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Exploring the therapeutic potential of honeybee venom in mitigating diabetic reproductive complications: insights from *in vitro* and *in vivo* studies in rats

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Abstract: Diabetes mellitus impairs male reproductive health through oxidative stress–mediated pathways. This study investigated the antioxidant capacity and reproductive effects of honeybee venom (apitoxin) in diabetic male rats. The *in vitro* antioxidant activity of bee venom showed a DPPH radical scavenging rate of 76.85 ± 1.55%, close to that of ascorbic acid (79.29 ± 1.82%, P < 0.001), and a metal chelating activity of 91.69 ± 2.55% (P = 0.227). For *in vivo* evaluation, streptozotocin-induced diabetic Wistar rats (n = 6/group) were assigned to control (C), apitoxin-treated (A), diabetic (D), and diabetic + apitoxin (DA) groups for 28 days. Diabetic rats exhibited markedly decreased sperm motility (61.4 ± 6.9%) compared to C (78.6 ± 3.8%) and A (82.9 ± 4.9%) (P = 0.007), whereas apitoxin treatment in DA rats improved motility to 77.1 ± 7.6%. Epididymal, vesicular, and prostatic weights were reduced in diabetic groups (P < 0.01). Oxidative stress was evident with elevated MDA in D (49.3 ± 31.1 nmol/mg protein) versus DA (27.7 ± 4.4) and C (29.0 ± 3.0) (P = 0.023), while GSH was significantly higher in DA (54.4 ± 18.4 µmol/mg protein) compared to D (35.2 ± 4.2) (P = 0.006). Overall, honeybee venom ameliorated diabetes-induced oxidative damage and improved sperm quality through redox modulation. These findings highlight its potential as a natural therapeutic agent against diabetic reproductive complications.
Keywords: Honeybee venom, diabetes, oxidative stress, sperm motility, antioxidant activity.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder that adversely affects quality of life in both humans and animals (Sharma et al., 2017). This detrimental effect primarily stems from the disruption of redox homeostasis within biological tissues, leading to an imbalance between oxidant generation and antioxidant defense (Korac et al., 2021). The excessive generation of reactive oxygen and nitrogen species (ROS and RNS) within tissues and organs disrupts metabolic pathways. It impairs cells sensitive to metabolic alterations, potentially leading to cellular and tissue demise. DM is characterized by significant disturbances in both glucose and fatty acid metabolism (Sivri, 2025). In managing DM, lifestyle modifications, dietary adjustments, and stress management strategies are recommended in conjunction with medications, including insulin tailored to the specific type of diabetes (Rosenfeld et al., 2025).

Given the established role of oxidative stress in diabetic complications, antioxidant-based interventions have gained interest, and bee venom presents a promising natural candidate due to its bioactive peptide components (Al-Hatamleh, 2020).
The male reproductive system is particularly vulnerable to diabetes-induced oxidative and metabolic alterations (Leisegang, 2022). Vascular, neural, and myopathic impairments contribute to reproductive dysfunction in males, thereby impacting sperm parameters as well. Perturbations in seminal activity parameters resulting from DM are often associated with infertility concerns (Gandhi et al., 2017). The susceptibility of spermatozoa membranes to peroxidative damage, owing to their lipid density, has been well-documented (Hwang, 2025), with previous reports outlining the effects of lipid peroxidation on semen quality in rats with experimental STZ-induced diabetes (Badejogbin, 2025).

Honeybee venom (apitoxin) from *Apis mellifera* is a complex mixture of peptides, enzymes, and bioactive molecules. Apitoxin has found therapeutic applications due to its recognized anti-inflammatory, antioxidant, and immune-modulating effects (Kasozi, 2020). Studies have indicated that administering apitoxin (0.1-0.3 mg per rabbit, subcutaneously) to male rabbits for 20 weeks enhances reproductive performance and antioxidant capacity (El-Hanoun et al., 2020). Similarly, research has shown that administering apitoxin at precise dosages, for specific durations, and via appropriate injection methods in female rats improves reproductive and immune performance (Elkomy et al., 2021). However, limited studies have examined the influence of bee venom on testicular redox balance and reproductive performance under diabetic conditions, particularly regarding semen quality, organ weights, and oxidative stress markers such as MDA, GSH, and NOx.

This study aimed to investigate the antioxidant capacity and reproductive effects of honeybee venom in diabetic male rats. Specifically, the objectives were:

1. To assess the *in vitro* antioxidant activity of honeybee venom using standard biochemical assays.
2. To evaluate the *in vivo* influence of honeybee venom administration on semen quality, testicular redox status, and reproductive organ morphology in streptozotocin-induced diabetic rats.
3. To examine the potential association between oxidative stress modulation and reproductive outcomes under diabetic conditions.

It was hypothesized that honeybee venom, owing to its bioactive constituents such as melittin and phospholipase A_2, may improve antioxidant status and testicular function by mitigating oxidative stress and supporting sperm quality in diabetic rats.

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2. Materials e Methods

2.1. Animals and the experimental stage

In our investigation, a cohort of 40 male Wistar Albino rats, aged 3 months, procured from the Afyon Kocatepe University Experimental Animals Research and Application Center (Ref No: 49533702/97-445-15), were employed. All procedures were designed and conducted in accordance with the ARRIVE 2.0 guidelines (Du Sert et al., 2020), ensuring appropriate animal welfare, minimizing the number of animals used, and providing detailed reporting of experimental design, randomization, and blinding. Before the commencement of the study, all rats underwent a ten-day acclimatization period in polycarbonate cages, maintained under a controlled environment of 12-hour light/dark cycles, 65% relative humidity, and a temperature of 20 ± 1°C. Throughout the study duration, *ad libitum* access to standard laboratory food and water was provided.

Rats were randomly assigned to experimental groups, and the investigators conducting injections and data collection were blinded to group allocation. Diabetes mellitus was induced in 20 rats by intraperitoneal injection of streptozotocin (STZ, CAS No. 18883-66-4; Sigma-Aldrich, Darmstadt, Germany) at a dose of 50 mg/kg body weight, freshly dissolved in citrate buffer (pH 4.5) at room temperature immediately before injection, ten days before the commencement of the study, following the protocol outlined by Furman (2015). Rats were fasted for 12 hours before STZ administration, and 10% sucrose was added to their drinking water on the first day to prevent hypoglycemia. Normal tap water was restored on the second day. Diabetes induction was confirmed by measuring fasting blood glucose levels 72 hours after STZ injection using a glucometer, with a threshold of ≥200 mg/dl indicating diabetic status (Zhang 2025). No mortality was observed among diabetic rats following STZ injection.

Apitoxin is a complex mixture of bioactive compounds (Aufschnaiter et al., 2020). The three main components of honeybee venom from *Apis mellifera* are melittin, phospholipase A_2, and apamin. Molecular structures of these components were obtained from the Protein Data Bank (PDB IDs: melittin 2MW6; phospholipase A_2 1POC; apamin 7OXF) and visualized using PyMOL (version 2.5.5, Schrödinger, LLC) (Figure 1). High-purity apitoxin (Sigma-Aldrich, V3375) was freshly prepared daily in saline (154 mmol/l NaCl) at 0.5 mg/ml (w/v) and administered intraperitoneally at 0.5 mg/kg per rat, following the protocol of Mousavi et al. (2012). This dose was selected based on previous studies demonstrating safety and efficacy in rodents.

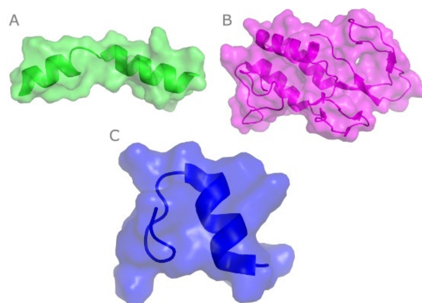

Figure 1 – Three main bioactive components of honeybee venom. The main three bioactive components of honeybee venom are presented in the figure. A depicts melittin, B shows phospholipase A_2, and C represents apamin. Peptide structures of these components were generated using PyMOL, employing the alpha-helix and beta-sheet methods, with the addition of a transparent outer layer using the molecular surface technique.

The experimental groups comprised 10 rats each: control (C), apitoxin-treated (A), diabetes-induced (D), and diabetes-induced apitoxin-treated (DA). The experiment lasted 28 days. Control and diabetes-induced groups received daily intraperitoneal saline injections, whereas apitoxin-treated groups received daily injections of freshly prepared apitoxin.

The *in vitro* antioxidant activity of apitoxin was assessed using DPPH radical scavenging activity (RSA) and metal chelating activity (MCA) assays. Apitoxin at 0.5 mg/ml and ascorbic acid, a standard antioxidant, were analyzed in triplicate, with three independent samples per assay. Samples were prepared in 200 µL volumes per well in a 96-well plate, and the reactions were incubated at room temperature for 30 minutes before measuring absorbance. The DPPH assay evaluates antioxidant activity by measuring the ability of antioxidants to neutralize 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (Sigma-Aldrich), which are quantified spectrophotometrically at 520 nm (Munteanu and Apetrei, 2021; Pinto et al., 2021). The MCA assay assesses metal-chelating activity by measuring competition with 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate (FerroZine®, Sigma-Aldrich) for Fe^{2+} ions. Chelation was quantified at 562 nm after a 30-minute incubation at room temperature (Dontha, 2016; Gulcin and Alwasel, 2022).

At the end of the experiment, rats were fasted overnight and anesthetized via intramuscular injection of Xylazine HCl (10 mg/kg) followed by Ketamine HCl (50 mg/kg). Rats were monitored for loss of reflexes and absence of pain response for 5 minutes to ensure adequate anesthesia. Euthanasia was performed by exsanguination under anesthesia, with continuous monitoring to confirm complete cessation of cardiac and respiratory activity.

2.2. Measurement of reproductive organ parameters

Following euthanasia, the testes, epididymides, seminal vesicles, and prostate glands were promptly extracted intact and rinsed with phosphate-buffered saline. Reproductive organ weights were measured using an analytical balance with 0.01 g precision.

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Epididymal sperm samples were obtained from the cauda epididymis, and a small aliquot was placed on a preheated microscope slide at 37°C containing Tris buffer solution (20 mM), as previously described by Li et al. (2022).

Sperm motility was assessed under a phase-contrast microscope at 200x and 400x magnification, examining three different fields per sample to calculate average forward-progressive motility percentages. Abnormal spermatozoa were evaluated as percentages, based on standard morphological criteria (head, midpiece, and tail defects). To assess abnormal spermatozoa rates, samples were treated with Hancock solution and evaluated using the liquid fixation method detailed by Güngör et al. (2023). Specifically, at least three drops (~50 µL each) of sperm sample were combined with 1 ml of Hancock solution in Eppendorf tubes and gently mixed for 30 seconds. A drop of the mixture was transferred to a microscope slide, covered with a coverslip, and examined under oil immersion (1000x magnification, immersion oil with refractive index 1.515). Four hundred spermatozoa per field were counted, and abnormalities in the head, midpiece, and tail were recorded to calculate the abnormal spermatozoa percentage (Yeni, 2022).

2.3. Measurement of testicular redox parameters

To measure testicular redox parameters, approximately 0.5 g of testis tissue was homogenized in 0.05 M phosphate buffer (pH 7.0) using a glass-Teflon homogenizer at 10,000 rpm for 60 seconds and centrifuged at 3000×g for 15 minutes. The resulting supernatants were collected for subsequent assays.

Lipid peroxidation was determined as malondialdehyde (MDA) equivalents using the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al., 1979; Moradi, 2024). For this assay, 0.1 ml of supernatant was mixed with 0.2 ml of 8.1% sodium dodecyl sulfate and 1.5 ml of 0.8% thiobarbituric acid. Tubes were vortexed for 10 seconds, boiled in a 95°C water bath for 60 minutes, and cooled under running tap water for 5 minutes. Then, 5 ml of a n-butanol: pyridine (15:1) mixture and 1 ml of distilled water were added, and the samples were centrifuged at 2200×g for 10 minutes. The absorbance of the colored supernatant was measured spectrophotometrically at 532 nm using a Shimadzu UV-1201 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Reduced glutathione (GSH) levels were measured using previously described methods (Tietze, 1969; Keshta, 2023). Briefly, 0.2 ml of supernatant was mixed with 3 ml of ice-cold metaphosphoric acid solution (5 g metaphosphoric acid, 1 g EDTA, 90 g NaCl in 300 ml distilled water) and vortexed. After 5 minutes, the mixture was filtered using Whatman No. 1 filter paper (Cytiva, Maidstone, UK), and 2 ml of filtrate was combined with 8 ml of 0.05 M phosphate buffer and 0.5 ml of DTNB. Absorbance was measured at 412 nm against a blank containing 2 ml of distilled water and 3 ml of metaphosphoric acid solution using a UV–Vis spectrophotometer (Shimadzu UV-1201, Shimadzu Corporation, Kyoto, Japan).

Nitric oxide (NOx) levels were measured according to the Griess method (Cortas and Wakid, 1990; Saka, 2024). Proteins were removed using Somogyi's method (Sadeghi, 2022). For the assay, 50 µl of homogenate was mixed with 200 µl of Somogyi reagent (1 g NaOH and 5 g ZnSO4 in 50 ml distilled water each) and incubated overnight. Samples were centrifuged at 3000g for 15 minutes, then 100 µl of $VaCl_3$ solution (160 mg $VaCl_3$ dissolved in 20 ml 1 M HCl), 50 µl sulphanilamide, and 50 µl N-(1-Naphthyl) ethylenediamine were added. Mixtures were incubated at 40 °C for 30 minutes, and the absorbance was read at 545 nm against distilled water using a UV–Vis spectrophotometer (Shimadzu UV-1201, Shimadzu Corporation, Kyoto, Japan).

2.4. Statistical analysis

The results are presented as mean ± standard deviation (S.D.). Normality of data distribution was assessed for each parameter (e.g., AOA, reproductive organ weights, sperm motility, abnormal sperm rates, testicular MDA, GSH, NOx) using Shapiro-Wilk and Kolmogorov-Smirnov tests. For the *in vitro* antioxidant activity (AOA) comparisons, two-tailed unpaired t-tests were applied. When the assumption of homogeneity of variances was violated (as assessed by Levene's test), statistical comparisons were conducted using Welch's ANOVA, followed by post hoc Games-Howell tests to determine differences between groups. All analyses were performed using SPSS software (version 20, IBM Corp., Armonk, USA), with statistical significance set at $P < 0.05$.

3. Results

In vitro antioxidant activity (AOA) of bee venom was high. Bee venom exhibited significantly lower DPPH radical scavenging activity (76.85 ± 1.55%) compared to ascorbic acid (79.29 ± 1.82%, $P < 0.001$). In contrast, no significant difference was observed in metal chelating activity (MCA) between bee venom (91.69 ± 2.55%) and ascorbic acid (91.14 ± 2.57%, $P = 0.227$) (Table 1).

	DPPH	MCA
Bee venom	76.85 ± 1.55^a	91.69 ± 2.55
Ascorbic acid	79.29 ± 1.82^b	91.14 ± 2.57
p	< 0.001	0.227

Table 1 – Average RSA and MCA percentages of venom and ascorbic acid diluted in saline solution. The values presented in the table indicate the mean ± S.D. The letters "a" and "b" within the respective columns denote significant statistical disparities between groups ($P < 0.05$).

Reproductive organ measurements and sperm parameters are summarized in Table 2. Epididymis weights were significantly reduced in the diabetic (D, 0.45 ± 0.09 g) and diabetic + apitoxin (DA, 0.46 ± 0.08 g) groups compared to control (C, 0.64 ± 0.06 g) and apitoxin-treated (A, 0.59 ± 0.05 g) groups ($P = 0.003$). Vesicula seminalis and prostate weights showed significant decreases in group D (0.26 ± 0.09 g and 0.16 ± 0.04 g, respectively) and DA (0.38 ± 0.15 g and 0.23 ± 0.06 g) compared to control and A groups ($P = 0.002$ and $P = 0.005$, respectively).

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Parameters	C	A	D	DA	P
Testis weight (g)	1.55 ± 0.12	1.45 ± 0.06	1.36 ± 0.16	1.45 ± 0.13	0.08
Epididymis weight (g)	0.64 ± 0.06^{a}	0.59 ± 0.05^{a}	0.45 ± 0.09^{b}	0.46 ± 0.08^{b}	0.003*
Vesicula seminalis weight (g)	0.84 ± 0.21^{a}	0.67 ± 0.08^{b}	0.26 ± 0.09^{c}	0.38 ± 0.15^{c}	0.002*
Prostate weight (g)	0.40 ± 0.11^{a}	0.32 ± 0.03^{b}	0.16 ± 0.04^{c}	0.23 ± 0.06^{c}	0.005*
Motility (%)	78.57 ± 3.77^{a}	82.85 ± 4.87^{a}	61.42 ± 6.90^{b}	77.14 ± 7.55^{a}	0.007*
Abnormal spermatozoon rate	12.35 ± 1.28^{a}	7.78 ± 1.18^{b}	12.92 ± 2.04^{a}	11.50 ± 0.95^{a}	0.009*

Table 2 – Means of reproductive organ parameters. Significant statistical variances were observed among groups, as denoted (data presented as mean ± S.D., n = 6; determined via Welch ANOVA, posthoc Games-Howell test, *P < 0.05; a,b,c: distinct letters on the same line signify intergroup disparities). Abbreviations used: C for control, A for apitoxin-treated, D for diabetic, and DA for apitoxin-treated diabetic groups.

Sperm motility was significantly lower in the diabetic group (D, 61.42 ± 6.90%) compared to control (C, 78.57 ± 3.77%), A (82.85 ± 4.87%), and DA (77.14 ± 7.55%) groups (P = 0.007). The lowest abnormal spermatozoon rate was observed in the apitoxin-treated control group (A, 7.78 ± 1.18%), significantly lower than control (C, 12.35 ± 1.28%) and diabetic (D, 12.92 ± 2.04%) groups (P = 0.009).

Analysis of testicular redox parameters revealed significant differences among groups (Figure 2). MDA levels were highest in group D (49.30 ± 31.06 nmol/mg protein), significantly higher than in the DA (27.72 ± 4.39) and C (28.98 ± 3) groups (P = 0.023). GSH levels peaked in group DA (54.4 ± 18.4 µmol/mg protein), which was significantly higher than group D (35.24 ± 4.21) and statistically similar to control (44.06 ± 9.25) and A (40.57 ± 8.43) groups (P = 0.006). NOx levels were significantly elevated in the apitoxin-treated control group (A, 8.33 ± 1.49 µmol/mg protein) compared to all other groups, while the diabetic group (D, 1.51 ± 0.25) had the lowest levels (P < 0.001).

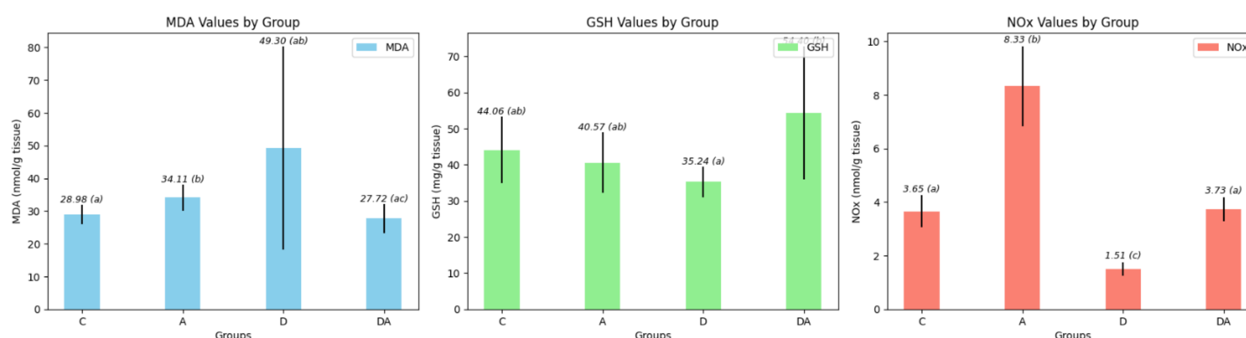

Figure 2 – Testicular tissue redox parameters. Bar graphs display the quantification, represented with various units and ±S.D., of malondialdehyde (MDA), reduced glutathione (GSH), and nitric oxide (NOx) parameters assessed among study groups (statistical analysis conducted using Welch ANOVA, posthoc Games-Howell test, P < 0.05). Group abbreviations: C for control, A for apitoxin-treated, D for diabetic, and DA for apitoxin-treated diabetic groups. Variances between groups are indicated by distinct letters.

4. Discussion

The results of this study indicate that bee venom exhibits considerable *in vitro* antioxidant activity. When assessed using DPPH radical scavenging activity, bee venom showed slightly lower activity compared to ascorbic acid (P < 0.001), whereas no significant difference was observed in metal chelating activity (P = 0.227). Similar trends have been reported in *Apis mellifera syriaca* venom, showing dose-dependent DPPH activity with slightly lower radical-scavenging activity than ascorbic acid. In contrast, metal chelation activity remained high, highlighting the potential of venom peptides in mitigating metal-induced oxidative damage (Frangieh et al., 2019). As observed in our previous study (Denk, 2023), the validity of optimal AOA for bee venom dissolved in saline solution has been confirmed in this study.

In terms of testes weights, no significant differences were observed among groups. This aligns with previous studies in which diabetic conditions did not significantly alter testicular weight, whereas accessory sex organs such as the epididymides, seminal vesicles, and prostate glands exhibited reductions (Atta et al., 2017; Soudamani et al., 2005; Zhao et al., 2021). Notably, apitoxin administration in healthy animals also led to decreased weights of seminal vesicles and prostate, which may be attributed to potential redox modulation or mild pro-oxidant effects of apitoxin (Denk and Fidan, 2021). These findings indicate that changes in organ weights may result not only from diabetic oxidative stress but also from apitoxin dosage and treatment duration.

Sperm motility was significantly reduced in diabetic rats, consistent with previous observations linking hyperglycemia-induced oxidative stress and lipid peroxidation to impaired sperm function (Khoei et al., 2019; Zhao et al., 2021; Zhu et al., 2021). Interestingly, apitoxin treatment partially restored motility in diabetic rats, suggesting a modulatory effect on the *in vivo* antioxidant capacity of the testes and epididymides, improving sperm function.

Regarding abnormal spermatozoa, the rate increased in diabetic rats as expected, reflecting oxidative damage to sperm membranes and DNA (Minas, 2024). Apitoxin treatment in healthy rats reduced the incidence of abnormal spermatozoa, consistent with previous reports highlighting its protective and antioxidant effects in reproductive tissues (El-Hanoun et al., 2020). Differences in species, dosage, and administration route may explain discrepancies between studies.

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Testicular redox parameters were affected differently across groups. MDA levels were elevated in diabetic rats, while GSH levels were decreased, reflecting oxidative and nitrosative stress associated with diabetes (Szabo, 2009; Tousoulis et al., 2012). Apitoxin administration in diabetic rats increased GSH and normalized NOx levels, consistent with reported antioxidant and redox-modulatory properties of melittin and phospholipase A_2 (Yoon et al., 2008). However, in healthy rats, apitoxin increased MDA and NOx levels without significantly affecting GSH, suggesting organ-specific and dose-dependent responses.

Overall, the study demonstrates that bee venom possesses notable *in vitro* antioxidant capacity and modulatory effects on reproductive parameters *in vivo*. These effects are consistent with previous studies indicating that apitoxin peptides can regulate intracellular oxidative stress and modulate redox balance (Denk, 2021) and can improve sperm function (Suleiman, 2021; Abdelhamid, 2023), although the magnitude of these responses may vary with metabolic status and dosage. While *in vitro* assays provide insight, *in vivo* outcomes highlight complex interactions between bee venom components, diabetic oxidative stress, and reproductive function.

5. Conclusion

This study demonstrates that honey bee venom exhibits notable antioxidant activity and modulates oxidative stress and reproductive parameters in diabetic rats. Although its *in vitro* radical scavenging capacity was slightly lower than that of ascorbic acid, bee venom maintained comparable metal chelating activity. It also exhibited the ability to modulate redox balance *in vivo*. The observed increase in reduced glutathione and normalization of nitric oxide levels in apitoxin-treated diabetic rats indicate its potential to mitigate oxidative and nitrosative stress. Moreover, improvements in sperm motility and reductions in abnormal spermatozoa rates suggest a protective influence of apitoxin on reproductive function under diabetic conditions. Collectively, these findings highlight the capacity of bee venom to influence redox homeostasis and reproductive health, supporting its possible role as a bioactive modulator within the context of diabetes-induced oxidative damage.

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Briefing notes: All animal experimental procedures were approved by the local Ethics Committee of Afyon Kocatepe University Experimental Animals Research and Application Center (Approval No: 49533702/97-445-15) and conducted in accordance with institutional guidelines.

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