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Original article

Olive leaves extract attenuates type II diabetes mellitus-induced testicular damage in rats: Molecular and biochemical study

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ABSTRACT

Diabetes mellitus (DM) has emerged as a public healthcare problem. Sustained hyperglycemia has been linked with many complications including impaired male fertility Olive tree (*Olea europaea* L.) leaves have been extensively used in traditional remedies worldwide to control blood glucose level in DM. In this study, the beneficial role of olive leaves extract (OLE) was investigated to combat diabetes-induced adverse effect on testicular tissues. Thirty male Wistar rats were divided into 5 equal groups: normal control group, streptozotocin (STZ)-diabetic group and diabetic groups which were given glibenclamide (GLB) or OLE at 250 and 500 mg/kg for 9 weeks to investigate the efficiency of olive leaves extract (OLE) in reducing the deleterious effect of diabetes on the reproductive system of male rats. Rats were checked for serum glucose, insulin, testosterone and gonadotropins. Also, testicular antioxidants, epididymal sperm characteristics and testicular histopathology were assessed. Expression of the testicular steroidogenic enzymes, cholesterol side-chain cleavage enzyme (P450 scc) and 17β-hydroxysteroid dehydrogenase (17β-HSD) was examined. Moreover, androgen receptor and proliferating cell nuclear antigen (PCNA) protein immunohistochemistry were assessed in testes. STZ-induced diabetes significantly increased serum glucose. However, STZ significantly decreased serum levels of insulin, testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Marked reductions in testicular antioxidants with elevated malondialdehyde (MDA) parallel with deterioration of the testicular histoarchitecture and epididymal sperm characteristics were recorded. Administration of GLB or OLE (250 and 500 mg/kg) resulted in a significant recovery of the above mentioned parameters in STZ-diabetic rats. Interestingly, OLE shows greater glycemic improvement and testicular protection than GLB with the highest percentage protection exhibited by the OLE high dose. Furthermore, OLE significantly induced testicular steroidogenesis in diabetic rat as evidenced by elevated P450 scc and 17β-HSD mRNA expression. The study proves that OLE possesses a potential protective role against diabetes-induced reproductive disorders, which may be due to its antioxidant activity and its ability to normalize testicular steroidogenesis.

1. Introduction

DM is a common metabolic disorder characterized by hyperglycemia. Oxidative stress associated with hyperglycemia plays a key role in the onset and progression of diabetes and diabetic complications including neuropathy, nephropathy retinopathy and cardiovascular diseases (Melendez-Ramirez et al., 2010). In addition, DM is associated with reproductive impairment and infertility in

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both sex. DM affects male reproductive functions at multiple levels due to the impairment of the endocrine control of spermatogenesis, steroidogenesis and sperm maturation. Up to 90% of the diabetic patients suffer from disturbances in sexual function including decreases in libido, impotence and infertility (Feng et al., 2001). Currently, insulin and oral hypoglycemic agents are commonly used for DM. GLB is one of the most frequently prescribed hypoglycemic agents belonging to sulfonamides. GLB reduces blood glucose by stimulating insulin secretion and reducing hepatic glucose production (Rendell, 2004). Although, several options of hypoglycemic medications are available to provide long term management of diabetic conditions and consequently ameliorate the sexual dysfunction in part, still the improvement is only marginal.

About one third of type 2 diabetes patients actively use alternative medicine to manage their disease (Yeh et al., 2003). Herbal preparations are gaining popularity in the control of diabetes and its complications. The leaves of the olive plant (*Olea europaea* L.), family: *Oleaceae*, have been used for centuries in folk medicine to treat diabetes (Komaki et al., 2003). Previous reports on OLE have demonstrated hypoglycemic, hypotensive, antimicrobial and antioxidant activities (Wainstein et al., 2012; Sarbishegi et al., 2017).

Although big effort has been made for controlling the blood glucose, the complication of diabetes is still the major reason to cause organ dysfunction and death. The present study was designed to evaluate the possible protective effects of OLE against the deleterious effect of diabetes on the reproductive system of male rats.

2. Materials and methods

2.1. Plant material and extraction

Leaves of *Olea europaea* L. subsp. cuspidata (Wall. ex G. Don) Cif. F. Oleaceae was purchased from the local market in Riyadh city, Saudi Arabia. The plants were identified by Dr. Mohammad Atiqur Rahman, taxonomist of the Medicinal, Aromatic and Poisonous Plants Research Center (MAPPRC), College of Pharmacy, King Saud University, Riyadh, Saudi Arabia by comparison with Voucher specimen at the herbarium of this center. The ground leaves (1000 g) were extracted to exhaustion by percolation at room temperature with 90% ethanol (15 L), and the extract was evaporated under reduced pressure to leave 160.82 g of the total extract.

2.2. LC-MS study of the extract

ESI-MS in both positive and negative ion acquisition mode was carried out on a XEVO TQD triple quadruple mass spectrometer (Waters Corporation, Milford, MA01757, USA). LC preformed on ACQUITY UPLC - BEH C18 1.7 μm-2.1 × 50 mm Column at flow rate of 0.2 mL/min. Mobile phases started with 90% water containing 0.1% formic acid/10% methanol containing 0.1% formic acid in gradient system ended with 10% water containing 0.1% formic acid/90% methanol containing 0.1% formic acid. Run time was 32 min. Mobile phase and sample solution were prepared by filtering using 0.2 μm filter membrane disc and degassed by sonication before injection. The sample (100 μg/mL) solution was prepared using high performance liquid chromatography (HPLC) analytical grade MeOH and volume of 10 μL was injected into the UPLC instrument. The parameters for analysis were carried out using both positive and negative ion mode as follows: source temperature 150 °C, cone voltage 30 eV, capillary voltage 3 kV, desolvation temperature 440 °C, cone gas flow 50 L/h, and desolvation gas flow 900 L/h. Mass spectra were detected in the ESI positive and negative ion mode between m/z 100–1000. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively identified by comparing its mass spectrum with reported data for known components of olive extract.

2.3. Animals

Male Wistar rats weighing between 200 g and 220 g were obtained from the Animal House Colony at the National Research Centre (NRC), Egypt. The rats were acclimated to the laboratory environment for 7 days prior to the study. All animals were housed under standard conditions at (26 ± 2) °C, 12 h day and night cycle, and with food and water ad libitum. Animal procedures were performed according to the protocol approved by the NRC Ethics Committee (approval number: MREC-17-142), the Institutional Animal Care and Use Committee at Cairo University (approval number: CU-II-F-14-18), and following the guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.4. Acute toxicity study

Acute toxicity study of OLE was performed according to Organization for Economic Co-operation and Development-423 guidelines (OECD, 2001). The rats were kept on fasting overnight, being provided only water prior to oral dosing of OLE at different dose levels (0.1, 1, 3 and 5 g/kg of body weight). Rats were allowed food and water *ad libitum* and were kept under observation for symptoms of toxicity and/or mortalities during the first 0.5 h and periodically during 24 h, then daily for a total of 14 days.

2.5. Induction of DM

DM was induced by a single intraperitoneal (ip) injection of freshly prepared STZ (Sigma-Aldrich Corp, St. Louis, MO, USA) in ice-cold citrate buffer (0.1 M, pH 4.5) at a dose of 45 mg/kg (Mestry et al., 2017). Seventy-two h after STZ injection, DM induction was confirmed by determination of FBG levels in blood samples collected from the tail vein using a blood glucose meter (Accu-Check Performa, Roche Diagnostic, Germany). The FBG levels over than 200 mg/dL were considered as diabetic model rats.

2.6. Experimental design

Rats were weighed and divided into five groups (n = 6). The first group received 3% Tween 80 (5 mL/kg) and served as normal control (NC). The second group was STZ-diabetic control (DC). The third group was diabetic rats, supplemented orally with GLB at 0.6 mg/kg. The fourth and fifth groups were diabetic rats, supplemented orally with OLE at 250 and 500 mg/kg body weight, respectively. The standard (GLB) and the tested extract were suspended in 3% Tween 80 and administered once daily using gastric gavage for 9 weeks.

2.7. Biochemical estimation

At the end of weeks 0, 3, 6 and 9 of the treatment period, blood samples were withdrawn from the overnight fasted rats by retro orbital puncture into sampling tubes. Blood samples were centrifuged at 3500 rpm for 15 min to separate serum. The FBG levels in serum were measured using the commercially available kits (Spinreact, Spain) while serum insulin levels were determined by using ELISA kits (Cobas, Belgium) according to the manufacturer's instructions. In addition, serum levels of testosterone and gonadotropins (FSH and LH) were estimated at the end of the experiment using ELISA kits (Cobas, Belgium) according to manufacturer instructions. After 9 weeks of treatment, rats were sacrificed with

an ip overdose of pentobarbital sodium and blood samples were collected in tubes containing EDTA as anticoagulant. Total hemoglobin (Hb) and glycosylated hemoglobin (HbA1c) were estimated in whole blood using the commercially available kits (QCA, Spain).

2.8. Assessment of testicular oxidative stress markers

Specimens from testis were homogenized (Heidolph Diax 900 homogenizer, Germany) in ice-cold phosphate buffer solution at 4 °C and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatants were collected and stored at −80 °C until analysis. Activities of the antioxidant enzymes as SOD, GPx and CAT, and levels of GSH and MDA in testicular homogenates were estimated using the corresponding assay kits purchased from Biodiagnostic (Egypt) according to the standard procedures in the manufacturer's instructions.

2.9. Assessment of epididymal sperm characteristics

2.9.1. Sperm count

For determination of sperm count, semen was sucked from the cauda epididymis using a red blood pipette to 0.5 mark. Semen was diluted using normal saline to 101 mark. One drop of the diluted semen fluid was added to the Neubauer counting chamber, counted microscopically (Kruss MBL2000, A. Kruss Optonic, Germany) using the magnification of ×40 and results were expressed as 10^6 cells/mL (Sönmez et al., 2005; Tatli-Çankaya et al., 2014).

2.9.2. Sperm motility

The cauda epididymis on one side was exposed and incised with a surgical blade. The epididymal semen was obtained by squeezing cauda epididymis in a sterile clean watch glass. Semen was then diluted 10 times with 2.9% sodium citrate dehydrate solution. After thorough mixing, a drop of dilute semen was transferred on to a cover glass with the help of Pasteur pipette. The cover glass was inverted over the cavity slide to obtain a hanging drop. The edges of the cover slip were sealed using Vaseline. The hanging drop preparation was observed under a light microscope at 40× magnification. For each animal, two separate hanging drop preparations were made. Sperm motility was assessed by two independent observers and data expressed as percentages (Tatli-Çankaya et al., 2014; Chenniappan and Murugan, 2017).

2.9.3. Sperm viability and sperm morphology

Sperm viability was determined by mixing one drop of Eosin–Nigrosine stain and one drop of semen in an Eppendorf tube using a Pasteur pipette. A drop of the mixture was placed on a glass slide, covered with coverslip and observed for at least 200 spermatozoa under light microscope (Kruss MBL2000, A. Kruss Optonic, Germany) at 40× magnifications. Dead spermatozoa appeared red, while live spermatozoa were not stained. The results were determined by counting both stained and non-stained sperms and are presented as a percentage.

Sperm morphology was evaluated adopting Eosin–Nigrosin method. One drop of Eosin–Nigrosin stain and a drop of semen were separately placed on the one end of a clean warm glass slide. The semen and the stain were mixed well and drawn out with the edge of another slide which served as a spreader so that, a thin film was and air-dried. The stained smears were observed under a light microscope (Hund Wetzlar H600/12, Germany, fitted with digital camera, canon EOS 550D) at ×40 magnification. At least 200 spermatozoa from different fields of the slide were examined for their morphological features. The total sperm abnormalities were expressed as percentage incidence.

2.9.4. Weights of reproductive organs

The reproductive organs (testis, epididymis, seminal vesicle and prostate) were dissected out and weighed (Analytical Balance; Shimadzu AUW220D; Kyoto-Japan; range: 0.1 mg to 220 g). Relative weight of each organ [(organ weight/body weight) × 100] was calculated.

2.9.5. Mating test

Ten days prior to the end of the experiment, each male rat was paired in a separate cage with two virgin female rats for 5 days (Chaturapanich et al., 2008). Successful mating was assured by detection of spermatozoa in the vaginal smears taken every morning. Mating success was calculated from the ratio of the number of females mated to the number of females paired. The female rats showing spermatozoa in their vaginal smears were separated for 10 days, and then euthanized with ip overdose of pentobarbital sodium for confirmation of pregnancy. The numbers of fetuses and corpora lutea (CL) were determined. Male fertility was expressed as the percentage fertility, calculated from the ratio of the number of fetuses to the number of CL.

2.10. Histopathological examination

The testes were collected from the different experimental groups and were fixed in 10% formalin and routinely processed for paraffin embedding (El-Alfy et al., 2012; Hetta et al., 2013). Slices of 5 μm were obtained with rotary microtome, stained with hematoxylin-eosin (H&E) and examined under a light microscope (Hund Wetzlar H600/12, Germany, fitted with digital camera, canon EOS 550D).

2.11. Immuno-histochemical analysis of androgen receptor (AR) and proliferative cell nuclear antigen (PCNA) expression in the testis

The immune-histochemical analysis of AR and PCNA were done according to Abdel-Rahman et al. (2017). The tissue specimens were deparaffinized and rehydrated then pretreated with citrate buffer pH 6 for 20 min for antigen retrieval. The sections were incubated with primary antibodies of rabbit polyclonal anti-androgen receptor antibody (ab133273; Abcam, Cambridge, UK) diluted 1:500 and Rabbit polyoclonal anti-PCNA antibody (ab18197; Abcam, Cambridge, UK) diluted 1:4000 for three hours in a humidified chamber. The sections were incubated with secondary (HRP) antibody (ab205718; Abcam, Cambridge, UK). DAB (Sigma) was used as chromogen to visualized reaction. Finally, the slides were counterstained with Mayer hematoxylin and mounted with DPX. The images were analyzed by Lieca Qwin 500 Image Analyzer (Leica, Cambridge, England). In each group, five sections were examined. Color density of the immunopositive cells (dark brown) was evaluated.

2.12. Gene expression analysis

Total RNA was purified from testicular tissues using Rneasy Mini Kit (Qiagen) following the manufacturer's protocol. Frist strand cDNA was synthesized from RNA (1 μg) by reverse transcription using cDNA synthesis kit (Thermo Scientific). Primers for hydroxysteroid 17-beta dehydrogenase 3 (17β-Hsd3) and cytochrome P450, family 11, subfamily a, polypeptide 1 (Cyp11a1) and beta actin were designed (Table 1). For quantitative real-time PCR of each gene, 2 μL of cDNA and 30 pg/ml of the specific primer set were added to a SYBR Green master mix (Qiagen). The PCR temperature conditions for each set of primers were as follows: initial denaturation (95 °C, 10 min), 40 cycles of denaturation (95 °C, 15 sec), annealing (the indicated tm, 30 sec), and extension (72 °C, 40 sec). Following the last amplification cycle, one cycle of

Table 1
Primers sequences.

Gene	Primer sequence		Accession number
CYP11a1	Forward	AGAAGCTGGGCAACATGGAGTCAG	NM_017286.3
	Reverse	TCACATCCCAGGCAGCTGCATGGT	
17β Hsd3	Forward	TTTCTTCGGGAGTAGGGGTTC	NM_054007.1
	Reverse	TCATCGGCGGTCTTGGTCG	
B-actin	Forward	ATGGTGGGTATGGGTCAG	NM_031144.3
	Reverse	CAATGCCGTGTTCAATGG	

extension (72 °C for 1 min) then heating to 95 °C at a rate of 1 °C per min were done. The specificity of PCR products was ensured by a single peak in the melting curve analysis. The results of each target gene expression level were normalized to that of beta actin reference gene and the relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.13. Statistical analysis

All data are presented as means ± SE. for multiple groups comparison, analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test (two-sided) at $p \leq 0.05$ were applied using GraphPad prism® software (version 6.00 for Windows, San Diego, California, USA).

3. Results

3.1. LC-MS study

The LC-MS study of the OLE in both positive and negative mode enable the identification of the key phenolic compounds and their approximate abundance base on the peak area of each compound. Results are presented in Table 2 and Fig. 1.

3.2. Acute toxicity study

The acute toxicity study revealed the nontoxic nature of the OLE. The LD_{50} value by oral route could not be determined as no lethality was observed up to 5000 mg/kg of OLE in the rats. As a result, one-tenth of the maximum tolerated dose of OLE was selected as therapeutic dose (500 mg/kg) and half of this dose was considered as low dose (250 mg/kg) for this study.

3.3. Effect on FBG and insulin levels

The rats treated with GLB and OLE showed a significant decrease ($p \leq 0.05$) in FBG and increase ($p \leq 0.05$) in insulin levels than the diabetic control rats (Fig. 2). Both doses of OLE were more effective than GLB in controlling FBG and insulin levels in diabetic rats. The antidiabetic effect of OLE was confirmed as it was successfully reversed the decreased Hb and the increased HbA1c levels in blood of STZ-diabetic rats toward their normal values (results not shown).

3.4. Effect on testicular antioxidant profile

Levels of the antioxidant enzymes and GSH in the testicular homogenates of different groups are displayed in Fig. 3. Our findings indicate that the levels of SOD (23.4 U/mg protein), GPx (1.7 U/mg protein), CAT (3.6 U/mg protein) and GSH (4.6 μmol/g tissue) were lower in diabetic control rats as compared to normal group (45.6 U/mg protein, 5.9 U/mg protein, 10.4 U/mg protein and 9.4 μmol/g tissue, respectively). GLB administration resulted in 20.09, 52.94, 30.56 and 19.57% higher SOD, GPx, CAT and GSH levels, respectively as compared to diabetic control rats. Treatment of diabetic rats with OLE at 250 and 500 mg/kg for 9 weeks resulted in significantly higher activity levels of SOD (56.41% and 79.06%, respectively), GPx (182.35% and 211.76%, respectively), CAT (127.78% and 163.89%, respectively) and GSH (82.61% and 93.48%, respectively) as compared to non-treated diabetic rats. The 500 mg/kg OLE was 3.93, 4.00, 5.36 and 4.77 time more potent than GLB in preventing the decrease in testicular levels of SOD, GPx, CAT and GSH, respectively in diabetic rats. Fig. 3E shows the effect of GLB or OLE on the amount of LPO product (MDA) in diabetic rats' testes. Testicular MDA levels were significantly higher (47.5 nmol/g tissue) in non-treated diabetic rats as compared to normal group (25.4 nmol/g tissue). However, the level of MDA was 16.42% lower following GLB treatment as compared to diabetic control rats. In addition, testicular MDA levels were 35.79% and 41.89% lower in 250 and 500 mg/kg OLE-treated diabetic rats, respectively as compared to diabetic control group. The 500 mg/kg OLE treatment was 2.55 time more potent than GLB in preventing the increase in testicular MDA levels in diabetic rats.

Table 2
Identified compounds in OLE and their relative percentage obtained from LC-MS.

Name	Molecular weight	M+ and/or M−	Percentage	Retention time
Apigenin-7-glucoside	432	431	0.47	6.87
Cyanidin-3-rutinoside	630	629	0.27	7.07
Rutin	610	611/609	0.28	7.39
Nuzhenide, dihydro[b]	688	687	0.14	7.48
Oleuropin aglycon	378	401[a]/377	0.07	7.61
Luteolin-7-glucoside	448	449/447	2.15	8.86
Hespridin	610	611/609	0.04	9.12
Oleuropein + glucose[b]	702	725[a]/701	0.34	9.57
Oleuropein	540	563[a]/539	14	10.29
Ligustroside	524	547[a]/423	3.29	11.39
Caffeic acid	180	181	1	11.71
Luteolin rutinoside	594	595/593	0.57	12.46
Ligustroside + glucose[b]	686	709[a]/685	0.40	12.73
Diosmin	608	609	0.09	12.94
Verbacoside	624	625/623	1.2	13.12
Oleanolic acid methyl ester	470	471/469	2	20.58
Oleanolic acid	456	479[a]/455	7	23.58

[a] M^+ + Na.
[b] Compounds are proposed from MS data but never isolated before.

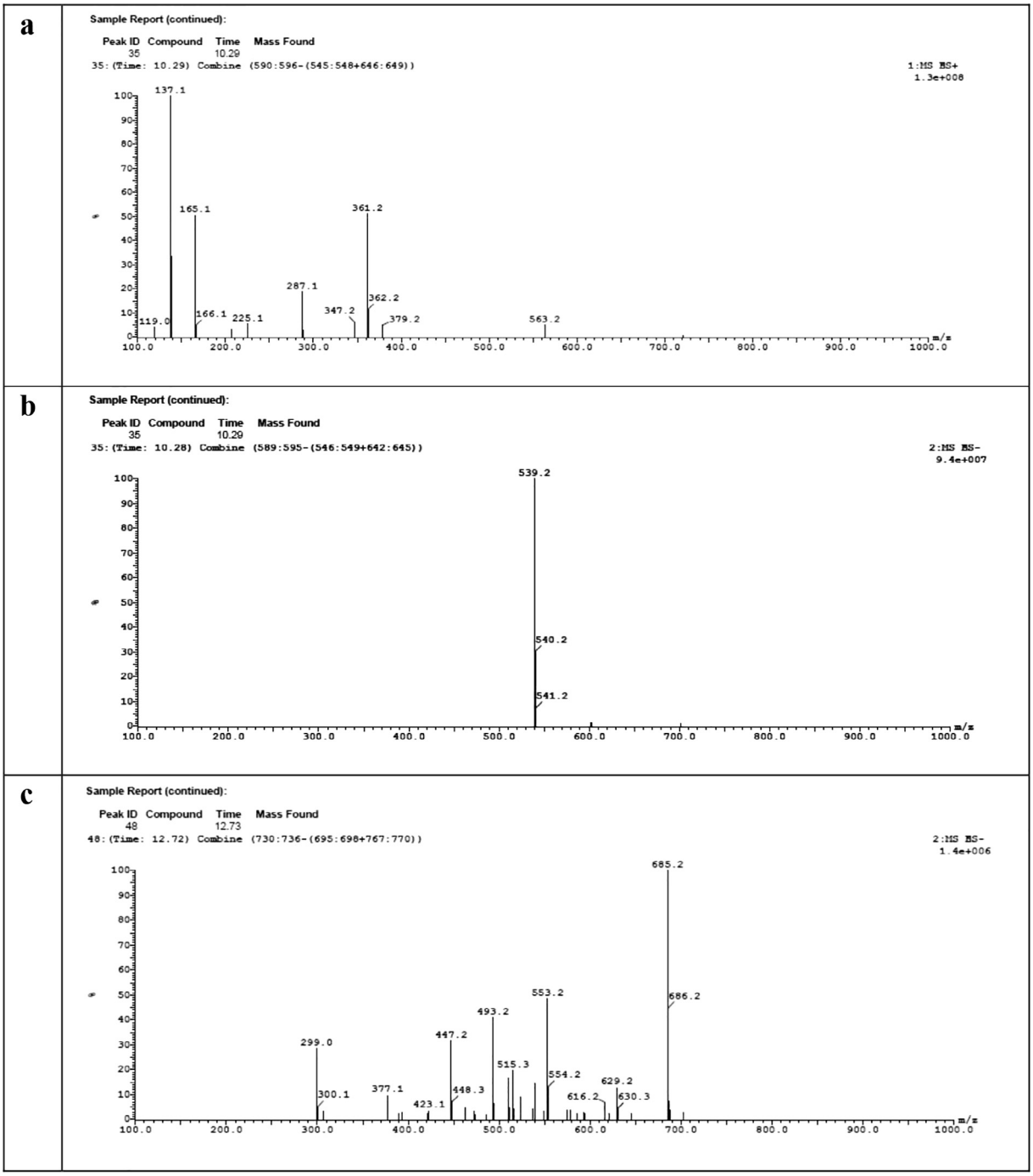

Fig. 1. A. LC-MS spectrum of oleuropein in positive mode, B. LC-MS spectrum of oleuropein in negative mode, C. LC-MS spectrum of proposed dihydro nuzhenide.

3.5. Effect on serum levels of testosterone and gonadotropins

The effects of OLE on serum levels of testosterone and gonadotropins are shown in Fig. 4. Serum levels of testosterone and gonadotropins were significantly lower in diabetic control rats (0.94 ng/mL, 1.87 mIU/mL and 0.16 mIU/mL, respectively) compared with normal group (2.47 ng/mL, 4.9 mIU/mL and 0.74 mIU/mL, respectively) at the end of experimental period. Alterations in the serum levels of these hormones in diabetic rats were significantly reversed ($p \leq 0.05$) by treatment with GLB or OLE (250 and 500 mg/kg). The 500 mg/kg OLE was 1.44, 1.32 and 1.38 time more potent than GLB in preventing the decrease in serum levels of testosterone, FSH and LH, respectively.

3.6. Effect on epididymal sperm characteristics

Biomarkers for monitoring semen quality, including sperm count, sperm motility, sperm viability and sperm abnormality are provided in Fig. 5. Diabetic control rats showed significant decrease in the count, percentages of motility and viability of their sperms in comparison with the normal control group. Moreover, the percentage of sperm abnormalities was significantly increased and the majority of spermatozoa showed coiled and bent tails (Fig. 6). Nine-week treatment with GLB produced a significantly higher sperm count, motility and viability when compared to the diabetic control group. Meanwhile OLE at 250 and 500 mg/kg produced 59.8% and 68.1% higher sperm count, respectively;

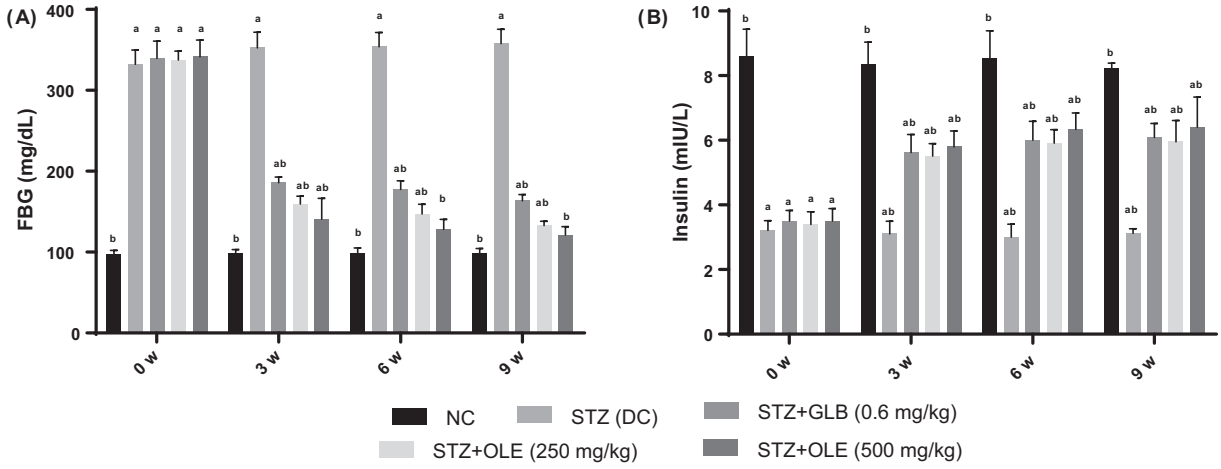

Fig. 2. Effect of GLB and OLE on serum levels of FBG (A) and insulin (B) of STZ-diabetic male rats. Values are expressed as mean ± SE (n = 6). [a] $P \leq 0.05$, statistically significant from the normal control (NC) group. [b] $P \leq 0.05$, statistically significant from the diabetic control (DC) group. Multiple group comparisons were performed by analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test at $p \leq 0.05$.

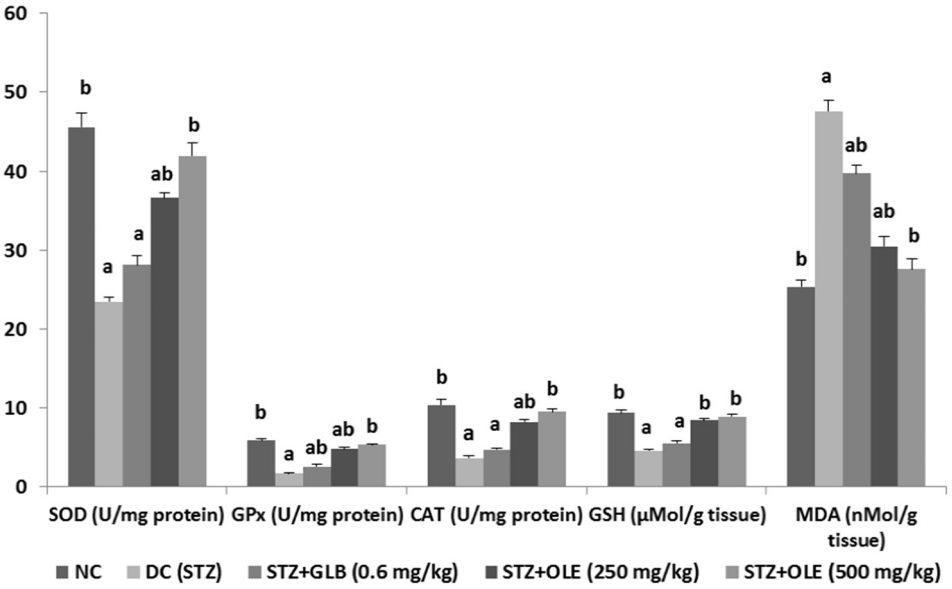

Fig. 3. Effect of GLB and OLE on antioxidant profile; SOD, GPx, CAT, GSH and MDA in the testicular homogenate of STZ-diabetic male rats. Values are expressed as mean ± SE (n = 6). [a] $P \leq 0.05$, statistically significant from the normal control (NC) group. [b] $P \leq 0.05$, statistically significant from the diabetic control (DC) group. Multiple group comparisons were performed by analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test at $p \leq 0.05$.

38.7% and 64.2% higher sperm motility, respectively and 91.4% and 123.9% higher sperm viability, respectively when compared to the diabetic control group. The spermatozoa in GLB and OLE groups were found to be of markedly better morphology in comparison to the control diabetic rats. OLE at 500 mg/kg was more effective than GLB in increasing the sperm count, motility and viability in diabetic rats.

3.7. Effect on sexual organs weights

The effects of GLB and OLE on the relative weights of testis, cauda epididymis, seminal vesicles and ventral prostate of rats are shown in Fig. 7. Marked reductions in relative weights of these organs were found in diabetic control animals at the end of the experimental period compared to normal rats. However, the administration of GLB and the extract (250 and 500 mg/kg) increased the relative weights of testis, cauda epididymis, seminal vesicles and ventral prostate when compared to the diabetes-only group.

3.8. Mating test

According to the results of mating test, the fertilizing capability of STZ-diabetic male rats was markedly reduced. The mating success of diabetic males was decreased (33.3%) as compared to 100% in normal group (Table 3). Significant improvement in the fertility was noted in male rats medicated with GLB or OLE (250 and 500 mg/kg) in a dose-dependent fashion. The ability of these males to mates was increased as manifested by the number of impregnated female rats (7/12, 7/12 and 10/12, respectively) in comparison to 4/12 in diabetic control group. Furthermore, the rates of pregnancy in normal females that mated with diabetic male rats exposed to GLB or OLE at 250 and 500 mg/kg were increased. Fertility of male rats, as expressed by the ratio of the

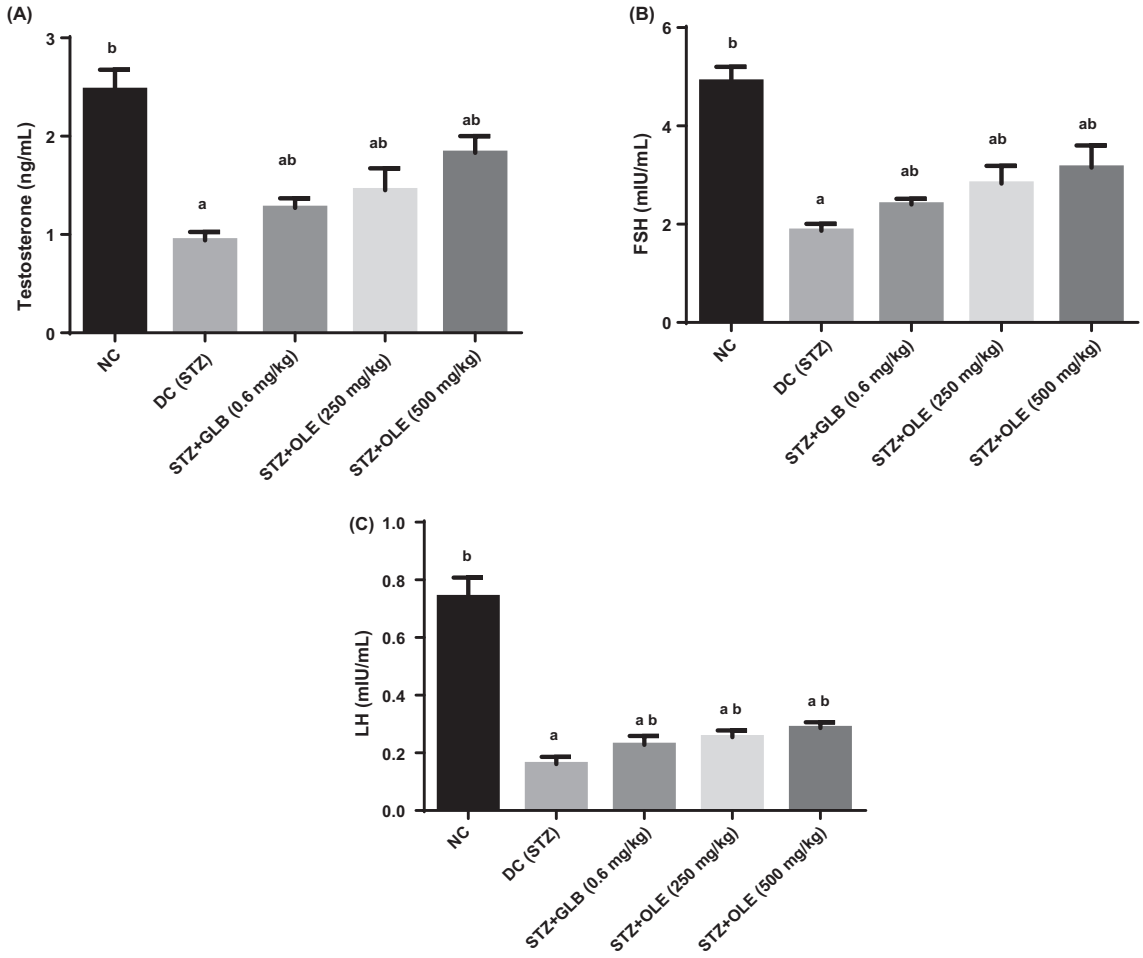

Fig. 4. Effect of GLB and OLE on serum levels of testosterone (A), FSH (B) and LH (C) of STZ-diabetic male rats. Values are expressed as mean ± SE (n = 6). [a] $P \leq 0.05$, statistically significant from the normal control (NC) group. [b] $P \leq 0.05$, statistically significant from the diabetic control (DC) group. Multiple group comparisons were performed by analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test at $p \leq 0.05$.

number of fetuses to the number of CL per mated female, appeared to be below normal in the diabetic control rats. However the male fertility percentage was 49.20% in diabetic-control males, it increased to 62.12, 78.63 and 88.80% in diabetic rats medicated with GLB or OLE (250 and 500 mg/kg), respectively. OLE at 500 mg/kg was 1.42 times more effective than GLB in increasing the fertility % in diabetic rats.

3.9. Histopathological examination of the testis

The normal control group revealed normal histological architecture of mature active seminiferous tubules with normal main cells (Fig. 8A). The diabetic control group showed severe testicular degeneration that characterized by marked reduction of the spermatogonial cells, primary and secondary spermatocytes (Fig. 8B) moreover, the presence of spermatid giant cells in the degenerated tubules. Some seminiferous tubules appeared with complete loss of spermatogenic series associated with one or two layer of vacuolated sertoli cells. Intense intertubular edema was also observed. The epithelial lining epididymal ducts were severely degenerated and vacuolated together with absence or only few numbers of spermatozoa were seen inside their lumina. In the other hand, the groups treated with GLB (Fig. 8C) or OLE (250 mg/kg) revealed moderate to mild testicular degeneration with increase the number of the main cells (Fig. 8D). The group treated with OLE (500 mg/kg) showed marked improvement in the testicular and epididymal lesions with restoration of the normal spermatogenic series (Fig. 8E). Fig. 8F represented the testicular lesion scoring of different experimental groups.

3.10. Effect of on steroidogenic genes expression

The expression levels of genes encoding two rate limiting steroidogenic enzymes, P450cc and 17β-HSD were analyzed in testes of the different experimental groups (Fig. 9). CYP11a1, Cytochrome P450 Family 11 Subfamily A Member 1 encodes P450scc, was significantly down-regulated in the STZ-induced diabetic group to 0.3 fold of its expression level in normal control rats. Groups treated with GLB or OLE (500 mg/kg) showed a significant increase in CYP11a1 mRNA levels to approximately 70%, and 60% of normal control level, respectively (Fig. 9A).

Moreover, the testicular 17β-HSD mRNA level was markedly decreased in diabetic controls to nearly one-half of its normal expression level. The administration of GLB or OLE 250 mg/kg restored 17β-HSD gene expression. Rats of OLE 500 mg/kg group showed overexpression of 17β-HSD to about 3 folds of normal controls (Fig. 9B).

3.11. The immunohistochemistry of AR and PCNA

The PCNA immunostained cells were the spermatogonial cells and the primary spermatocytes that showed strong immunopos-

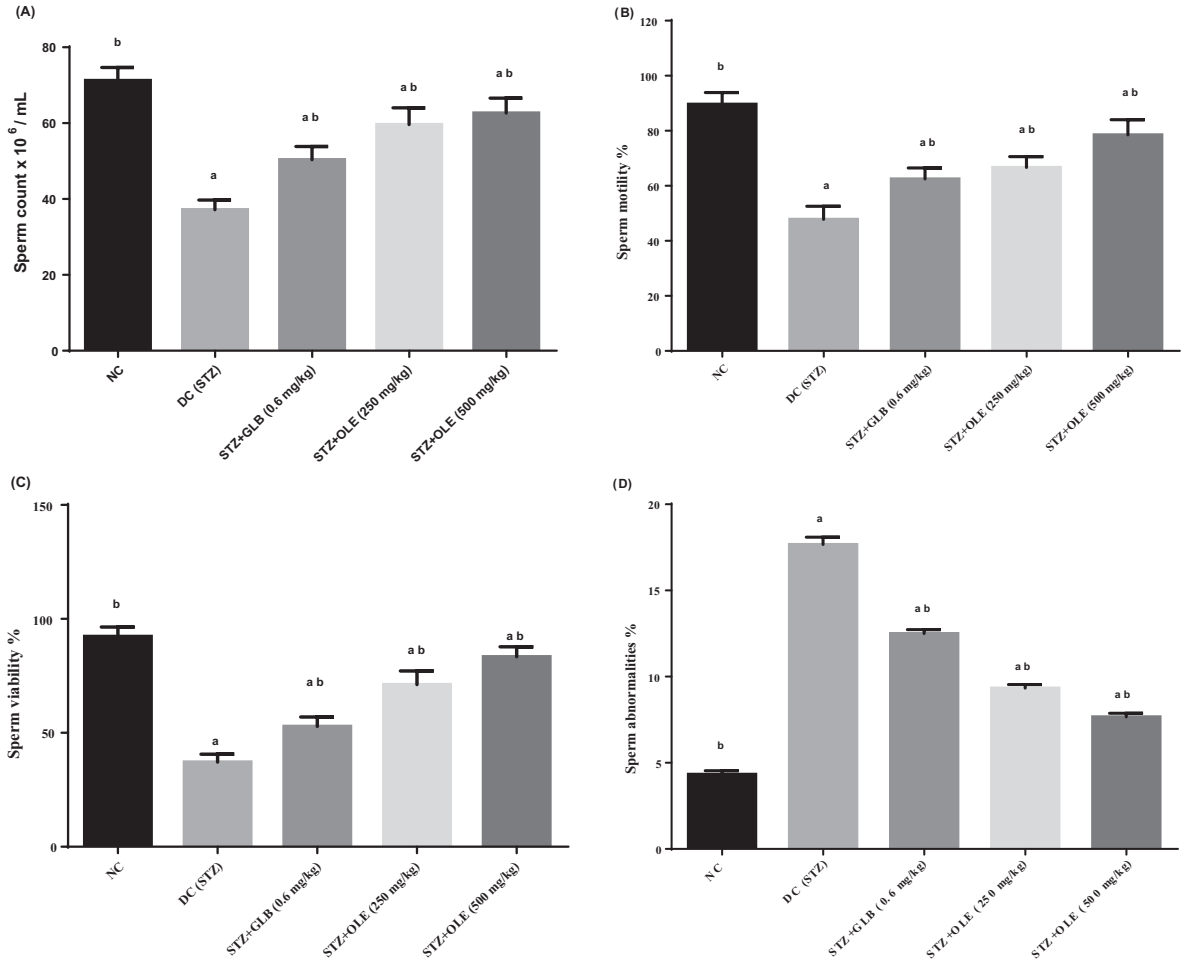

Fig. 5. Effect of GLB and OLE on sperm count (A), motility (B), viability (C) and abnormalities (D) of STZ-diabetic male rats. Values are expressed as mean ± SE (n = 6). [a] $P \leq 0.05$, statistically significant from the normal control (NC) group. [b] $P \leq 0.05$, statistically significant from the diabetic control (DC) group. Multiple group comparisons were performed by analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test at $p \leq 0.05$.

tive reaction (Fig. 10A). While the secondary spermatocytes and the spermatid showed moderate PCNA immunopostive reaction. The diabetic control group revealed significant reduction of the PCNA immunostained cells (Fig. 10B) in comparing to the normal control group. The treated groups with GLB or OLE (250 and 500 mg/kg) showed significant elevation in the PCNA immunostained cells compared with diabetic control group as shown in Fig. 10F. No significant difference was observed between the normal control and OLE (500 mg/kg). The AR immunostained cells were the leydig cells in the intertubular region (Fig. 11A). The diabetic control group showed weak positive reaction in the intertubular region (Fig. 11B). GLB or OLE (250 and 500 mg/kg) revealed intense immunostating reaction in the intertubular area (Fig. 11C, D, and E). Diabetic rats medicated with GLB, OLE (250 mg/kg) and OLE (500 mg/kg) showed significant elevation in the AR immunostained cells compared with diabetic control group as shown in Fig. 11F.

4. Discussion

LC-MS study of OLE in both positive and negative mode enables the identification of the key phenolic compounds in the extract with their relative abundance through correlating the peak area to the total area of the extract. As expected, the major compound was the anti-diabetic secoiridoid glycoside oleuropein (14%) (Cumaoğlu et al., 2011). Oleuropein was identified via its MS spectra in both positive and negative mode. All compounds were identified by compare their MS data with literature. Compounds were isolated before from olive extracts (Cardoso et al., 2011; Ghanbari et al., 2012). Both oleuropein glucose and oleuropein aglycon were proposed to be present in olive mill wastewaters studied by LC-MS (Cardoso et al., 2011). Our results suggested the presence of the dihydro derivative of the secoiridoid nuzhenide, however, the suggestion needs further confirmation.

In our study, STZ-diabetic rats showed elevated FBG level and decreased insulin level, which are indicative of hyperglycemia. However, treatment with GLB or OLE improved the altered FBG and insulin levels in the diabetic rats. This implies that OLE is able to increase the ability of insulin to lower serum glucose, confirming its anti-diabetic activity. These results are consistent with other studies (Choudhury et al., 2017; Elsaid et al., 2018). Effect of DM on male reproductive function can also be explained through the impact of oxidative stress, caused by the inequality between reactive oxygen species production and antioxidant defense mechanisms (Temidayo and Stefan, 2018). Further, the induction of oxidative stress in the testis and sperm in the diabetic state might be due to a hypoxic state as diabetes results in HbA1c formation that interferes with oxygen delivery at the testicles (Cabrales et al., 2008). Monitoring of the levels of the cellular antioxidants in biological samples is widely used to determine the state of oxidative stress. Depletion of these cellular antioxidants leaves the cell vulnerable to oxidative stress.

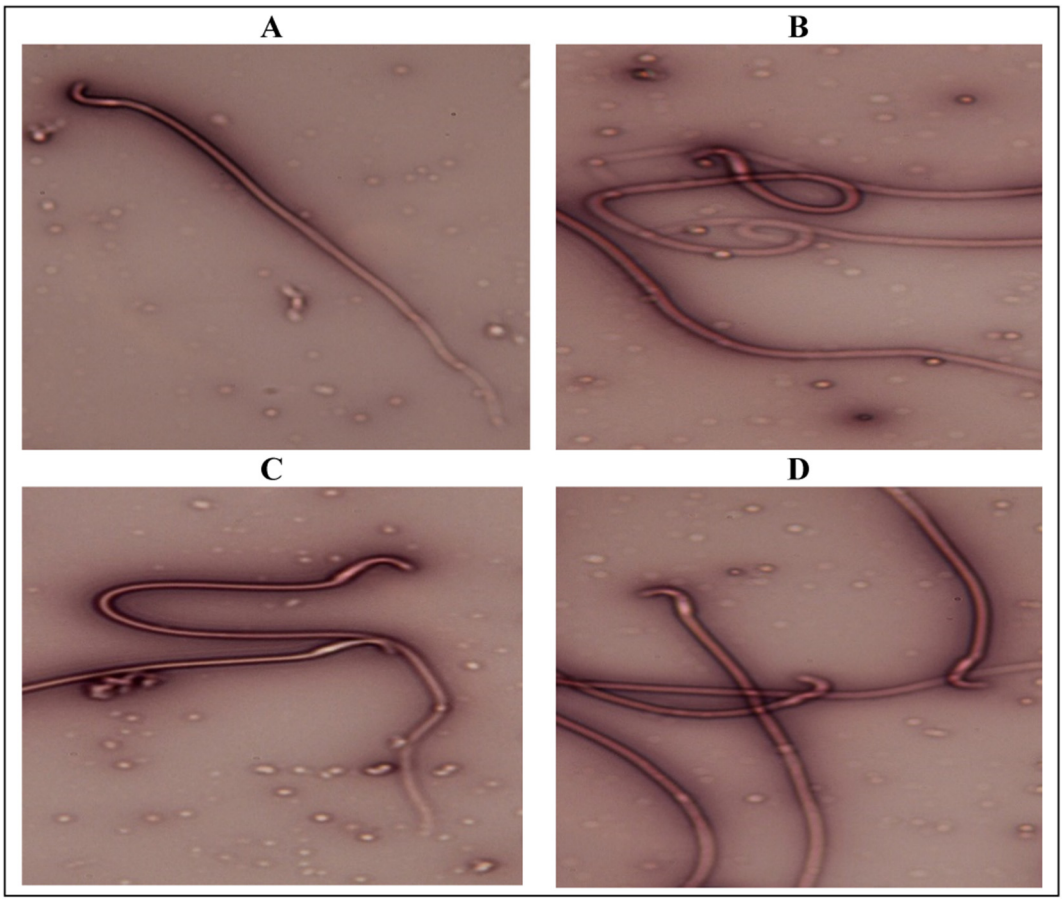

Fig. 6. Photomicrographs (×400) of sperm morphology of normal control rats (A), sperms with coiled tails of diabetic control rats (B) sperm with bent tail of diabetic rats treated with GLB (C) and normal morphology of sperms of diabetic rats treated with OLE at 500 mg/kg (D).

In this study, the activities of SOD, GPx and CAT and the level of GSH were significantly reduced in the testicular tissue homogenate of the diabetic control group. Following treatment of STZ-diabetic rats with OLE, the testicular levels of SOD, GPx, CAT and GSH were significantly restored to established values that were not different from normal control rats, suggesting OLE's ability to scavenge and neutralize STZ induced oxidative stress. The significantly raised level of MDA, a by-product of lipid peroxidation, during uncontrolled stage of diabetes indicated free radical stress induced LPO (Chugh et al., 2001). In the present study, MDA levels increased significantly in the testes of diabetic rats when compared to the normal control. Subsequent treatment with OLE led to a significant decrease in MDA levels in diabetic rats. The significant fall of MDA and increased levels of SOD, GPx, CAT and GSH in testicular tissues of diabetic rats in response to OLE treatment revealed beneficial effect of the extract against oxidative stress. On intergroup comparison, OLE (500 mg/kg) showed a better improvement in oxidative status than GLB. Phytochemicals such as polyphenols are important non-toxic sources of antioxidants (Valdez-Solana et al., 2015). The high levels of polyphenolic compounds in OLE; particularly oleuropein and hydroxytyrosol suggest its protective ability against oxidative stress and cellular damage (Choudhury et al., 2017). Polyphenols also has an anti-hyperglycemic activity, which may suppress glucose production in the liver and improve glucose uptake in peripheral tissues (Prabhakar and Doble, 2009). The amelioration of blood glucose level in OLE-treated diabetic rats and the antioxidant property of OLE reduced ROS generation and subsequently reduces the LPO (Kim et al., 2016). In another model, OLE treatment protected rats against cisplatin-induced testicular toxicity owing to its antioxidant and antiapoptotic properties (Almeer and Abdel Moneim, 2018).

Many studies have documented an association between low testosterone concentrations and type 2 diabetes (Bhasin et al., 2010; Salimnejad et al., 2017). As shown in the current study, the serum level of testosterone is remarkably reduced in STZ-diabetic rats. In this respect, Zhao et al. (2014) reported that the low level of testosterone in type 2 DM might be due to diabetic induced-oxidative stress that causes a significant reduction in testicular and adrenal androgen productions. Furthermore, the reduced serum testosterone concentration in STZ-diabetic rats might be a direct effect of excess glucose or its metabolites on the Leydig cell function, defective gonadotropins, or resistance to these hormones (Navarro-Casado et al., 2010). The results of the present work also demonstrated a significant decrease in serum gonadotropins levels in STZ-diabetic group in comparison to the normal control group. These results are in line with many investigators who attributed gonadotropin reduction to interference of diabetes with the hypothalamo-pituitary–testicular axis. In this study, OLE at doses of 250 and 500 mg/kg decreased the levels of testosterone, FSH and LH compared to diabetic control values. The 500 mg/kg OLE was 1.33, 1.22 and 1.67 time more potent than GLB in preventing the decrease in serum levels of testosterone, FSH and LH, respectively. Serum testosterone concentration is negatively correlates with the blood glucose level (Kim et al., 2014). Thus, the increase in testosterone levels could be associated with the anti-diabetic effect of OLE. Derouiche et al., (2013) added that the marked increase in the serum testosterone of diabetic rats treated with OLE is attributed to the androgen releasing activity of the

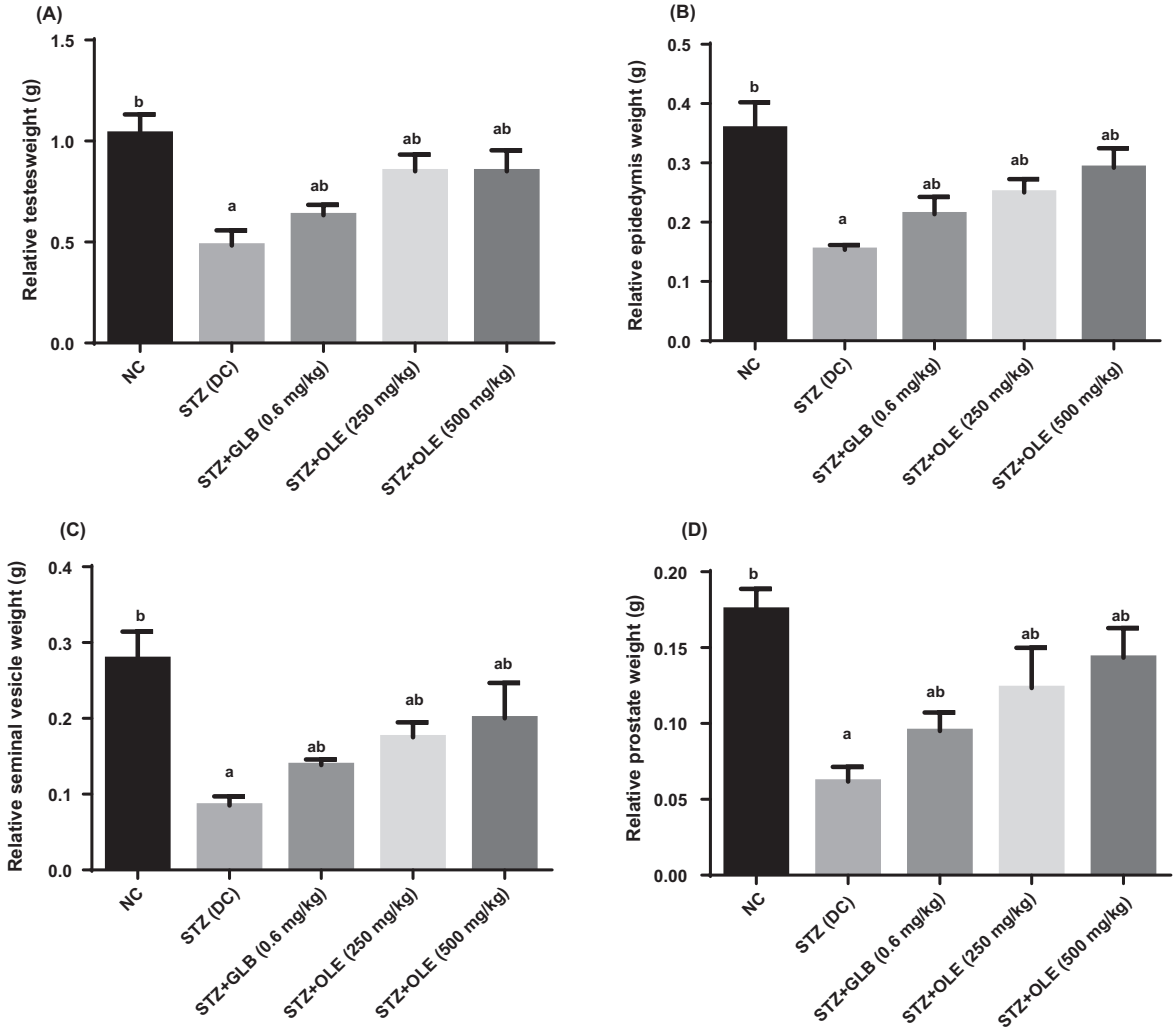

Fig. 7. Effect of GLB and OLE on relative weight of testis (A), epididymis (B), seminal vesicle (C) and prostate (D) of STZ-diabetic male rats. Values are expressed as mean ± SE (n = 6). [a] $P \leq 0.05$, statistically significant from the normal control (NC) group. [b] $P \leq 0.05$, statistically significant from the diabetic control (DC) group. Multiple group comparisons were performed by analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test at $p \leq 0.05$.

Table 3
Effect of GLB and OLE on the mating success and male fertility % of STZ-diabetic male rats.

Groups	No. of females mated	Mating success (%)	No. of females pregnant	No. of fetuses/rat	No. of CL/rat	Male fertility (%)
NC	12	100	12	12.1 ± 0.54[b]	12.4 ± 0.67	97.58 ± 4.27[b]
STZ (DC)	4	33.3	3	6.2 ± 0.49[a]	12.6 ± 0.48	49.20 ± 2.53[a]
STZ + GLB (0.6 mg/kg)	7	58.3	6	8.2 ± 0.60[a,b]	13.2 ± 0.43	62.12 ± 3.77[a,b]
STZ + OLE (250 mg/kg)	7	58.3	7	9.2 ± 0.42[a,b]	11.7 ± 0.43	78.63 ± 3.49[a,b]
STZ + OLE (500 mg/kg)	10	83.3	10	11.1 ± 0.43[b]	12.5 ± 0.48	88.80 ± 4.12[b]

Data are expressed as numbers and percentage of six males and 12 females.
Multiple group comparisons were performed by analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test at $p \leq 0.05$.
[a] $P \leq 0.05$, statistically significant from the normal control (NC) group.
[b] $P \leq 0.05$, statistically significant from the diabetic control (DC) group.

extract. This finding is similar to that obtained by Derouiche et al. (2013). They reported that consumption of virgin olive oil increased blood levels of testosterone and LH among adult men. In addition, the improvement in serum testosterone level in diabetic rats treated with OLE may be related to the antioxidant effect of polyphenols content of OLE that can counteract free radicals. Although the level of testosterone has increased in the blood of OLE-treated rats compared to the diabetic control group, but it did not reach the normal level. This is closely supported by the fact that LH is responsible for functional Leydig cells and testosterone production (La Vignera et al., 2012). As a consequence, the previous observation can be explained by the failure of LH to reach to normal level, leading to a decrease in the testicular weight and thus lack of testosterone secretion.

Steroid hormones biosynthesis, in response to LH hormone and other steroidogenic stimuli, begins with cholesterol to form the first steroid, pregnenolone, in all steroidogenic tissues (Stocco and Clark, 1996). Subsequently, testosterone is synthesized under

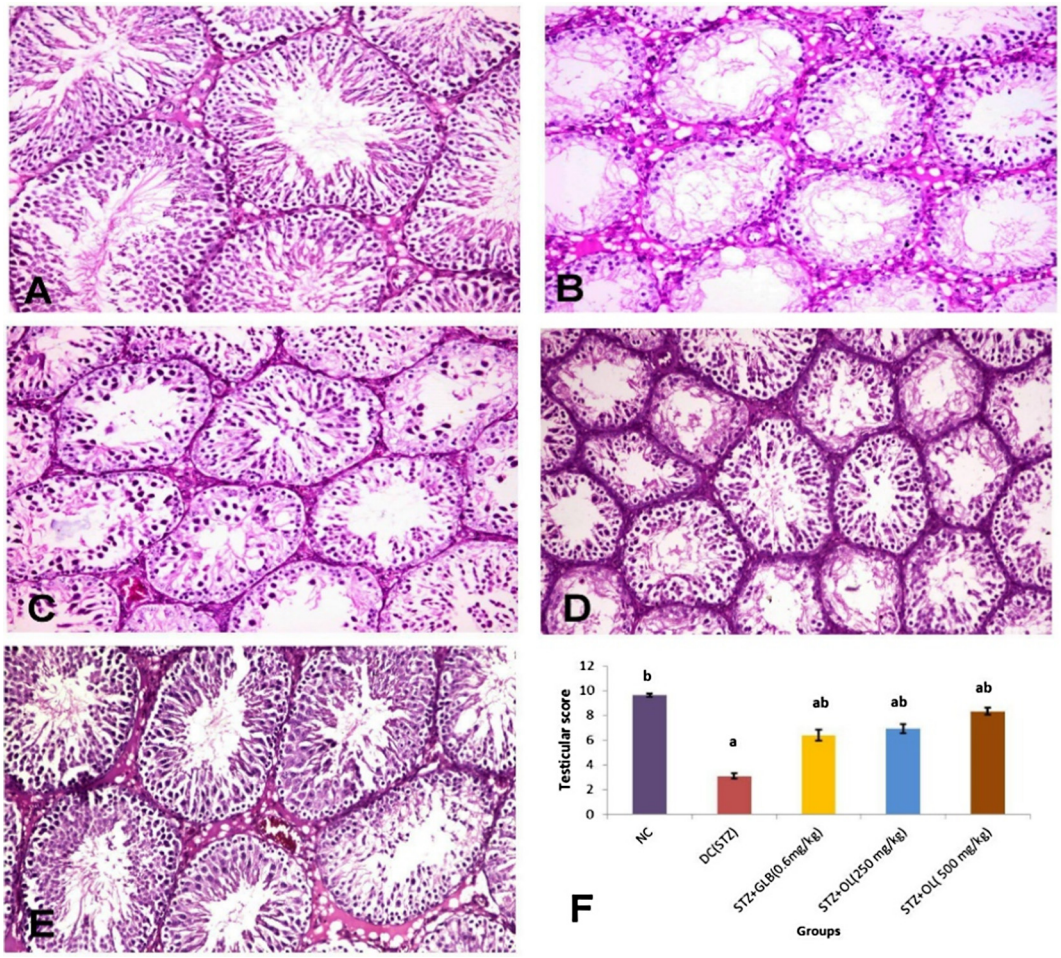

Fig. 8. Histopathological examination of the testis in the different experimental groups (H&E ×200). (A) Normal control group showing normal histological architecture of mature active seminiferous tubules. (B) Diabetic control group showing severe testicular degeneration with intertubular edema. (C) GLB-treated group showing moderate testicular degeneration. (D) OLE (250 mg/kg) treated group showing mild testicular degeneration. (E) OLE (500 mg/kg) treated group showing with restoration of the normal spermatogenic series. (F) The bar chart represents testicular lesion score. [a] is significantly different from diabetic control group, [b] is significantly different from normal control group ($p \leq 0.05$).

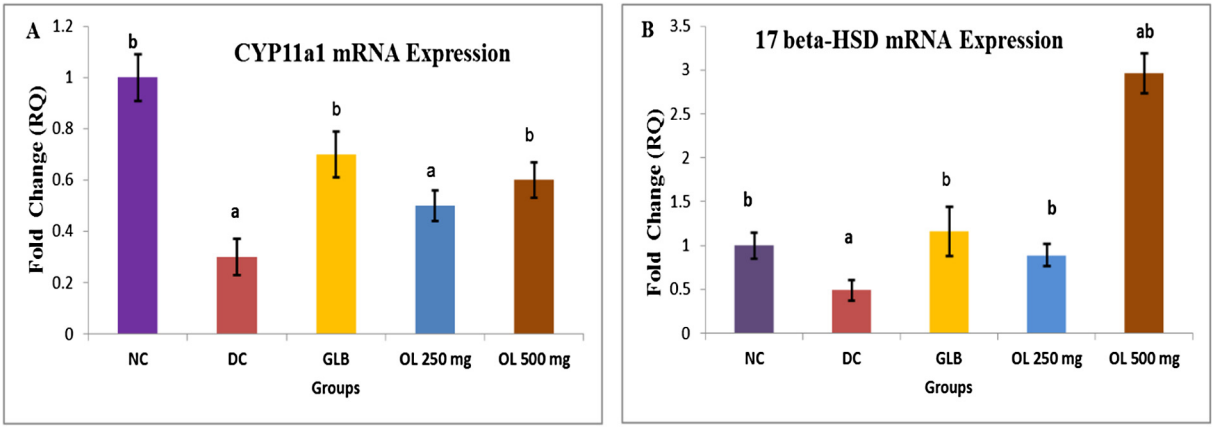

Fig. 9. mRNA expression of CYP11a1 and 17β-HSD genes in testis of the different experimental groups. Values represent the fold changes (RQ) in mRNA levels (means ± SE). Beta actin was used as an invariant control gene. [a] is significantly different from normal control. [b] is significantly different from diabetic control ($p \leq 0.05$).

the influence of 3β- and 17β-hydroxy steroid dehydrogenases. Leydig cell dysfunction is detected by a decrease in testosterone production as a consequence of suppressed CYP11A1, 3b-HSD and 17b-HSD activities (Saradha et al., 2008; D'Cruz et al., 2010). The intra-tissue steroid concentration is implicated as a significant biomarker in evaluating the steroid-metabolizing enzymes expression (Page, 2011). The present study was designed to elucidate the effects of olive extract on testicular steroidogenesis in STZ-induced diabetic rats. Accumulating evidences have verified that dysfunction of testicular steroidogenesis and spermatogenesis resulted

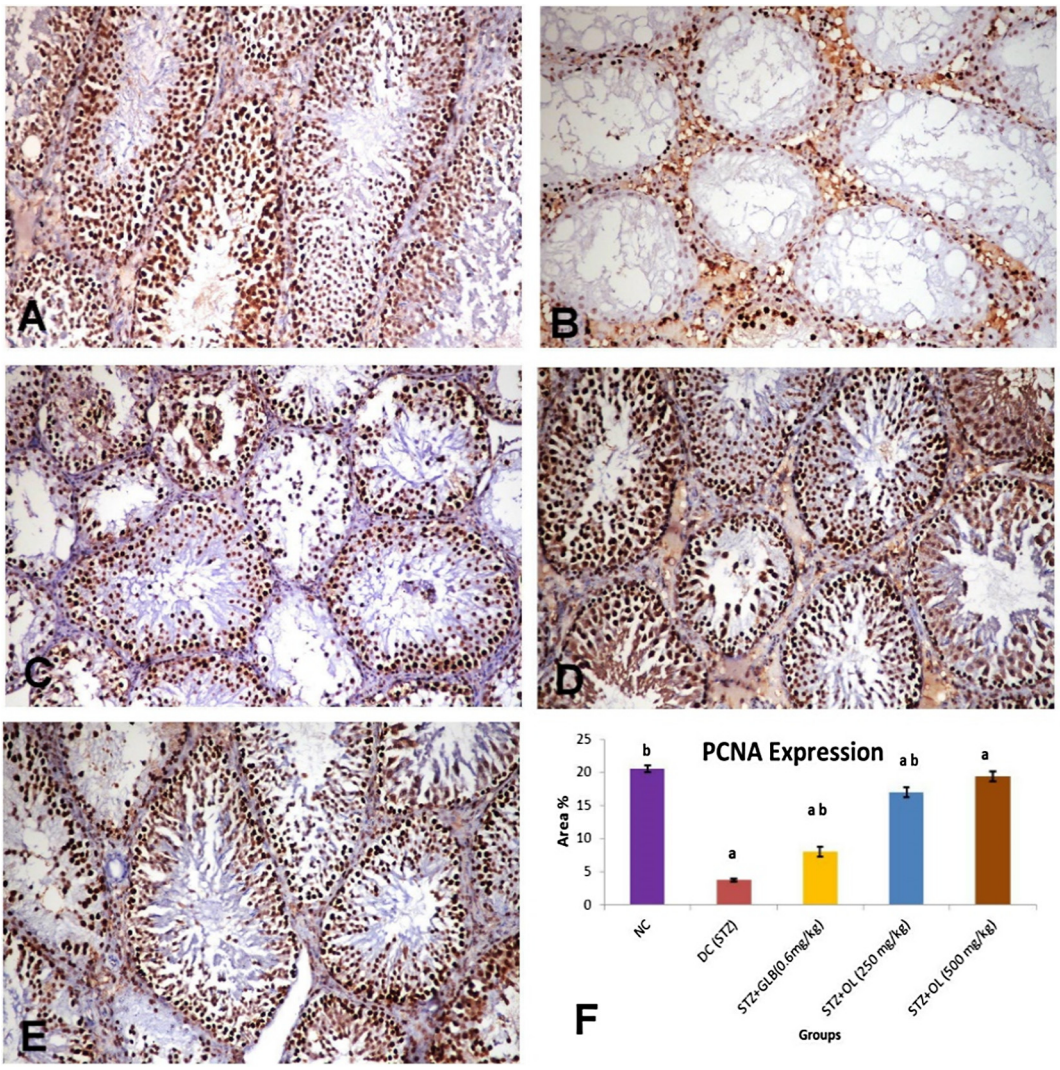

Fig. 10. Immunohistochemical analysis of PCNA in the different experimental groups (×200). (A) Normal control group. (B) Diabetic control group. (C) GLB-treated group. (D) OLE (250 mg/kg)-treated group. (E) OLE (500 mg/kg)-treated group. (F) The bar chart represents PCNA immunopositive cells expressed as area %. [a] is significantly different from diabetic control group, [b] is significantly different from normal control group ($p \leq 0.05$).

from hyperglycaemia-induced oxidative stress and insulin deficiency is associated with male reproductive impairment (Premalatha et al., 2013).

Testicular steroidogenesis is a highly regulated signaling pathway that depends on the availability of cholesterol within testicular mitochondria besides the activities of steroidogenic acute regulatory protein (StAR), P450scc, 3β-HSD and 17 β-HSD (Miller, 1988). CYP11A1 gene, encodes P450scc that catalyzes the first step of cholesterol metabolism to form pregnenolone (a precursor for all steroid hormones) (Stocco and Clark, 1996). Since CYP11A1 is essential for steroid synthesis, abnormal CYP11A1 expression affects the steroid levels (Chien et al., 2013).

To investigate the protective influence of the extract on the fertility of male diabetic rats, we examined the expression level of two key steroidogenic genes Cyp11a1 and 17 β-HSD in testis. Total RNA was isolated from control and treated testis to monitor the steady-state mRNA levels by real time RT-PCR. As shown in Fig. 9; the testicular Cyp11a1 and 17 β-HSD mRNA expression was obviously suppressed in diabetic rats indicating an impaired testicular steroidogenesis. A significant recovery was noted ($p \leq 0.05$) in the gene expression for Cyp11a1 in GLB and OLE (500 mg/kg)-treated groups (Fig. 9A). Also, mRNA levels of 17 β-HSD were restored towards the control or even more with OL administration at dose of 250 or 500 mg/kg, respectively (Fig. 9B). These results could explain the low testosterone and gonadotropins in serum of diabetic rats (Fig. 4). Besides, deficiency of insulin, a positive regulator of de novo steroidogenesis in STZ model could be considered as additional cause of impaired steroidogenesis (Lubik et al., 2011).

Count, progressive motility and morphology of spermatozoa are considered as markers for testicular function. Previous investigations have reported diabetes as a devastating element for testicular tissues and functions (Ding et al., 2015; Salimnejad et al., 2017). Diabetes disrupts the process of spermatogenesis by mechanisms that involved the generation of ROS and induction of LPO. Subsequently, progressive motility and morphology of spermatozoa may be impaired and sperm cell death may occur depending on ROS levels. In the present study, sperm count, motility and viability were significantly decreased, while sperm abnormalities were statistically increased in STZ-diabetic rats. Ding et al. (2015) explained that spermatids and mature sperms are highly susceptible to ROS due to the high content of polyunsaturated fatty acids in their membranes. The improvement in these sperm parameters following medication of diabetic rats with OLE could relevant to

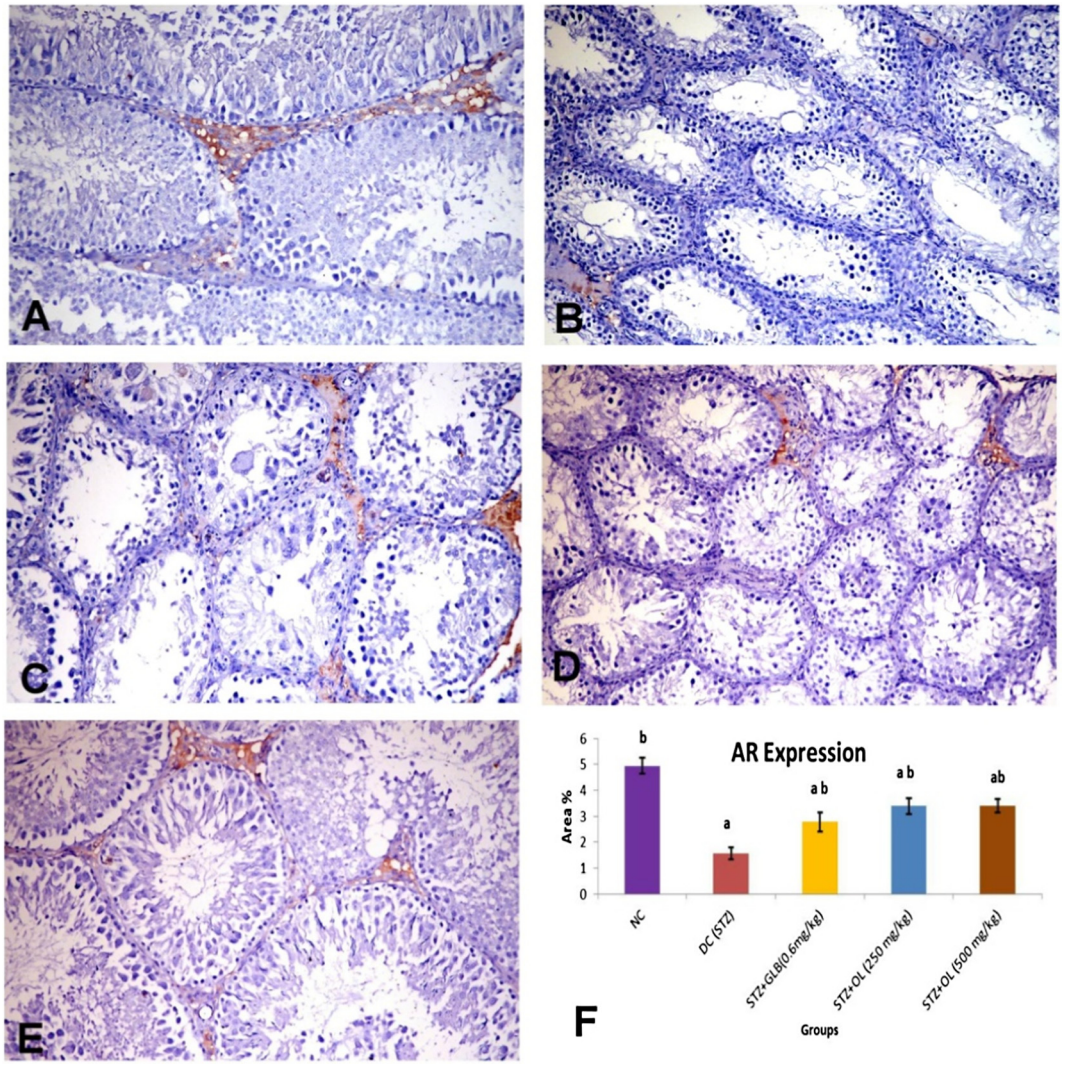

Fig. 11. Immunohistochemical analysis of AR in the different experimental groups (×200). (A) Normal control group. (B) Diabetic control group. (C) GLB-treated group. (D) OLE (250 mg/kg)-treated group. (E) OLE (500 mg/kg)-treated group. (F) The bar chart represents AR immunopositive cells expressed as area %. [a] is significantly different from diabetic control group, [b] is significantly different from normal control group ($p \leq 0.05$).

its capability to protect against the oxidative stress and LPO. This explanation is consistent with Alirezaei et al. (2012) who reported that oleuropein of OLE improves the epididymal sperm parameters in ethanol induced oxidative stress in rat testis. Another study demonstrated that oral administration of OLE improved the sperm qualitative parameters in rats exposed to rotenone (Sarbishegi et al., 2017). In addition, some studies have shown a direct link between blood glucose levels and sperm quality (Khaki et al., 2009). Accordingly, our results could be explained on the basis of the glucose lowering effect of OLE and its capability to protect testicular germ cells against the oxidative stress and LPO. OLE at 500 mg/kg is more effective than GLB in increasing the sperm count, motility and viability in diabetic rats. Adequate level of testosterone is necessary for regulating spermatogenesis and maintaining the normal physiological state of seminiferous tubules. Furthermore, insulin is one of the regulators of testicular steroidogenesis (Pasquali et al., 1995). Thus, the improvement in sperm quality of OLE-treated rats could be related to the increase in testosterone and insulin levels observed in the present study.

Regarding to mating test, experimental studies reported that the induction of diabetes in animal models has impaired testicular function and decreased male fertility based on impotency, retrograde ejaculation, and reduced libido (Navarro-Casado et al., 2010). In the present study, the percentages of mating success and male fertility were significantly decreased in STZ-diabetic rats. Some previous studies implicated that the diabetes decreased male fertility through decreased sperm counts, motility and morphology (Bartak et al., 1975) and disruption of seminiferous tubular morphology (Murray et al., 1983). Moreover, DM induced male infertility via decreasing serum levels of testosterone, LH and FSH (Fedail et al., 2016). The effect of DM on male reproductive function can also be explained through the impact of oxidative stress, caused by the inequality between ROS production and antioxidant defense mechanisms (Agarwal et al., 2014). In our study, the increased rate of pregnancy in female rats after mating with diabetic males treated with OLE is an indicator for the protective effect of the extract against the deleterious effect of diabetes. In this respect, Donnelly et al. (1998) mentioned that sperm count and motility are directly consistent with the successful fertilization and pregnancy rates. Moreover, Jannini et al. (1999) reported that erection of penis and sexual desire of males depends on concentration of testosterone in blood. Accordingly, the increase in mating success of OLE-medicated males could be due to increased level of testosterone that improves androgen dependent parameters such as penile erection and sexual desire. Additionally, the observed improvement in the percentages of mating success and fertility

of STZ-diabetic rats given OLE can be attributed to the ability of the extract to improve sperm quality, gonadotropin levels and spermatogenesis. OLE at 500 mg/kg was 1.42 times more effective than GLB in increasing the fertility percentage in diabetic rats.

The decrease in the weight of reproductive organs of STZ-diabetic rats is mainly due to the decrease in testosterone level associated with the oxidative stress induced by diabetes. Treatment of diabetic rats with OLE recovered the weights of reproductive organs towards control level. The protective effects of OLE can be explained by the prevention of cellular damage occurring because of oxidative stress in testicular tissues. The normal growth and function of male reproductive organs depend on different hormones such as androgens. The testosterone is the main androgen that is very important for the development of male reproductive organs and maintenance of their structural and functional integrity (O'Hara and Smith, 2015). Thus, the marked elevation in the testicular and epididymal weights could be due to the elevated level of testosterone in blood of OLE-medicated groups.

Prolonged and constant exposure to free radicals in diabetic state leads to destruction of testicular tissue (Ding et al., 2015). In the present study, the protective effects of OLE against deleterious effect of diabetes on the reproductive organs of males were confirmed by the histopathological results in the testicular tissues of treated animals. The protective effects of this extract may be due to its anti-diabetic and free radical scavenging properties, which leads to the reduction in the oxidative damage of testis.

5. Conclusion

In conclusion, the present study demonstrated that OLE has a potential to inhibit hyperglycemia and oxidative stress induced by diabetes. In addition, our results suggest that administration of OLE may be helpful in the protection against diabetes associated male reproductive disorders through up-regulating P450scc and 17β-HSD expression and via the enhancement of the antioxidant capacity.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgment

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