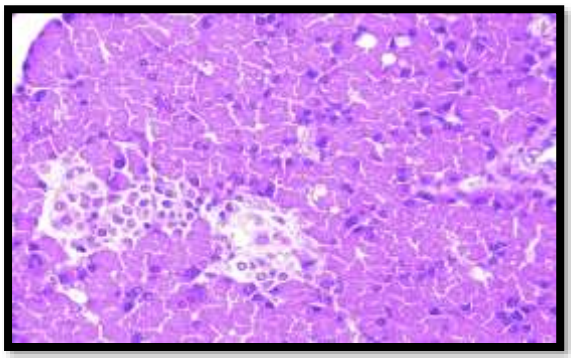

Figure-3: Pancreas of rat from alloxan treated group at the end of the 8 weeks. Prominence of an atrophic appearance islets that are poorly delineated, with **extensive cellular vacuolation and a severe reduction in β-cells.** (H&E stain 20X)

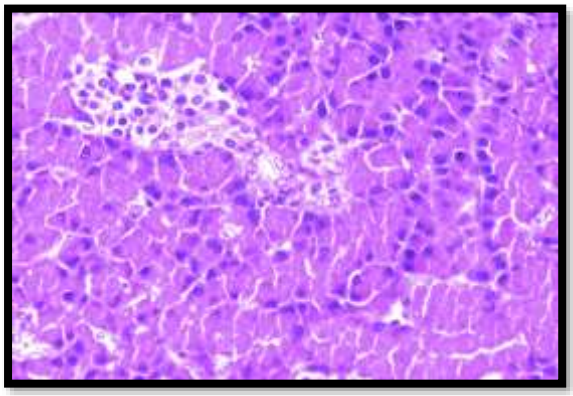

Figure-4: Reversal of islet degeneration in diabetic rat maintained on normal rat diet and co-administrated orally with aqueous olive leaves extract. Treated rats are illustrating semi-characteristic appearance of normal islets with significant regenerative features. (H&E stain 20X)

LIVER HISTOPATHOLOGICAL FINDINGS

The liver of the rats from both of the control and the aqueous olive leaves extract groups revealed normal tissue architecture and appeared to be divided into the classical hepatic lobules; each was formed of cords of hepatocytes radiating from the central vein to the periphery of the lobule. The hepatic cords were separated by narrow blood sinusoidal spaces (Figure-5).

Liver of alloxan injected rats at the end of the 4th week exhibits reversible degenerative changes ranged between hydropic\ and or fatty changes with few and individual necrotic hepatocytes (Figure-6).

The histopathologic examination of liver of alloxan injected rats at the end of the 8th week showed severe centrilobular coagulative necrosis of the most hepatocytes, prominence of Kupffer cells and some degree of pericentral glycogen depletion (Figure-7).

Examination of liver tissue of alloxan injected rats that treated with aqueous olive leaves extract at the end of experimental period indicated that the hepatic lobules appeared more or less like the control group with mild reversible regenerative changes and active looking hepatocytes (Figure-8).

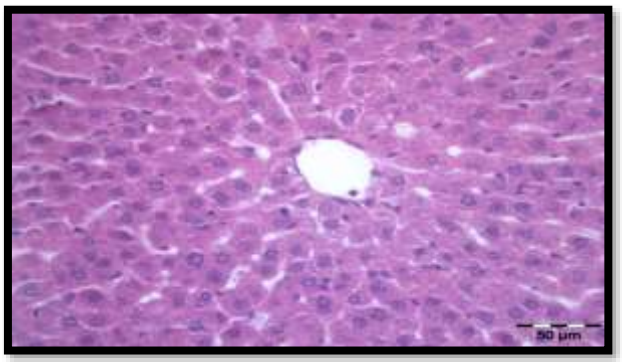

Figure-5: liver of rats from both control and aqueous olive leaves extract groups revealed normal tissue architecture of hepatocytic cords radiating from the central vein to the periphery of the lobule. The hepatic cords were separated by narrow blood sinusoidal spaces (H&E 20X).

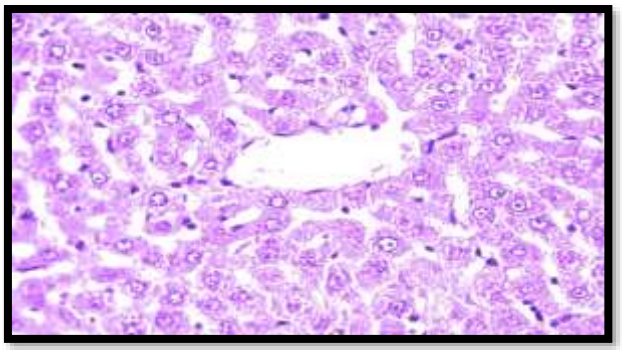

Figure-7: Liver of rat from alloxan injected group. Centrilobular coagulative necrosis of the most hepatocytes and some degree of pericentral glycogen depletion (H&E 20 X).

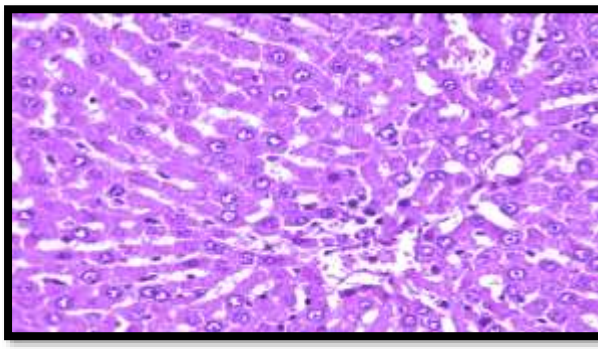

Figure-8: liver of rat from alloxan injected- aqueous olive leaves extract treated group reveals almost active looking regenerative hepatocytes with frank hepatic architecture (H&E 20X).

DISCUSSION

Alloxan ($C_4H_2N_2O_4$) is a toxic glucose analogues is well known to initiate diabetes mellitus in various rodent models [21] .Different dosing protocols were used for the induction of the alloxan induced diabetes. Most of the studies implemented the single dosing protocol using alloxan at 150-200 mg/kg (body weight) while smaller doses were used in the multiple dosing protocols. In this study, 75 mg/kg of alloxan were given in two doses leaving a period of 28 days between them. The dosing protocol has an impact on the mechanism of the induced diabetes as it was found previously that the pathogeneses of both type I and type II may appear with the alloxan. Type I is more predominant with the former while type II is more with the later. [22].

The pathogenesis of its diabetogenicity relies on its ability to generate reactive oxygen species in a cyclic redox reaction in the presence of thiols rich glutathione. The cycle includes a continuous swap of the alloxan between its original form and its metabolite (Dialuric acid which is its final reduction product). Upon its autooxidation can generate superoxide radicals, hydrogen peroxide and hydroxyl radicals. Obviously, these hydroxyl radicals were blamed for the necrosis and or apoptosis of the beta cells of Islets of Langerhans[7] .

The current study was designed to create a diabetes model characterized by the incidence of the cumulative deleterious effects of two separated sub-diabetogenic doses of alloxan. Furthermore, it aimed to find the prophylactic impact of adding olive leaves extract at a concentration of 5% to the drinking water to explore its ameliorative potential against alloxan induced diabetes mellitus. Previous studies revealed that this model has a stronger impact on the insulin sensitivity in comparison with the single dose model and may lead to creation of a combined effect of insulin deficiency and reduction of the insulin sensitivity [23]. This model is advantageous in screening the antidiabetogenic effects of drugs as it shows their aptitude to enhance insulin insensitivity or release. Both targets are crucial for the treatment of diabetes mellitus

In this study, a biphasic fasting hyperglycemic response were noticed after the 1st and 2nd alloxan injections. Frankly, the hyperglycemia during the first 4 weeks was of regressive type in contrast to the progressive type after the second dose of alloxan injection. Despite these two types of hyperglycemia it is well known that diabetes does not occur without progressive β-cell dysfunction.

Lots of studies have pointed out to the multitude of health and life-extending benefits of the olive leaves extract. It is endowed with plenty of phytochemicals with wide range of prophylactic and therapeutic effects against obesity, hypertension and hyperlipidemia, [11] [24] [25] & [26]. Oleuropein; a polyphenolic monoterpenoid glycoside, constitutes the main content of olive and is responsible for the bitter taste for olive. It is found in the seeds, flesh and leaves of the olive [25] [27] & [28]. Furthermore, the extract is rich in hydroxy tyrosine which is a polyphenol with a potent antioxidant effect [29] & [30]. This study aimed at determining its beneficial effect against the progression of the two-dose model of alloxan mediated diabetes mellitus

The regressive type of the hyperglycemia; that was noticed during the first 28 days and before the second dose of alloxan, can be ascribed to the ability of the regenerative system to restore the glucose homeostasis after the first injection of alloxan. Meanwhile, the progressive profile that was noticed during the second month indicates the incapability of the regenerative system to restore the homeostasis and to counteract the flow of free radicals. this observation can be linked to results of the antioxidant enzyme profile on days 28 and 56. The profile showed a mild decline in the glutathione peroxidase, superoxide dismutase and catalase on day 28 while the decline was stronger on day 56. These enzymes are crucial for prevention of the oxidative stress which plays an important role in progression of the alloxan induced diabetes. Furthermore, the longer period of exposure to the oxidative stress can be set as another enhancement factor for the deterioration of the glucose homeostasis.

The incidence of the mild hyperglycemia during the first 4 weeks of the experimental period was associated with increase in fatty acid flux and led to enhancement of the substrate delivery to mitochondria, leading to enhanced superoxide production that results in mitochondrial dysfunction [31]. As a compensatory mechanism, most Islets of Langerhansduring this period underwent hypertrophy and or hyperplasia due to hyperphagia, insulin resistance, or most commonly, a combination of these two criteria.

The histology study revealed a compensatory mechanism in most Islets of Langerhansduring this period as they underwent hypertrophy and or hyperplasia. This can be ascribed to the incidence of hyperphagia, insulin resistance, or most commonly, a combination of these two criteria.

Previous studies showed that during diabetes or metabolic syndrome, the source of energy in different cells shift from carbohydrates to fats. The overflow of more fatty acids into the mitochondria results in generation of more free radicals as fatty acids are metabolized by the process of β- oxidation. Firstly, this stress triggers the cells to undergo the compensatory mechanisms as free radicals can act as signaling molecules for some biological processes but then at their excessive level, they turn deleterious and lead to cellular degeneration and necrosis [32].

An advance-stage of diabetes was developed after 56 day suggesting the incidence of a time and dose dependent necrotic or apoptotic lesions of the pancreatic beta cells leading to insulinopenia and subsequently to hyperglycemia.

Apparently, the alloxan treated-rats at this time showed marked reduction in the β-cells with atrophic appearance of islet architecture together with fat deposition as a sequela of the pronounced dyslipidemia. Unfortunately, the deposition of intra-islet amyloid that described by one of the previous studies (Elizabeth, *et. al.,* 2019) [33] has not been seen in current study. The regenerative capacity in both liver and pancreas treated with olive leaves extract may attributed to the evoking of intermitotic activity of these organs by the antioxidative capacity and restoring of redox system in favor of reduction status done by this plant. The discrepancy between day 28 and 58 can be ascribed to the longer period of exposure to the oxidative stress and the observed decline in the antioxidant enzymes that protect against the flow of free radicals.

The excess superoxide production as an intrinsic property of mitochondria of insulin-sensitive tissues does not result from conditions mimicking the pathophysiology of diabetes. In the current study, it is important to distinguish between the concepts of antioxidant activity and capacity. while, antioxidant activity refers to the rate constant of a reaction between an antioxidant and an oxidant. The antioxidant capacity is a measure of the amount of a certain free radical captured by an antioxidant sample [25].

Besides, the condition was associated with a prominent decline in the liver function. The decline was higher after the second dose as compared with that after the first. This stronger deterioration can be ascribed also to the longer period of exposure to the oxidative stress along with the prominent dysfunction of the endogenous antioxidant system that is represented by the antioxidant enzymes. Although the oxidative stress parameters on day 56 were slightly less than that on day 28 (Table 5) but this persistent decline suggests a longer period of exposure to the free radicals which may enhance the dysfunction in different organs.

The liver function profile showed a prominent increase in the serum level of alkaline phosphatase enzyme in the ALX-Gr. It was increased by about 1.7 times on day 28 and 2.6 time on day 56. This increase suggests a deterioration in the integrity of the biliary hepatocytes whose membrane microvilli is endowed with a plenitude of this enzyme. Alkaline phosphatase helps in breakdown of the organic phosphates and facilitates their uptake by cells. It is found abundantly in bone, placenta, biliary hepatocytes and renal tubules. Since, it is a nonspecific indicators for the liver dysfunction, the study does not exclude the presence of other dysfunctions that might have enhanced its over-release into the plasma [34]. The results suggest the incidence of an intrinsic damage in the plasma membrane of the cells that bear this enzyme that biliary hepatocytes constitute an important type of such cells

Furthermore, the study also showed a significant increase in the plasma levels of the hepatic enzymes; ALT and AST. Both enzymes are important for transamination of the amino acids and creation of metabolites important for the citric acid cycle. Their presence is crucial for the progression of different metabolic reactions. AST is found abundantly inside the mitochondria while ALT is found more in the cytosol. In most of the cases of the liver dysfunction, there is a higher increase in the level of ALT as compared to AST (lower AST/ALT ratio) [35]. With smaller ratios, the muscle rather the hepatic dysfunction should be considered. In this study, the ratio was about 4.2 in the control rats but it declined to about 3.4 after 28 days of the first dose of alloxan and showed a further decline on day 56 of the study to about 1.7. This progressive decline in the ratio suggests the incidence of the hepatic dysfunction and the liver has experienced a prominent loss of the integrity of its plasma membrane that led to leakage of the hepatic enzymes into the plasma. This observation can be linked to the higher oxidative stress that was noticed on days 28 and 56. The higher dysfunction on day 56 can also be ascribed to the longer exposure period to the oxidative stress.

In the current study, after 28 days of the first dose, alloxan triggered excessive release of free radicals due to their excessive generation in β cells of islets of langerhaz and due to the incidence of diabetes [36], which may be sublethal yet and remained diabetogenic. Steatosis was the prominent histopathological change in the livers of alloxan treated rats [36]. The accumulation of fat droplets displaced the cytoplasm of the liver which occurred in two forms; (microvesicular steatosis) which appear after day 28 that then progressed to the macrovesicular on day 56 wherein multiple small fat droplets coalesce to form one large well delineated droplet of neutral fat. The droplet was sufficient enough to distort and push the nucleus aside.

Histologically, the fatty steatosis that was seen as a cardinal degenerative change of alloxan injected rats is induced by the hypertriglyceridemia that the alloxan treated rats had experienced [37]. Beside steatosis, hydropic degeneration with wide accumulation of water vacuoles with hazy boundaries was seen as well. It is a reversible change that hydropic degeneration was developed due to the partial cessation of energy production as a consequence of the oxidative stress mediated mild dysfunction of mitochondria.

The study also showed a prominent hyperlipidemia characterized by hypertriglyceridemia and hypercholestremia after the alloxan treatment. The deterioration was stronger on day 56 as compared with that on day 28. The results of the lipid profile showed that each of the triglyceride, cholesterol and LDL were increased by (1.6 and 2.55), (2.1 & 2.7) & (2.75 & 4.5) times as compared to that of the negative control on days 28 and 56 respectively. This progressive increment can be ascribed to the deterioration in the glucose homeostasis and to the persistent decline in the oxidative stress. The longer period of exposure to the oxidative stress might have played a role in the stronger deterioration in the lipid profile. The lipid profile study showed also a decrease in the serum level of HDL; the beneficial form of cholesterol. The decline was more prominent for the LDL as it was seen previously that LDL is highly affected by the oxidative stress as it transforms into the oxidized form LDLox which has a high propensity to deposit in the blood vessels and induce atheroma.

Co-administration of the oleuropein rich olive leaves extract hindered the progression of the alloxan induced metabolic changes. The most important regenerative effect was seen on the serum levels of the oxidative stress parameters. The extract could have significantly reduced the serum level of MDA in comparison with the positive control but the values were still higher than that of the negative control. This suggests that the extract could have produced a partial restoration of the body defensive system against the deterioration in the oxidative stress. On day 28, it succeeded

to restore the TOC (total antioxidant capacity) to a level comparable to that of the negative control but it failed to do that on day 56 as the regenerative antioxidant system was still active during the first month but its ability was more compromised during the latter period. The extract showed a capacity to restore the antioxidant enzymes and the restorative capacity was stronger on day 56. Alloxan produced a milder effect after the first dose as its impact was somehow regressive so the impact of the extract was not clearly visible as that on day 56 wherein the subjects of the positive control group experienced a stronger decline in antioxidant capacity.

The impact of the extract on the oxidative stress was reflected on the other metabolic and histology parameters. The study showed a success of the extract to hinder the progression of hyperglycemia, hyperlipidemia as well as the liver dysfunction. Maged *et al* ., (2019) detected a considerable drug herb interaction when the extract is co-administered with glyburide via modulation of insulin receptor , glucose transporter 2 and peroxisome proliferator-activated receptor-a genes expression in the liver of diabetic rats [38].

The antioxidant effect of the extract might have played a role in the maintenance of the integrity of the plasma membrane of the hepatocytes [39]. This effect was seen obviously in the results of the serum levels of each of AST and the ALT enzymes. It succeeded to restore the AST/ALT ratio to 2.5. Meanwhile, it was 1.7 in the positive control group. This indicates that the efflux of ALT from the hepatocytes was decreased and the integrity of the hepatocyte's plasma membranes was improved. On the other hand, this impact was not seen for the alkaline phosphastase enzyme and the results shows a persistent increment in the serum level of the enzyme. This can be ascribed to the inability of the extract to counteract the damage in the biliary hepatocytes or due to the longer half-life of the enzyme that gives a persistent elevation in its level.

Previous studies highlighted the antihyperlipidemic effect of the olive leaves extract and this was seen in this study as well. The highest impact was seen on day 56 and this can be ascribed to the capacity of the extract to decrease the overall oxidative stress induced by alloxan. Not merely does the antioxidant mechanism be involved. The extract might have mediated that through a non-antioxidant mechanism. Previous studies revealed that phytochemicals might act as Janus molecules that they can interfere with functions of a multitude of cellular enzymes resulting in changing their functional activities [40]. It has been suggested that such molecules can interfere with the cascade pathway of lipids biosynthesis resulting in mediation of their antihyperlipidemic effect.

CONCLUSION

Overall, the study suggests the presence of a detrimental effect of the alloxan induced diabetes against the integrity and the functional characteristics of liver and pancreas. The alloxan induced changes in the pancreas are both time and dose dependent that it a reversible degenerative changes were evoked by the first dose meanwhile an irreversible coagulative necrosis was obtained after the second dose. Future studies are recommended to determine the type of diabetes mellites (type I or II) along with different doses in the multiple dosing protocols of alloxan induced diabetes.

Phyto-chemicals of the olive leaves extract can counteract this detrimental effect and can be suggested as safe and prophylactic dietary supplements against this dilemma. The study also showed that the oxidoreductive mediated hyperglycemia leads to hepatic and pancreatic cellular degenerative changes (steatosis and hydrops) that may augment the incidence of hyperglycemia. Furthermore, there is a close cross talk between the augmentation of the oxidative stress and the incidence of the diabetes induced metabolic changes and this crosstalk indeed has an impact on the functional characters of the liver and the pancreas. The study highlights the impact of the prolonged oxidative stress on progression of the histopathological lesions in liver and pancreas.

The study also suggests implementation of the olive leaves extract as potent antioxidative stress adjuvant therapy along with diabetes management. Furthermore, the pathophysiologic aspects of the inhibitory role of olive leaves extract against oxidoreductive stress necessitate more advanced studies at the molecular and ultra-structural levels.

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CONFLICT OF INTEREST

All the authors confirm that there is no any conflict of interest regarding publication of this manuscript.

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