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Potential of ant nest extract (*Hydnophytum formicarum*) for protection of testicular morphometry, epididymal functions, and sperm quality in male rats with alloxan-induced diabetes

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Abstract

Medicinal herbs, such as the ant nest plant (*Hydnophytum formicarum*), are promising for the management of diabetes mellitus-associated infertility. The aim of this study was to evaluate the biological activity of the ant nest plant and its capacity to mitigate the adverse effects of alloxan-induced diabetes on testicular morphology, epididymal function, and sperm quality in male rats. The tuber of the ant nest plant was extracted using methanol and then subjected to phytochemical screenings. For the experiment, 20 male white rats (*Rattus norvegicus*), aged 3–4 months and weighing 150–200 g, were equally divided into four groups. The ant nest extract was administered orally using oral gavage over 14 days. The testes, epididymis, and sperm were collected for weighing, morphometric measurements, and quality evaluation. Qualitative testing of phytochemical compounds indicated the presence of flavonoids, tannins, steroids, terpenoids, and phenolic compounds in the plants. The results revealed the protective effects of ant nest extract against the adverse fertility effects induced by alloxan and a high-fat diet, as observed in testicular weight (p=0.045), epididymal weight (p=0.041), and sperm quality (p>0.05).

Keywords: Ant nest, epididymis, methanol, spermatozoa, testes

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by the dysregulation and impaired function of insulin which consequently causes disruptions in carbohydrate, fat, and protein metabolisms [1]. This condition is divided into types 1 and 2. Diabetes mellitus represents a group of physiological dysfunctions characterized by hyperglycemia resulting directly from insulin resistance (in the case of type 2 diabetes mellitus), inadequate insulin secretion/production, or excessive glucagon secretion (in type 1 diabetes mellitus) [2]. As of 2021, the global prevalence of diabetes is 1.9%, ranking as the seventh leading cause of death worldwide [3]. The prevalence of

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DM is expected to reach up to 4.4% globally by 2030 [4]. Based on estimation, there will be 593 million diabetes patients in 2035 [5,6].

Elevated glucose levels in DM patients can produce free radicals, inducing oxidative stress [7,8]. High oxidative stress in the body can cause infertility due to damage to the nucleus and mitochondrial DNA of sperm [9,10]. Furthermore, DM results in changes in testicular morphology and epididymis [11,12]. The epididymis plays an important role in fluid absorption from the seminiferous tubules of the testes, maturation, storage, and distribution of spermatozoa to the ductus deferens before ejaculation into the female reproductive tract [13]. Increased reactive oxygen species (ROS) cause oxidative stress and organ imbalance in male animals, potentially resulting in sperm damage and infertility [12,14]. A similar report also proved that DM can cause infertility in men [15]. The infertility due to DM is likely related to a decrease in semen quality such as volume, concentration, motility, and viability but not to damage the acrosome. However, another study revealed that glycemia profiles did not affect sperm DNA fragmentation in men [16]. More studies are needed to explain how diabetes mellitus affects infertility.

Antioxidants are needed to prevent excessive free radical production, as they are essential for improving spermatogenesis [17,18]. DM is commonly treated using oral antidiabetic drugs or insulin injections. However, chemical medications have several drawbacks, including side effects, long-term estimations, and high treatment costs [19]. Medicinal plants provide an alternative for addressing DM-induced infertility. Previous studies have reported the use of medical plants to alleviate DM-induced infertility has been reported previously, such as using *Morinda tinctoria* Roxb fruit extracts [20], curcumin [21], methanolic extracts of seeds and leaves of *Strychnos potatorum* [22], *Pedilanthus tithymaloides* extracts [23], coriander [24], *Psidium guajava* Lin. [25], ethanolic extracts of *Cassia auriculata* L. [26], and *Gymnema sylvestre* extracts [27].

The potential of ant nest plants, specifically those from Aceh, in overcoming infertility due to DM has never been reported. These medicinal plants are widely used for their effectiveness in treating various diseases, specifically metabolic conditions [28]. In the last decade, many such plants have been discovered in various regions of Indonesia, including in Aceh. Research and scientific publications on ant nest plants from Aceh are still very limited, despite their long-standing use as traditional medicine in the community. Ethanol extracts of ant nests collected from different parts of Indonesia, such as Central Maluku Regency (Maluku) and Fakfak Regency (Papua), have shown strong antioxidant activity in vitro [29,30]. Environmental factors such as carbon dioxide, temperature, ozone, lighting, soil salinity, soil water, and soil fertility significantly impact plants' physiological and biochemical responses, including secondary metabolite production [31]. Therefore, the objective of this study was to determine the biological activity of ant nest plants originating from Aceh and to evaluate the capacity of ant nest extract in mitigating the adverse effects of alloxan-induced hyperglycemia on testicular morphology, epididymal function, and sperm quality in male rats.

Methods

Sample preparation and extraction process

The plant specimen was collected from tropical forests in Aceh, specifically Aceh Besar Regency. The taxonomic classification was examined at the Directorate of Scientific Collection Management, National Agency for Research and Innovation (BRIN), Indonesia, confirming that the specimen belongs to the Kingdom *Plantae*, within the Subkingdom *Tracheobionta*, specifically the genus *Myrmecodia* and the species *Hydnophytum formicarum* Jack.

The tuber of the ant nest plant (*H. formicarum*) was cleaned, washed, cut into small pieces, and air-dried for approximately 10 days. Thereafter, the dried tubers were crushed into fine powder. The extraction was performed by macerating the dried ant nest fine powder (250 g) with methanol (2.5 L) in a 5-L container. The mixture was occasionally stirred in a covered container and left to stand for five days, shielded from light, with occasional stirring. After five days, the sample was filtered, and the residue was re-macerated with 25 parts of methanol solvent. This mixture was left to stand for two days. Subsequently, the macerate was evaporated using a rotary evaporator at a temperature of 45°C until a concentrated extract was obtained.

Phytochemical screening

Phytochemical screening was conducted at the Natural Pharmaceutical Laboratory, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala, Banda Aceh, Indonesia. The analysis followed the guidelines in a previously reported study [32], where the Thermo Scientific ISQ 7000 single quadrupole gas chromatography-mass spectrometry (GC-MS) was used to identify compounds. The gas chromatography (GC) used was equipped with column TG-5MS, where the inlet temperature was set at 220°C and spitless injection mode was selected (split flow rate 50 mL/min at 0.8 min). The septum purge flow was set at 5 mL/min, with helium as the carrier gas at a 1.2 mL/min flow rate. The temperature gradient was set at 4°C/min, with a ramp of 30°C/min to 150°C, followed by a ramp of 100°C/min to 250°C. The emission current was 50 µA, with the MS transfer line temperature at 250°C and the ion source temperature at 200°C.

Experimental animals

A total of 20 male white rats (*Rattus norvegicus*) were acclimatized to experimental animal cages for two weeks. The inclusion criteria in this study were male white rats of Wistar strain, aged 3–4 months and weighed 150–200 g. The rats should not have macro anatomical abnormalities or any prior treatment. Sick rats during the adaptation period and dead rats during the treatment were excluded. Before treatment initiation, rats were weighed, and their rectal temperature was measured. The cage, constructed from plywood and wire mesh, provided drinking water and feed. The animals were daily fed 20 g of pellets and provided ad libitum access to drinking water. Throughout the preparation phase, the cages were maintained under standard room conditions at a constant temperature of 25–27°C, humidity levels between 30–70%, and a 12-hour light-dark cycle.

The rats were divided equally into four groups of five each (block randomization). These groups included the normal group, alloxan-induced, ant nest 100 mg/kg BW, and ant nest 200 mg/kg BW. The ant nest extract was administered orally using a gastric tube for 14 days. The rats were placed on a high-fat diet starting seven days prior to alloxan induction, and this feeding regimen was continued for 14 days. The diet consisted of 50 g egg yolk, 1 kg pellet (T-794), and 100 g fat which had been converted into oil and mixed homogeneously.

Diabetes induction with alloxan

Diabetes induction in rats was conducted using alloxan monohydrate, following the instructions outlined by a previous report [21]. Alloxan was administered intraperitoneally at a single dose of 120 mg/kg BW. It is important to note that diabetes induction with alloxan was conducted on day 7 after a high-fat diet.

Examination of blood sugar levels

Blood glucose levels were measured using Easy Touch GCU® (measuring range of 20–600 mg/dL) on day 0 (before alloxan injection) to determine normal sugar levels in each treatment group. The diagnosis of diabetes was made 72 hours after alloxan induction, following a 12-hour fast, with rats being classified as diabetic when their glucose levels were above 200 mg/dL [21]. Blood samples were collected from the veins in rats' tails. Following the administration of ant nest extract, subsequent glucose measurements were taken on days 7 and 14.

Sample preparation and collection

Following 14 days of treatment with ant nest extract, rats were euthanized using Zoletil® 100 with a dose of 40 mg/kg BW after overnight fasting. The testes and epididymis were collected for weighing and morphometric measurements. The cauda epididymis was placed in 2 mL of phosphate-buffered saline (PBS) and gently sliced using a scalpel. Subsequently, the suspension was stirred gently and allowed to stand for five minutes, facilitating the release of sperm. This suspension was used for sperm quality evaluation [33].

Testicular and epididymal morphometric measurements

The reproductive organs of the white rats were excised through surgery, and euthanasia was conducted using a determinant method. After a few minutes, the animals were retrieved and placed on a board in a dorsal recumbent position. Subsequently, they were dissected to obtain the

testes, and the testicular organs were rinsed with physiological NaCl solution and placed in a petri dish for morphometric observations comprising of length, width, weight, and volume. The weight of the testes/epididymis was measured using a digital scale, and their volume was determined through immersion in a graduated cylinder filled with NaCl solution. Finally, the length and width were measured using a caliper with a millimeter scale.

Statistical analysis

Data from the phytochemical screening were reported descriptively. Meanwhile, data on testicular morphometry, epididymis morphometry, and spermatozoa quality were analyzed using one-way analysis of variance (ANOVA) and followed by the Tukey test (DMRT). Statistical significance was achieved if $p<0.05$. The statistical analysis was carried out on SPSS version 20.0 (SPSS Inc., Chicago, USA).

Results

Phytochemical profile and blood glucose lowering effect of ant nest

Qualitative phytochemical screening showed that ant nest extract was positive for flavonoids, tannins, steroids, terpenoids, and phenolics (**Table 1**). Negative results were found for alkaloids and saponins (**Table 1**). The results of blood glucose levels in rats before alloxan induction and after treatment with ant nest extract are presented in **Figure 1**. Based on observations, the highest blood glucose levels were discovered in rats induced with alloxan without receiving ant nest extract on days 7 and 14 after treatment. The administration of ant nest extract on day 7 after treatment showed that 200 mg/kg BW was more effective in reducing blood sugar levels than 100 mg/kg BW ($p=0.022$). However, on day 14 after treatment, both dose groups exhibited no significant difference ($p=0.079$).

Table 1. Results of phytochemical screening of methanol extract of ant nest

Secondary metabolites	Observation
Flavonoids	Detected
Terpenoids	Detected
Steroids	Detected
Tannins	Detected
Phenolic	Detected
Saponins	Not detected
Alkaloids	
Dragendroff	Not detected
Meyer	Not detected
Wagner	Not detected

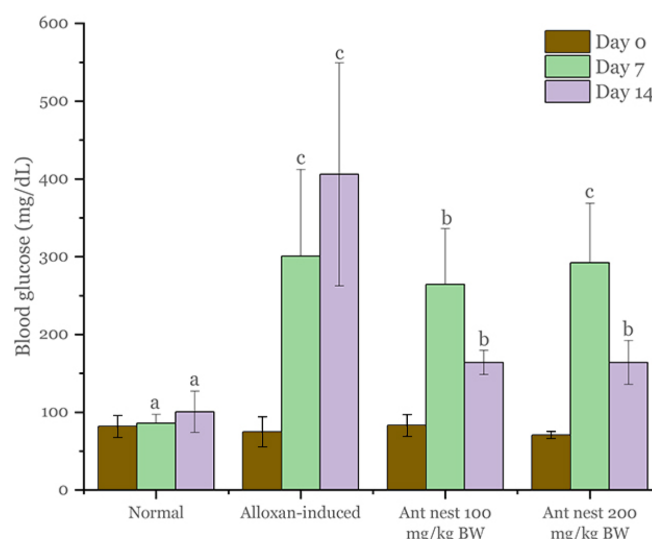

Figure 1. Effect of ant nest extract on blood glucose level of alloxan-induced rats. Different alphabets indicate the statistical significance at $p<0.05$ on the same observation day.

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Effect of ant nest extract on testicular and epididymal morphometry

The group that was solely alloxan-induced had the lowest average rats' testicular weight (0.34±0.05 g), followed by normal (*p*=0.048), ant nest 100 mg/kg BW (*p*=0.044), and ant nest 200 mg/kg BW (*p*=0.038) at 0.65±0.03 g, 0.66±0.04 g, and 0.67±0.04 g, respectively. The lowest length was observed in the alloxan-induced group at 1.26±0.11 cm, followed by normal (*p*=0.035), ant nest 100 mg/kg BW (*p*=0.038), and ant nest 200 mg/kg BW (*p*=0.048) at 2.00±0.19 cm, 2.02±0.23 cm, and 2.34±0.034 cm, respectively. The alloxan-induced group exhibited the lowest width at 0.26±0.11 cm, followed by normal (*p*=0.041), ant nest 100 mg/kg BW (*p*=0.032), and ant nest 200 mg/kg BW (*p*=0.029), at 1.10±0.16 cm, 1.04±0.11 cm, and 1.08±0.15 cm, respectively (*p*=0.030). Moreover, this group also had the lowest volume at 0.28±0.08 mL, followed by normal (*p*=0.040), ant nest 200 mg/kg BW (*p*=0.037), and ant nest 100 mg/kg BW (*p*=0.027), with values of 1.00±0.14 mL, 1.40±0.39 mL, and 1.56±0.43 mL, respectively. Similarly, the alloxan-induced group recorded the lowest diameter, followed by the ant nest 100 mg/kg BW, ant nest 100 mg/kg BW, and normal group, with diameters of 1.14±0.15 cm, 1.80±0.23 cm, 2.94±0.11 cm, and 3.04±0.11 cm, respectively (*p*=0.021).

The results of morphometric measurements, including length, width, and weight of the epididymis of the Wistar strain white rats treated with alloxan and various groups of ant nest tuber extract treatments are presented in **Table 2**. Alloxan-induced rats had the lowest length, followed by ant nest 100 mg/kg BW (*p*=0.033), normal (*p*=0.038), and ant nest 200 mg/kg BW (*p*=0.043), measuring 1.07±0.74, 3.52±1.24, 3.86±1.21, and 3.66±0.58 cm, respectively. The lowest width was also observed in the alloxan-induced group, followed by normal (*p*=0.022), by ant nest 100 mg/kg BW (*p*=0.037), and by ant nest 200 mg/kg BW (*p*=0.041), measuring 0.28±0.08, 0.58±0.08, 0.72±0.06, and 1.08±0.11 cm, respectively (*p*=0.032). The lowest average rat's testicular volume and diameter were recorded in the P1 group alloxan-induced, followed by normal (*p*=0.012), ant nest 200 mg/kg BW (*p*=0.020), and ant nest 100 mg/kg BW (*p*=0.044), at 0.28±0.08, 1.00±0.14, 1.40±0.39, and 1.56±0.43 mL, as well as diameters of 1.14±0.09, 1.52±0.08 (*p*=0.044), 1.56±0.45 (*p*=0.047), and 2.06±0.67 cm (*p*=0.049), respectively. Overall, these results showed that administration of ant nest extract effectively counteracts the decline in length, width, volume, and diameter of the testes in white rats induced by alloxan and a high-fat diet.

Table 2. Effects of the ant nest administration on the morphometric parameter of testes and epididymis of the rat model

Groups	Weight (g)	Length (cm)	Width (cm)	Volume (mL)	Diameter (cm)
Testicular morphometry					
Normal	$0.65±0.03^{b}$	$2.00±0.19^{b}$	$1.10±0.16^{b}$	$1.00±0.14^{b}$	$3.04±0.11^{c}$
Alloxan-induced	$0.34±0.05^{a}$	$1.26±0.11^{a}$	$0.26±0.11^{a}$	$0.28±0.08^{a}$	$1.14±0.15^{a}$
Ant nest 100 mg/kg BW	$0.66±0.04^{b}$	$2.02±0.23^{b}$	$1.04±0.11^{b}$	$1.56±0.43^{c}$	$2.94±0.11^{c}$
Ant nest 200 mg/kg BW	$0.67±0.04^{b}$	$2.34±0.34^{c}$	$1.08±0.15^{b}$	$1.40±0.39^{c}$	$1.80±0.23^{b}$
ANOVA *p*-value	*p*=0.045	*p*=0.038	*p*=0.030	*p*=0.026	*p*=0.021
Epididymal morphometry					
Normal	$1.00±0.14^{b}$	$3.86±1.21^{a}$	$0.58±0.08^{a}$	$0.64±0.02^{a}$	$1.52±0.08^{ab}$
Alloxan-induced	$2.8±0.08^{a}$	$1.07±0.74^{ab}$	$0.28±0.08^{b}$	$0.31±0.06^{b}$	$1.14±0.09^{a}$
Ant nest 100 mg/kg BW	$1.56±0.43^{c}$	$3.52±1.24^{a}$	$0.72±0.06^{a}$	$0.59±0.43^{ab}$	$2.06±0.67^{b}$
Ant nest 200 mg/kg BW	$1.40±0.39^{c}$	$3.96±0.58^{a}$	$1.08±0.11^{a}$	$0.49±0.03^{c}$	$1.56±0.45^{ab}$
ANOVA *p*-value	*p*=0.041	*p*=0.032	*p*=0.048	*p*=0.045	*p*=0.048

Different alphabets in the same column show significant differences ($p<0.05$)

The spermatozoa quality of DM-induced rats treated and untreated with ant nest extract is depicted in **Figure 2**. Based on statistical tests, the group receiving ant nest extract treatment exhibited significantly improved sperm quality compared to the group without treatment. According to the data obtained, ant nest extract dosage had a significant impact on sperm concentration (*p*=0.034) and viability (*p*=0.046), while sperm motility (*p*=0.909) and abnormality (*p*=0.996) remained unaffected. This underscored the treatment's potential to enhance sperm concentration, motility, and viability, while simultaneously reducing the percentage of abnormal sperm morphology in rats with DM.

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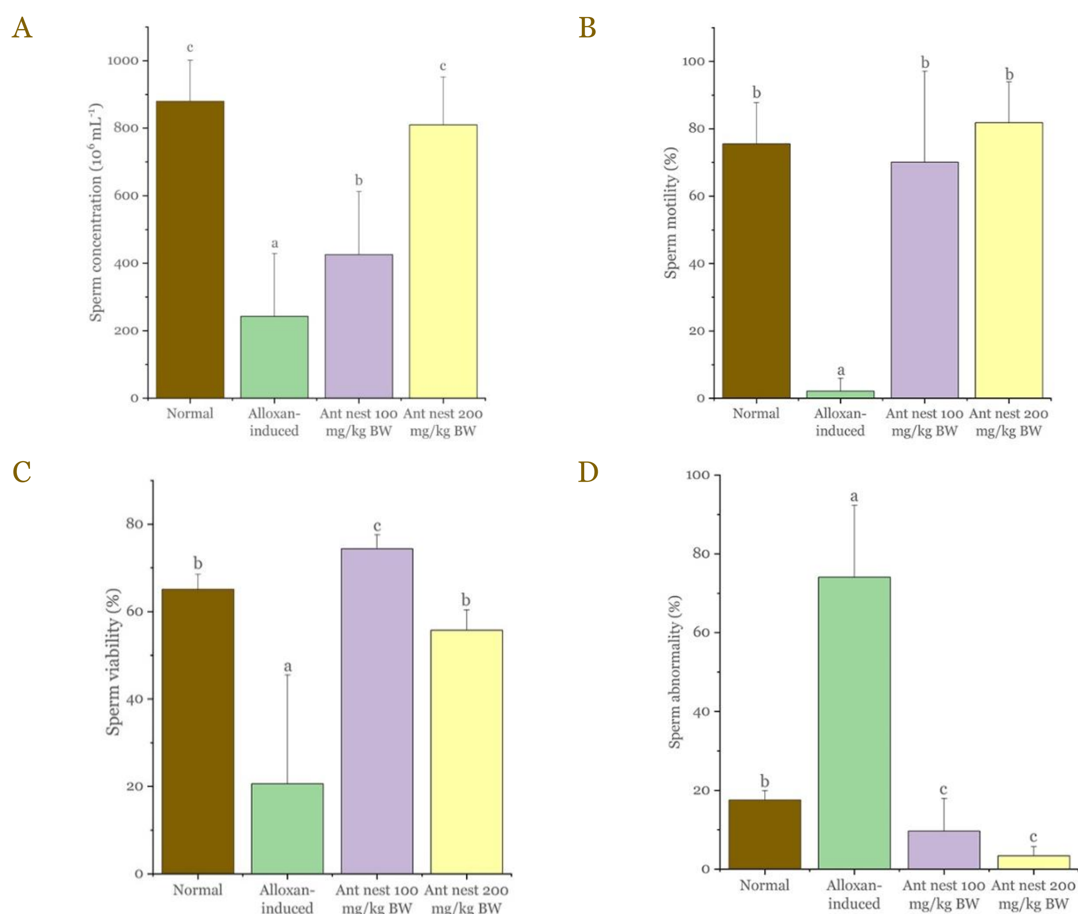

Figure 2. Effects of the ant nest administration on sperm concentration (A), motility (B), viability (C), and abnormality (D). Different alphabets indicate the statistical significance at $p<0.05$ on the same observation day.

Discussion

The present study revealed that administration of ant nest extract for seven days reduced the blood glucose levels of alloxan-induced rats. A more significant decrease was observed on day 14. In a previous study, the ability of ant nest extract to decrease blood glucose levels was attributed to the presence of flavonoids, terpenoids, steroids, tannins, and phenolics. [38]. These compounds have been suggested to regenerate pancreatic β-cells and stimulate insulin secretion [39]. Moreover, phytocompounds from ant nest extract were reported to regulate glucose absorption and enzyme expression related to carbohydrate metabolism [40]. The reduction of blood glucose could be associated with the induced phosphorylation of the insulin receptor by forming glucose transporter 4 (GLUT-4) by the compounds in ant nest extract [41]. The reduction in blood glucose levels in the ant nest extract-treated groups indicates its effectiveness in enhancing insulin action at the cellular level and its potential as an antidiabetic agent [42-45].

Findings from the present study indicate that the administration of ant nest extract effectively mitigates the reduction in testicular weight, length, width, volume, and diameter in white rats induced by alloxan and a high-fat diet. Similarly, the treatment appeared to restore epididymal morphology in diabetic rats, providing a protective effect against the disruption of spermatogenesis and resulting in conditions similar to those of normal rats. In terms of sperm quality, the extract could increase sperm concentration, motility, and viability. Increased sperm abnormality was significantly reduced by the administration of ant nest extract. This restorative effect is attributed to active compounds contained in the ant nest extract. Previously, ant nests were found to contain anthocyanins such as including delphinidin, cyanidin, and pelargonidin [46]. Additionally, the presence of tannins was thought to contribute to the strong antioxidant properties of the extract [47]. In addition to these polyphenolic compounds and tannins, ant nest extract also contains vitamin C, which acts as an additional antioxidant capable of suppressing

the activity of ROS [48]. These antioxidant-rich contents could protect Langerhans cells (responsible for insulin secretion) and Leydig cells (responsible for testosterone production) from oxidative stress. Moreover, the flavonoid contents in the extract could improve the activity of pancreatic β cells in insulin secretion [49]. In a previous study, flavonoids also affected the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), the two important hormones in testosterone synthesis [50].

Interpretation of the findings is limited by the small sample size and short study duration, which may not have been sufficient to capture the long-term effects and potential side effects of the ant nest extract. The present study also did not investigate the pure isolate from the ant nest extract. While the study demonstrated the extract's effectiveness in reducing blood glucose levels and improving sperm quality, it lacked detailed mechanistic insights into how these effects are achieved. Measuring endocrine hormones (such as FSH and LH) could explain the mechanism behind the effect on sperm quality. Moreover, the study was conducted on a single species (rats), limiting the generalizability of the findings to other species, including humans. Cross-species studies are necessary to validate the results and explore their relevance to human health. Future studies should employ larger sample sizes, observe for longer durations, perform compound isolation of ant nest extracts, and carry out cross-species studies with standardized methods.

Conclusion

It can be concluded that the methanol extract of ant nest holds the potential to shield against the deterioration of testicular morphology, epididymis function, and sperm quality in diabetic rats, particularly when induced by a high-fat diet and alloxan. To ensure better validation of the results obtained, further studies are needed to improve the limitations of this study by adding levels of extract doses, extending the duration of administration, recruiting a larger sample size, and measuring the concentrations of FSH, LH, and testosterone hormones after administration of ant nest extract.

Ethics approval

This study has been approved by the ethics committee of the Veterinary Medicine Faculty, Universitas Syiah Kuala, Banda Aceh, Indonesia, with certificate no: 255/KEPH/I/2022.

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

How to cite

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