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Original article

Artemisia judaica L. diminishes diabetes-induced reproductive dysfunction in male rats via activation of Nrf2/HO-1-mediated antioxidant responses

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ABSTRACT

Diabetes mellitus is a well-known danger element for the progression of male reproductive dysfunctions. Available evidence supports oxidative stress to be the underlying mechanism for the manifestation of testicular dysfunctions during diabetes, and this relation represents an attractive target to antagonize these complications. *Artemisia judaica* L. is known to have antidiabetic and antioxidant characteristics. The possible protective effect of *Artemisia judaica* against diabetes-induced testicular disorders was not explored. In this investigation, we planned to estimate the possible protective effect of *Artemisia judaica* extract against diabetes-induced testicular disorders in male rats. The blood levels of insulin, glucose, glycosylated hemoglobin, testosterone, luteinizing hormone and follicle stimulating hormone were evaluated in rats after 12 weeks of *Artemisia judaica* treatment. Further, oxidative stress markers were determined in their testicular tissue. Epididymal fluid and testicular histological changes were also assessed. Expression of proliferating cell nuclear antigen has been evaluated in testis. Testicular mRNA expression of nuclear factor erythroid 2-related factor 2 and heme oxygenase-1 as the significant transcription factors in controlling antioxidant system were evaluated by real-time polymerase chain reaction. *Artemisia judaica* extracts have the ability to ameliorate the elevation in the serum glucose and blood glycosylated hemoglobin and the reduction in insulin, testosterone, follicle stimulating hormone and luteinizing hormone caused by streptozotocin-induced diabetes. It induced a significant recovery of the testicular oxidative stress markers, sperm characteristics and improved histopathological findings of the testes. Treatment with *Artemisia judaica* extracts led to an increase in proliferating cell nuclear antigen protein expression. Reduction of testicular oxidative stress potential in streptozotocin-treated groups was confirmed by upregulation of nuclear factor erythroid 2-related factor 2 and heme oxygenase-1.

Abbreviations: AJ, *Artemisia judaica* L.; CAT, Catalase; DC, Diabetic control; EDTA, Ethylenediamine tetraacetic acid; ELISA, ELISA: Enzyme-linked immunosorbent assay; FBG, Fasting blood glucose; FSH, Follicle stimulating hormone; GSH, Reduced glutathione; GSH-Px, Glutathione peroxidase; H&E, Hematoxylin and eosin; HbA1c, Glycosylated hemoglobin; HO-1, Heme oxygenase-1; HPTLC, High-performance thin layer chromatography; LH, Luteinizing hormone; LPO, Lipid peroxidation; MDA, Malondialdehyde; NC, Negative control; Nrf2, Nuclear factor erythroid 2-related factor 2; PCNA, Proliferating cell nuclear antigen; ROS, Reactive oxygen species; RT-PCR, Real time polymerase chain reaction; SOD, Superoxide dismutase; STZ, Streptozotocin; TST, Testosterone.

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1. Introduction

Diabetes is one of the metabolic disorders characterized by complicated nature that influences multiple systems, leading to the development of various complications. The prevalence of diabetes has accelerated quickly. World Health Organization (WHO) statistics expect that the number of people with diabetes will reach 366 million by 2030 (Bahmanzadeh et al., 2016). Among the complication of diabetes is the spermatogenic dysfunction that characterized by abnormal spermatogenesis and sperm deformities (Han et al., 2019). Altered sperm morphology and reduced sperm motility and counts are the main factors implicated in the decreased fertility of men with diabetes (La Vignera et al., 2012). Testicular damage in diabetic individuals is usually associated with increased oxidative stress (Wang et al., 2014). Interestingly, Nrf2 is one of the key transcription factors in controlling the cellular antioxidative system (Wang et al., 2014). Expressions of antioxidant enzymes are mostly activated by Nrf2 (Singh et al., 2010). It is broadly expressed in tissues and has a critical role in preventing the development of testicular oxidative disruption (Bae et al., 2016).

In addition to the available antidiabetic drugs, many herbal medicines have been recommended for the management of diabetes. Ethnobotanical information suggests that approximately 800 plants may have significant antidiabetic effects (Nuckols et al., 2018). In Western and African folk medicine, several members of *Artemisia* genus are used for the management of diabetes (Eshetu, 2016). *Artemisia* L. is a genus of small herbs and shrubs found in northern temperate regions. It belongs to the important family Asteraceae, one of the most numerous plant groupings, which comprises about 1000 genera and over 20,000 species (Nigam et al., 2019). *Artemisia judaica* L. is a perennial fragrant shrub that grows widely in the deserts and on the Sinai Peninsula in Egypt, and is a very common anthelmintic drug in most North African and Middle-Eastern countries where it is known by the Arabic name of "shih" (Abad et al., 2012). AJ infusion is recommended by Bedouins in Jordan for management of diabetes and sexual weakness (Alzweiri et al., 2011). AJ is also used in traditional medicine as antibacterial, anti-inflammatory and analgesic (El-Sayed et al., 2013). Additionally, the water and alcohol extracts of AJ significantly reduced blood glucose levels in diabetic rats (Nofal et al., 2009). The genus Artemisia is known to contain many bioactive compounds; artemisinin exerts not only antimalarial activity but also profound cytotoxicity against tumor cells (Efferth, 2007) and arglabin is employed for treating certain types of cancer in the former Soviet Union (Wong and Brown, 2002).

Development of diabetic complications is extremely related to chronic sustained hyperglycaemia. Further, oxidative stress has been reported as a major pathway in the pathogenesis of diabetic complications. Therefore, the antidiabetic and antioxidant activities of AJ may successfully stop the development of diabetic complications. Hence, this study aimed to assess the potential effect of AJ to protect against diabetes-related sexual complications in male rats.

2. Materials and methods

2.1. Plant material

Aerial parts of *Artemisia judaica* L. were obtained from the herbal market in Al Kharj City, Saudi Arabia. The plant was identified by Dr. Mohammad Atiqur Rahman, taxonomist of the MAPPRC, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. A voucher specimen (#16723) was preserved in the MAPPRC.

2.2. Preparation of extracts

The dried powdered aerial parts of AJ (800 g) were extracted by maceration at room temperature with 90% ethyl alcohol. The ethanol was evaporated under reduced pressure leaving 50.66 of the total ethanol extract.

2.3. HPTLC densitometric quantification of vulgarin and epivulgarin

The extract was analyzed for vulgarin and epivulgarin (see Fig. 1) using HPTLC densitometric validated method as previously described (Foudah et al., 2018).

2.4. Animals

Male Wistar rats (160–180 g) drawn from the Animal House Colony of the National Research Centre, Egypt. Experimental procedures were approved by Institutional Animal Care and Use Committee, Cairo University (approval number: CU-II-F-86–18), complying with recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.5. Induction of diabetes

Diabetes was produced by single intraperitoneal injection of STZ (Sigma- Aldrich Corp) in 0.01 M citrate buffer (pH 4.5) at 60 mg/kg body weight. After three days, diabetes was checked by measuring fasting blood glucose (FBG) levels of rats using a blood glucose meter (Accu- Check Performa, Roche Diagnostic, Germany). Rats developed FBG greater than 250 mg/dL were considered diabetics and selected for the study.

2.6. Experimental design

Rats were divided into five groups (n = 6).

NC group: Negative control rats received the vehicle (3% Tween 80).

DC group: Diabetic control rats received the vehicle.

AJ-250 and AJ-500 groups: Diabetic rats treated with AJ at 250 and 500 mg/kg, respectively. The dose levels of 250 and 500 mg/kg of AJ used in this study were selected based on the study of Nofal et al. (2009). The vehicle and AJ had been given by gastric gavage for 12 weeks. Body weight, FBG (Trinder, 1969), insulin (Anderson et al., 1992), HbA1c (Chang et al., 1998), TST (Chen et al., 1991), and gonadotropins (Uotila et al, 1981) were measured at zero time and end of the experiment. Blood samples were obtained from overnight fasted animals through the *retro*-orbital venous plexus under mild ketamine anesthesia. One part of each blood sample was put in a tube with EDTA as anticoagulant for estimation of HbA1c. The second part was centrifuged at 3500 rpm for 15 min to separate serum. The animals were euthanized by cervical dislocation and the testes was carefully excised, weighed and prepared for sperm characteristics, histopathological and molecular analysis.

2.7. Biochemical estimation

FBG levels were measured in serum using commercial kits (Spinreact, Spain) while serum levels of insulin, TST and gonadotropins were estimated using ELISA kits (Cobas, Belgium) following the manufacturer's procedures. HbA1c was estimated using kits (QCA, Spain) in the whole blood.

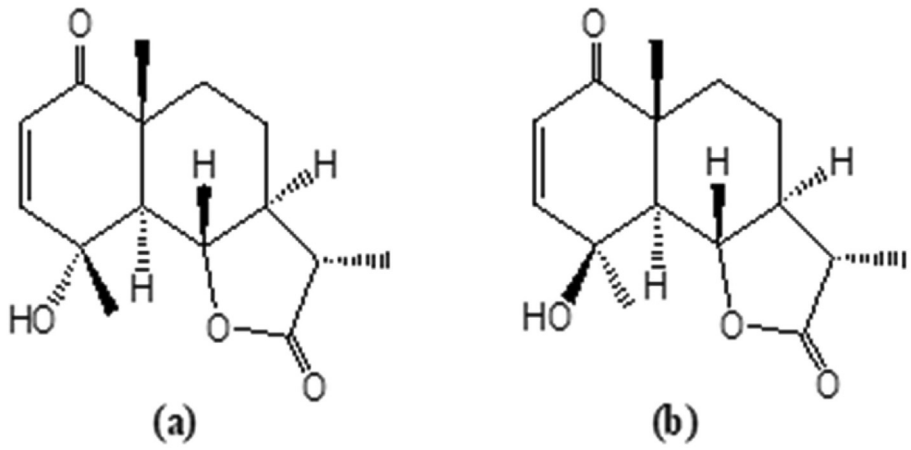

Fig. 1. Structure of vulgarin (**a**) and epivulgarin (**b**).

2.8. Assessment of oxidative stress markers

Testicular specimens were homogenized using Heidolph Diax 900 homogenizer, Germany, in 0.1 M phosphate buffer and centrifuged at 18,000 rpm for 30 min. The supernatants were collected and stored at −80 °C till analysis. The levels of reduced glutathione (GSH) (Ellman, 1959), malondialdehyde (MDA) (Ruiz-Larrea et al., 1994), superoxide dismutase (SOD) (Sun and Zigman,1978), glutathione peroxide (GSH-Px) (Mohandas et al., 1984), and catalase (CAT) (Chance and Maehly, 1955) were estimated using the assay kits obtained from Cayman Chemical Company (Michigan, USA) according to steps described by the manufacturer's guidelines.

2.9. Assessment of sperm characteristics

The testes were weighed and the cauda epididymides was carefully excised. The fluid obtained from the cauda epididymides was analyzed for sperm count, motility, viability and morphology (Soliman et al., 2019).

2.10. Histopathological examination

Testicular samples were kept in Bouin's solutions for 48–72 hr for fixation. Tissues were processed to obtain 3–4 μm thickness paraffin embedding sections. The tissue sections were stained with hematoxaylin and eosin stain (H&E). The scoring system of testes was established according to Soliman et al (Soliman et al., 2019).

2.11. Immunohistochemistry of PCNA

The immunohistochemical evaluation of PCNA was done according to reported method by Soliman et al (Soliman et al., 2019).

2.12. Real-time quantitative RT-PCR

Testicular total RNA was extracted using TRIzol reagent as directed by the manufacturer's (Invitrogen). RNA quantity and purity were determined using a Nanodrop-1000 spectrophotometer. Total RNA was used for reverse transcription and complementary DNA synthesis using the M−MLV first chain synthesis kits. The expression levels of testicular Nrf2 and HO-1 genes were analyzed in a Real-Time PCR System (Applied Biosystems, USA) using SYBR Green Mix (Invitrogen) and the following primers for Nrf2, forward: 5′- CACATCCAGACAGACACCAGT-3′, reverse: 5′-CTA CAAATGGGAATGTCTCTGC-3′ (XM_006234398.3); HO-1, forward: 5′-ACAGGGTGACAGAAGAGGCTAA-3′, reverse: 5′-CTGTGAGG GACTCTGGTCTTTG-3′ (NM_012580.2); and β actin, forward: 5′-AT GGTGGGTATGGGTCAG-3′, reverse: 5′- CAATGCCGTGTTCAATGG-3′ (NM_031144.3). Quantitative PCR was done in triplicate reactions for each cDNA sample. Each 20 μl PCR reaction consisted of 10 μl of SYBR Green Master Mix, 8 μl of cDNA, and 1 μl of each primer. Amplification of each target gene was monitored by measuring the fluorescence intensity of each sample. The fold changes of each gene expression were calculated by the comparative cycle time method (2-ΔΔCt) using β-actin (endogenous reference gene) and relative to a calibrator (Abdel-Rahman et al., 2020).

2.13. Mating study

A mating study was designed to the method described by Soliman et al (Soliman et al., 2019). Separate thirty male rats were assigned into 5 equal groups. Grouping of animals and dosing patterns were similar to those stated with protective effect against diabetes-related male sexual complications.

2.14. Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis using one-way analysis of variance (one-way ANOVA) followed by Tukey's test to determine the intergroup variability by using Graph Prism® were preformed. Pearson's correlation coefficients were used to assess the relationship between different variables in the study. A probability level<0.05 was designated as statistically significant.

3. Results

3.1. Effects on blood levels of insulin, FBG and HbA1c

After the experimental period elapsed, the blood insulin level was reduced significantly while blood glucose and HbA1c levels were increased in DC rats compared with that in the NC group (Table 1). AJ-250 and AJ-500 groups exhibited significant increase in blood insulin level and decrease in FBG and HbA1c levels compared with DC rats.

3.2. Effect on oxidative biomarkers

Efficacy of AJ against oxidative stress that induced by diabetes was assessed by estimation of oxidative enzymes, GSH and MDA in testicular tissue (Table 2). Induction of diabetes resulted in a sig-

Table 1
Effect of AJ on blood levels of glucose, insulin and HbA1c of STZ-diabetic male rats.

Groups	FBG (mg/dL)		Insulin (mIU/L)		HbA1c (%)	
	0-time	12 w	0-time	12 w	0-time	12 w
NC	98.3 ± 4.59	96.9 ± 4.57	7.40 ± 0.38	7.25 ± 0.32	6.5 ± 0.30	6.7 ± 0.33
DC	351.8 ± 10.23 [a]	349.7 ± 12.62 [a]	5.18 ± 0.30 [a]	3.1 ± 0.06 [a]	6.6 ± 0.31	11.5 ± 0.43 [a]
AJ-250	354.7 ± 17.81 [a]	117.6 ± 6.49 [a,b]	5.55 ± 0.37 [a]	6.0 ± 0.18 [a,b]	6.7 ± 0.32	7.9 ± 0.26 [a,b]
AJ-500	359.5 ± 13.09 [a]	106.6 ± 5.25[b]	5.18 ± 0.25 [a]	6.7 ± 0.38[b]	6.5 ± 0.34	7.2 ± 0.30[b]

Values are expressed as Mean ± SEM of six animals in each group.
Column with different letter means significant at $p \leq 0.05$.
[a] Significantly different from the values of the negative control rats.
[b] Significantly different from the values of the diabetic control rats.

nificant reduction in the activities of SOD, GSH-Px and CAT in the testis compared with NC rats. This reduction was improved following AJ treatments. Strong inverse correlations were observed between FBG of AJ-500 rats and the activities of their testicular antioxidant enzymes such as SOD (r = -0.82; P < 0.045), GSH-Px (r = -0.89; P < 0.018) and CAT (r = -0.83; P < 0.040). Level of GSH, a non-enzymatic antioxidant, decreased in testis of DC rats in comparable to normal rats. Conversely, testicular tissue of STZ-diabetic rats showed a significant increase in MDA level. Administration of AJ significantly increased GSH level and abolished the increase in MDA levels in testicular tissues. Interestingly, AJ-250 and AJ-500 were able to maintain SOD, GSH-Px, CAT, GSH and MDA levels in testicular tissue almost similar to the values of NC rats. Significant positive correlation was found between the FBG of the AJ-500 group and the testicular level of LPO (r = 0.84; P < 0.036).

3.3. Effect on serum levels of TST and gonadotropins

In table 3, serum level of TST in DC rats is lesser than normal value. Administration of AJ-250 and AJ-500 caused significantly higher serum levels of TST as compared to DC rats. In addition, STZ injection resulted in significant decrease in serum levels of gonadotropins in DC rats in relation to NC group. Administration of AJ-250 and AJ-500 to diabetic rats produced significant increases in blood levels of FSH and LH compared to DC group. In AJ-500 treated rats, there were positive correlations between TST and the activities of testicular antioxidant enzymes such as SOD (r = 0.84; P < 0.036], TST and GSH-Px (r = 0.89; P < 0.018), and TST and CAT (r = 0.87; P < 0.023).

3.4. Assessment of sperm characteristics

As expected, sperm characteristics were adversely affected in DC group (Table 4). DC rats showed significant decrease in count and percentages of motility and viability of their sperms compared with negative controls. Administration of AJ-250 and AJ-500 caused significantly higher sperm count, motility and viability. Blood TST levels and sperm count in AJ-500 rats were positively correlated (r = 0.85; P < 0.033). There was a marked significant positive correlation between TST level and sperm motility (r = 0.87; P < 0.025) and between TST level and sperm viability (r = 0.89; P < 0.017). Additionally, positive correlations were observed between sperm characteristics and the activities of the testicular antioxidants. Semen analysis revealed a significant higher percentage of abnormal spermatozoa in DC group in comparison with NC group. Administration of AJ at 250 and 500 mg/kg significantly lowered the percentage of abnormal-shaped sperm.

3.5. Effect on body and relative testis weights

DC rats exhibited reduction in their final body and relative testicular weights in contrast with NC group (Table 5). Treatment of diabetic rats with AJ-250 and AJ-500 significantly increased their final body and relative testicular weights. Interestingly, there were no significant differences between both AJ-500 and NC groups in terms of body and relative testes weights.

3.6. Histopathological examination

NC group revealed normal histological picture of the seminiferous tubules with main spermatogenic series and Sertoli cells (Fig. 2-A). DC rats showed marked testicular degeneration with decrease in numbers of spermatogonial cells, primary spermatocytes and secondary spermatocytes (Fig. 2-B&C). Large numbers of spermatid giant cells were seen in the degenerated seminiferous tubules. Vacuolated Sertoli cells were observed without any spermatogenic cells and marked intertubular edema was also recorded. Testis of AJ-250 (Fig. 2-D) and AJ-500 (Fig. 2-E&F) groups showed marked improvement with complete main spermatogenic and Sertoli cells. Some seminiferous tubules showed incomplete spermatogenic series. Large number of spermatozoa occupied the lumen of the epididymis.

Figure (3-A) summarized the results of the testicular scoring system. DC-treated groups showed a significant reduction in the score when compared to NC group. AJ-250 and AJ-500 treated groups showed no significant difference with NC group.

Table 2
Effect of AJ on the antioxidant profile; SOD, GSH-Px, CAT, GSH and MDA in the testicular homogenate of STZ-diabetic male rats.

Groups	SOD (U/mg protein)	GSH-Px (U/mg protein)	CAT (U/mg protein)	GSH (μmol/g tissue)	MDA (nmol/g tissue)
NC	49.4 ± 2.84	7.8 ± 0.49	12.3 ± 0.97	11.4 ± 0.82	32.8 ± 1.57
DC	27.7 ± 1.15 [a]	3.0 ± 0.21 [a]	4.8 ± 0.20 [a]	4.7 ± 0.27 [a]	58.1 ± 2.98 [a]
AJ-250	37.0 ± 1.47 [a,b]	5.8 ± 0.29 [a,b]	8.8 ± 0.40 [a,b]	7.9 ± 0.50 [a,b]	41.9 ± 1.23 [a,b]
AJ-500	42.7 ± 2.62[b]	6.6 ± 0.38[b]	10.3 ± 0.72[b]	9.6 ± 0.58[b]	35.8 ± 1.95[b]

Values are expressed as Mean ± SEM of six animals in each group.
Column with different letter means significant at $p \leq 0.05$.
[a] Significantly different from the values of the negative control rats.
[b] Significantly different from the values of the diabetic control rats.

Table 3
Effect of AJ on blood levels of testosterone, FSH and LH of STZ-diabetic male rats.

Groups	TST (ng/mL)		FSH (mIU/mL)		LH (mIU/mL)	
	0	12 w	0	12 w	0	12 w
NC	2.54 ± 0.21	2.80 ± 0.25	4.39 ± 0.27	5.14 ± 0.39	0.72 ± 0.06	0.87 ± 0.05
DC	2.62 ± 0.19	0.95 ± 0.07 [a]	4.50 ± 0.31	1.43 ± 0.11 [a]	0.76 ± 0.05	0.19 ± 0.01 [a]
AJ-250	2.45 ± 0.22	1.67 ± 0.15 [a,b]	4.57 ± 0.35	3.08 ± 0.21 [a,b]	0.77 ± 0.05	0.35 ± 0.03 [a,b]
AJ-500	2.55 ± 0.17	2.15 ± 0.18 [a,b]	4.48 ± 0.29	3.83 ± 0.27 [a,b]	0.75 ± 0.05	0.46 ± 0.03 [a,b]

Values are expressed as Mean ± SEM of six animals in each group.
Column with different letter means significant at $p \leq 0.05$.
[a] Significantly different from the values of the negative control rats.
[b] Significantly different from the values of the diabetic control rats.

Table 4
Effect of AJ on sperm cell characteristics of STZ-diabetic male rats.

Groups	Sperm characteristics			
	Count (x 10^6/mL)	Motility (%)	Viability (%)	Abnormalities (%)
NC	67.3 ± 3.83	82.0 ± 3.29	94.2 ± 4.80	7.2 ± 0.58
DC	35.3 ± 1.43 [a]	41.5 ± 1.73 [a]	52.3 ± 2.25 [a]	18.4 ± 0.78 [a]
AJ-250	52.3 ± 2.57 [a,b]	68.3 ± 3.26 [a,b]	77.5 ± 3.27 [a,b]	12.5 ± 0.84 [a,b]
AJ-500	58.8 ± 2.44 [a,b]	75.0 ± 3.91 [a,b]	84.0 ± 4.25 [a,b]	9.1 ± 0.77 [a,b]

Values are expressed as Mean ± SEM of six animals in each group.
Column with different letter means significant at $p \leq 0.05$.
[a] Significantly different from the values of the negative control rats.
[b] Significantly different from the values of the diabetic control rats.

Table 5
Effect of AJ on body weight of STZ-diabetic male rats.

Parameters	NC	DC	Diabetic		
			GLB	AJ-250	AJ-500
Initial weight (g)	168.8 ± 5.39	163.8 ± 6.15	167.1 ± 6.62	168.0 ± 5.69	165.6 ± 5.12
Final weight (g)	231.3 ± 8.89	147.2 ± 6.42 [a]	196.3 ± 5.77 [a,b]	202.6 ± 7.70 [a,b]	222.7 ± 8.14[b]
Weight gained/lost (g%)	37.03 ± 1.84	−10.13 ± 0.69 [a]	17.47 ± 0.81 [a,b]	20.59 ± 1.23 [a,b]	34.48 ± 1.88[b]
Testes relative weight (g)	1.48 ± 0.11	0.78 ± 0.04 [a]	0.97 ± 0.06 [a,b]	1.15 ± 0.08 [a,b]	1.23 ± 0.10[b]

Values are expressed as Mean ± SEM of six animals in each group.
Column with different letter means significant at $p \leq 0.05$.
[a] Significantly different from the values of the negative control rats.
[b] Significantly different from the values of the diabetic control rats.

3.7. Immunohistochemistry of PCNA

Fig. 3-B summarized the results of the image analysis of PCNA immunohistochemistry in the different groups. In the testis sections of NC group, spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids were strongly positive with PCNA staining (Fig. 4-A). Conversely, there were significantly less PCNA-positive germinal cells in DC group (Fig. 4-B&C). Interestingly, AJ-250 (Fig. 4-D) and AJ-500 (Fig. 4-E&F) treatments significantly increased number of PCNA immune-positive cells in spermatogonial cells, primary spermatocytes and secondary spermatocytes when compared with DC group. No significant difference was observed between AJ-250 and AJ-500 treated groups and NC group.

3.8. Effect on Nrf2 and HO-1 gene expression

The mRNA expression of two potent antioxidant proteins (Nrf-2 and HO-1) was examined by qPCR. As shown in Fig. 5-A, the results demonstrated that Nrf2 mRNA level was decreased in the testes of DC rats compared with negative controls. Similarly, mRNA level of HO-1 was reduced significantly in testes of DC rats compared with those in NC group (Fig. 5-B). Conversely, both Nrf2 and HO-1 transcriptional levels were significantly normalized in AJ-treated groups (250 and 500 mg/kg) compared with DC group. In AJ-500 rats, the expression level of testicular Nrf2 gene was positively correlated with sperm count ($r = 0.88$; $P < 0.020$), sperm motility ($r = 0.90$; $P < 0.013$), and sperm viability ($r = 0.91$; $P < 0.011$). Similar to Nfr2, the expression level of testicular HO-1 was positively correlated with sperm count ($r = 0.92$; $P < 0.009$), sperm motility ($r = 0.84$; $P < 0.037$), and sperm viability ($r = 0.86$; $P < 0.028$).

3.9. Mating study

In table 6, more than 90% of NC males successfully mated with females. All of these successful matings resulted in pregnancies. In contrast, number of successfully mated females exposed to DC males was significantly decreased. The reproductive ability of male rats was reduced by diabetes, as indicated by decreased fertility index compared with NC rats. Administration of AJ-250 and AJ-500 to male rats had significant increase on copulation, pregnancy, and fertility indices compared with DC rats.

4. Discussion

The used AJ extract was standardized for its vulgarin and epivulgarin contents. HPTLC technique was used for this quantification. The plates loaded with the standard vulgarin and epivul-

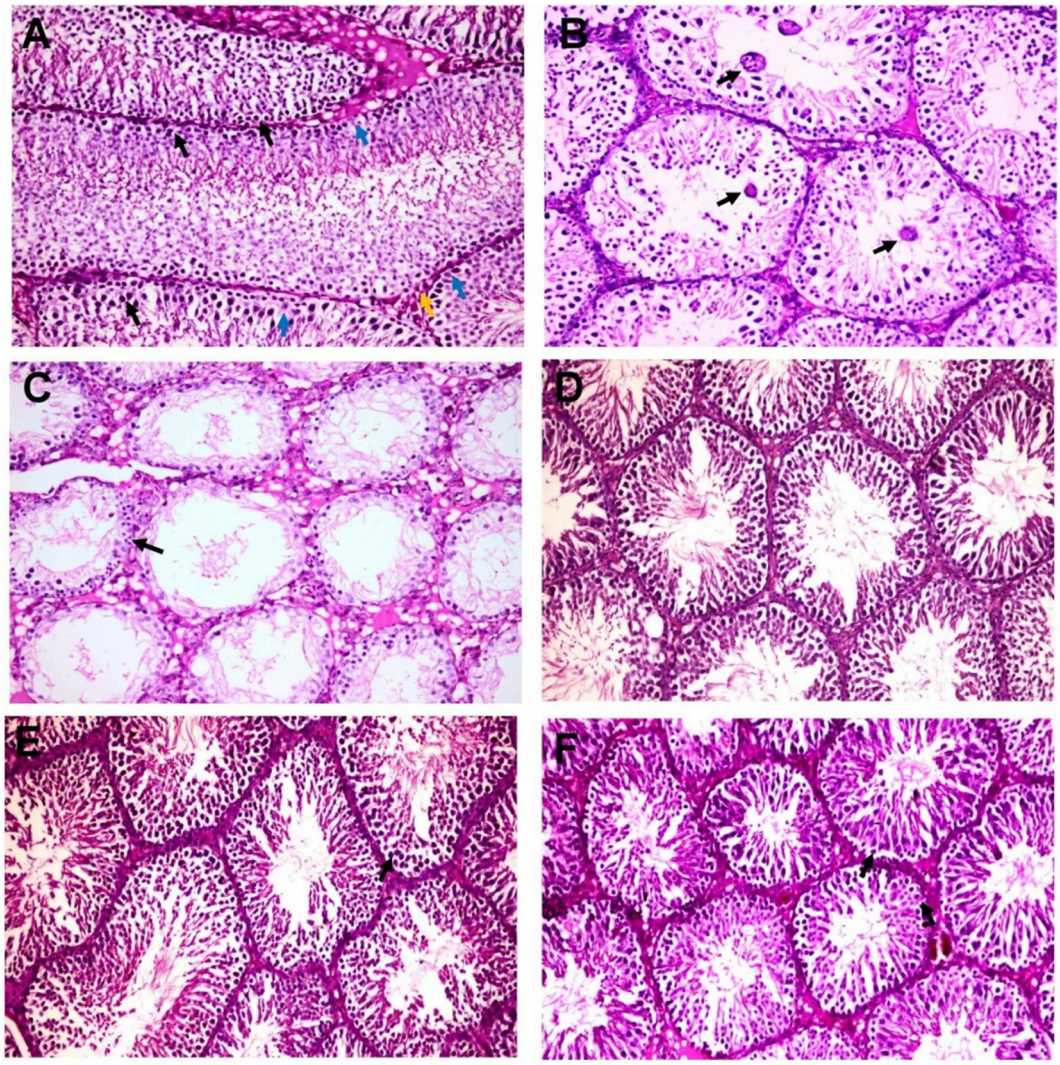

Fig. 2. Histopathological micrograph of the testes. (A) NC group showing normal histological picture of the seminiferous tubules with main spermatogenic series (black arrow), Sertoli (blue arrow) and Leyding cells (yellow arrow) (H&E X200). (B & C) DC group showing marked testicular degeneration with spermatid giant cells (arrow), intertublar edema and marked vacuolation of sertoli cell with complete absence of spermatogenic series in some tubules (H&E X200). (D) AJ-250 group showing normal seminiferous tubules with incomplete spermatogenic series in some tubules (H&E X200). (E & F) AJ-500 group showing normal seminiferous tubules with slight reduction in spermatogenic series (arrow) (H&E X200).

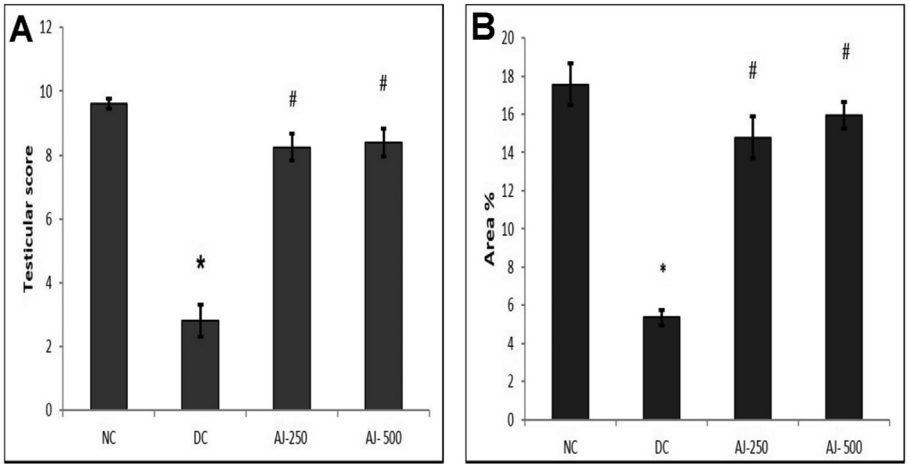

Fig. 3. (**A**) The bar chart represents testicular lesion score. (**B**) The bar chart represents PCNA immune-positive cells expressed as area %. * $p \leq 0.05$ compared with NC group; # $p \leq 0.05$ compared with the DC group.

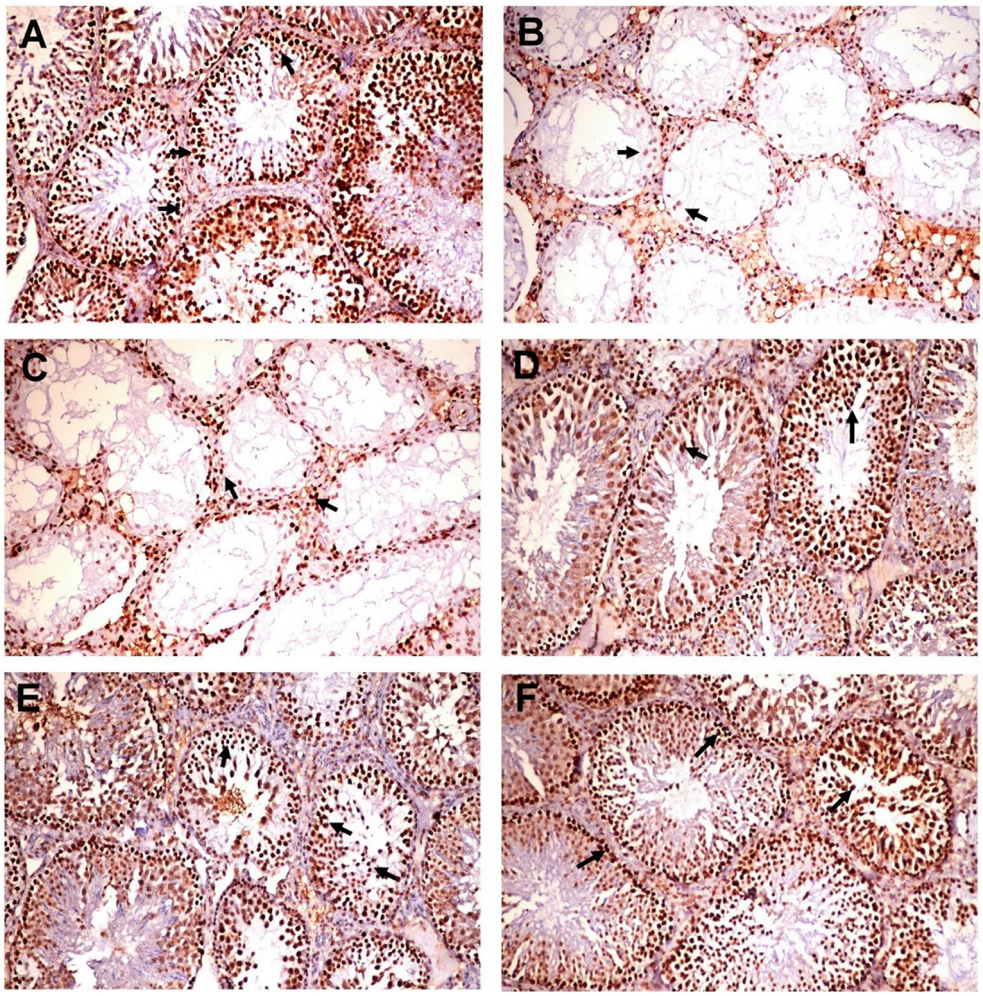

Fig. 4. Immunohistochemical analysis of PCNA expression in testes. (A) NC group showing strong immune-positive reaction in spermatogenic cells (arrow) (X200). (B & C) DC group showing very weak immune-positive reaction in spermatogenic cells and Sertoli cells (arrow) (X200). (D) AJ-250 and (E & F) AJ-500 groups showing strong immune-positive staining in the spermatogenic series (arrow) (X200).

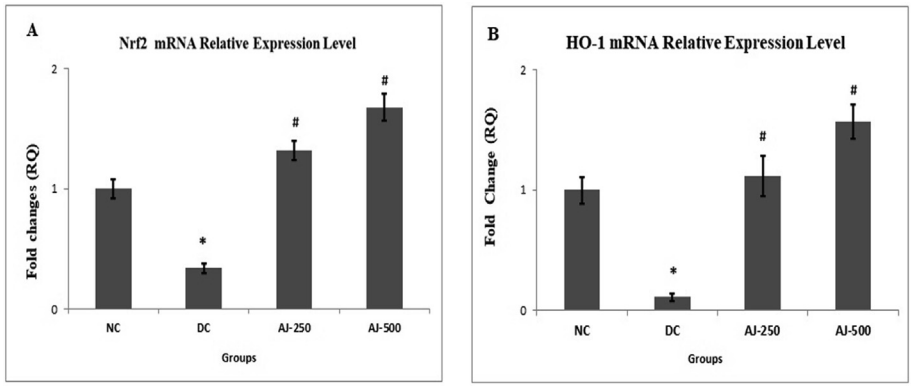

Fig. 5. Protective effect of AJ on relative mRNA expression of Nrf-2 and HO-1 against diabetes-induced reproductive injury in adult male rats. Fold-change of mRNA expression of Nrf-2 (**A**) and HO-1 (**B**) in the different experimental groups using qPCR. Data were expressed as mean ± SEM. $^{*}p \leq 0.05$ as compared to NC group. # $p \leq 0.05$ as compared to DC group.

garin and AJ extract were scanned and quantified at 224 nm for the two compounds using Camag TLC scanner III operated by WinCats software. The spots corresponding to vulgarin and epivulgarin in the sample were identified by comparing their single spot at R_f = 0.30 ± 0.02 and R_f = 0.36 ± 0.01 respectively with those of the standards. The concentration were calculated using the regression equations Y = 7.1282x + 6.2857 and Y = 6.2861x + 40.143 for vulgarin and epivulgarin, respectively obtained from the calibration curve of the standards. The percentages w/w based on the dried pant weight of vulgarin and epivulgarin were 0.241 and 0.180, respectively. The antidiabetic effect of AJ was traced to the eudesmanolide vulgarin (Rodriguez de Vera et al., 1976).

Table 6
Effect of AJ on the reproductive indices of STZ-diabetic male rats.

Groups	Copulation index	Pregnancy index	Fertility index
NC	11/12 (91.6%)	11/11 (100%)	11/12 (91.6%)
DC	4/12 (33.3%)	2/4 (50.0%)	2/12 (16.6%)
AJ-250	8/12 (66.7%)	7/8 (87.5%)	7/12 (58.3%)
AJ-500	10/12 (83.3%)	9/10 (90.0%)	9/12 (75.0%)

Copulation index = Number of sperm-positive females/Number of pairings.
Pregnancy index = Number of pregnancies/number of sperm-positive females).
Fertility index = Number of pregnancies/number of pairings.
The values in parentheses represent the percentages

AJ have been used to treat diabetes in Middle East since a long time ago. The obtained data showed that diabetic rats had elevated FBG and HbA1c levels after 12 weeks of diabetes induction. Treatment of diabetic rats with AJ reduced FBG and HbA1c levels compared with DC animals (Table 1). A lower HbA1c value confirms the antidiabetic activity of AJ and indicates good glucose control with a low risk of developing complications. Significant antidiabetic effects of AJ might be related to its bioactive components including flavonoids and saponins (Nofal et al., 2009).

Oxidative stress is one of the major mechanisms in initiation of diabetic spermatogenic disorders. Induction of STZ-diabetes encourages oxidative stress in the testicular tissue by increasing reactive oxygen species (ROS) generation and decreasing the cellular antioxidants activities. Oxidative stress occurs when extra generation of ROS in the cells overcome endogenous antioxidant defense system. Studies with both type-1 and type-2 diabetic animal models have shown significant decreases in antioxidant enzymes and a significant increase in LPO in the testis (Nna et al., 2017). In this investigation, decreased antioxidant enzymes activities in DC rats suggest that their testicular tissue were under oxidative stresses. Reports from studies using STZ-diabetic rats have shown significant decreases in testicular SOD, CAT and GSH-Px activities (Kanter et al., 2013). Meanwhile, AJ medication restored the activities of these antioxidants in testes of diabetic animals (Table 2). These results therefore show the possible efficacy of AJ in improving antioxidant defense system in testis of diabetic animals. In AJ-250 and AJ-500 treated rats, negative correlations were observed between the activities of their testicular antioxidant enzymes and FBG. Additionally, decreased tissue content of GSH has been considered as a marker of oxidative situation (Manna et al., 2010). The obtained data manifested that GSH content was significantly reduced in testis of DC rats. Administration of AJ ameliorates testicular content of GSH in diabetic animals to near-normal values. The significant role of AJ against GSH depletion in diabetic rats may attribute to its antidiabetic efficacy. The antioxidant activity of AJ was confirmed by changes in oxidative stress markers, such as LPO. The results manifested that LPO is elevated in testis of DC group. AJ significantly decreased testicular LPO, as manifested by decreased testicular MDA content of diabetic animals. This effect was the best following AJ-500 medication. In this group, there was a positive relationship between FBG and testicular MDA. Phytochemical analysis of AJ showed that it is a huge source of flavonoids including apigenin and cirsimaritin (Al-Wahaibi et al., 2018). Accordingly, the antioxidant potential of AJ may be due its flavonoids contents.

TST is the major androgen in the process of spermatogenesis and its improvement has a significant role in spermatozoal quantity and quality (Solomon et al., 2014). The outcome of the current investigation manifested that diabetes showed a remarkable suppression of FSH and LH levels in serum. Alterations in serum level of LH suppresses the release of TST from the Leydig cells (Cariati et al., 2019). Several reports have linked diabetes with disruption of the hypothalamic–pituitary–gonadal axis, thus altering the concentrations of TST, FSH and LH in males (Nna et al., 2017). The present data are in accordance with previous reports, which indicated that induction of diabetes in male rats lead to a shortage in TST level (Minaz et al., 2019). Reduced serum level of TST in DC group might be due to a direct impact of excess glucose or its metabolites on the function of Leydig cell and/or due to oxidative stress induced by diabetes. The serum levels of TST, FSH and LH in animals exposed to AJ-500 were noted to elevate in comparison with DC group (Table 3). In the correlation analysis, significant positive correlations were noted between the blood TST levels of AJ-250 and AJ-500 rats and their testicular antioxidant activities of SOD, GSH-Px and CAT. Accordingly, the rise in serum concentrations of TST in AJ-treated rats could be attributed to antioxidant and antidiabetic properties of the phytochemical contents of AJ that can counteract free radicals.

Some reproductive disorders, including decreases in the number and motility of spermatozoa, and blood level of TST are considered complications of diabetes (Kanter et al., 2013). As expected, induction of diabetes resulted in reduced spermatozoal count, motility and viability. The reason for spermatogenesis disorders is through diabetes-induced oxidative stress in testicular tissue. Oxidative stress is known to influence spermatozoa by inducing some changes in axoneme structure and consequently leads to reduction in spermatozoal motility (Syntin and Robaire, 2001). This may also elucidate the high percentage of spermatozoal abnormalities in DC group. In AJ-treated rats, sperm count, sperm motility, and sperm viability were positively correlated with the activities of testicular antioxidant enzymes. Thus, the enhancement in spermatozoal count, motility and viability observed in AJ-treated rats might be because of improvement in the antioxidant capacity, which could have suppressed oxidative stress in the testicular tissue (Ibrahim et al., 2009). Moreover, improvement in spermatozoal count and motility in diabetic rats exposed to AJ-500 may results from increased concentrations of their reproductive hormones such as TST that enhances spermatogenesis (Mylchreest et al., 2002). Additionally, ability of AJ to ameliorate hyperglycemia and HbA1c of diabetic rats could also assist to antagonize the chance of acquiring abnormal spermatozoa and sperm oxidative stress (Kanter et al., 2012).

In our study, histopathological examination demonstrated serious testicular degeneration and marked decrease in the testicular lesions score in DC group (Fig. 2). Whilst, groups that treated by AJ manifested marked amelioration in the testicular histopathological picture and lesion scoring. This improvement may be occurred as result of controlling of hyperglycemia as well as reduction of testicular oxidative stress.

As a valuable cellular proliferation marker, PCNA expression has been applied to identify spermatogonia and spermatocytes of the seminiferous tubules. PCNA is an intranuclear polypeptide and a cofactor of DNA polymerase delta that is necessary for replication and repair (Kanter et al., 2013). Some reports have explained that elevation of PCNA expression in testis is an evidence of high proliferative efficacy and enhancement of spermatogenesis, and this expression is downregulated in diabetes (Kanter et al., 2013). The present data confirmed that negative effect of reduced PCNA expression in diabetic animals is attenuated by AJ medication (Fig. 4). Therefore, upregulation of PCNA led to stimulation of cell cycle advancement and alleviation of apoptosis.

Nrf2 is a redox-sensitive transcription factor that acts as a master regulator in the cellular defense mechanism against oxidative stress-related tissue damage. Upon activation, Nrf2 translocates into the nucleus, binds to the antioxidant response elements sites in several oxidative stress response genes including HO-1 and upregulates their transcription (Wang et al., 2014). HO-1 is a cytoprotective gene regulated by Nrf2 and is expressed in the testicular tissue (Ewing and Maines, 1995). HO-1 of Leydig cells has been reported to be involved in testicular response to stress (Bae et al.,

2016). Spermatogenesis impairment induced by diabetes is associated with decreased Nrf2 and HO-1 expressions in testis. The obtained data demonstrated that AJ administration markedly restored Nrf2 and HO-1 expressions in testis comparable with NC group (Fig. 5). Several natural Nrf2 activators were reported including polyphenolic compounds and fungal secondary metabolites (Jiménez-Osorio et al., 2015). Interestingly, AJ extract was previously reported to have a high total polyphenolic content (Bhat et al., 2018). Hence, activation of Nrf2 expression in the current investigation may be attributed to these polyphenolic components. A strong positive correlation between sperm quality and TST was observed in rats treated with AJ-500. Also, a positive correlation was observed between sperm quality and Nrf2. This correlation revealed that the improved sperm quality observed following AJ administration is due to its antioxidant property. Antioxidant components such as phenolic compounds and flavonoids have been reported in *Artemisia* species (Afshar et al., 2012).

The results of mating study test showed that administration of AJ to diabetic male rats increased their ability to copulate females. The increased copulation index observed in AJ-treated groups is a reflection of recovery of erectile efficiency and capability to achieve effective copulation via increasing serum levels of TST (Fedail et al., 2016). Improvement in serum concentration of TST in AJ-treated rats could be considered as one of the reasons that increase their sexual performance, because TST regulates most components of sexual desire and penile erection (Kashif et al., 2018). Pregnancy index reflects ability of males to fertilize females. High value of pregnancy index observed in females exposed to AJ-treated males can be considered as evidence of successful fertilization. This effect can be attributed to the improved spermatozoal count and motility as Donnelly et al (Donnelly et al., 1998) reported that number and motility of sperms are directly correlated with the effective fertilization and successful pregnancy.

5. Conclusion

In conclusion, the current investigation demonstrates that AJ attenuates diabetes-associated sexual complications in male rats. Our results also suggest that AJ protects against male sexual complications through various mechanisms including improvement of hyperglycemia, activation of the antioxidant system, inhibition of LPO, maintenance of sperm quality, improvement of PCNA expression and regulation of Nrf2/HO-1 signaling pathways. The study indicates that AJ is a possible new protective agent for diabetes-related male sexual complications.

6. Recommendation

Artemisia holds a great potential for human health and its protective effect against diabetes-associated sexual complications should be more strictly and intensively analyzed. Preclinical and clinical studies need to be done on the use of this plant and further in-depth investigations are urgently necessary to study all bioactive compounds and their biomolecular mechanisms at the cellular and tissue levels.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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