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HISTOLOGICAL AND BIOCHEMICAL EFFECT OF *AZADIRACHTA INDICA* ON DIABETES-INDUCED TESTICULAR DAMAGE

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ABSTRACT

Introduction: Infertility is among the major health problems of people living with diabetes. The suggestion that herbal drugs has promising effects on the male infertility is contentious. This study aimed to determine the effect of *Azadirachta indica* on diabetes-induced testicular damage.

Materials and Methods: Twenty-four Wistar rats were allotted into six groups (n=4). Group I was normal control. Group II was induced with diabetes but not treated. Groups IV-VI were induced with diabetes and treated with 200, 300, and4300 mg/kg extracts while Group III was treated with Metformin once daily for 14 days. Animals were euthanized, and blood samples were collected for Glucose and Testosterone assays while testes were excised and processed by the paraffin wax method.

Result: Oral administration of *Azadirachta indica* extract significantly ($P<0.05$) reduced blood glucose, and increased testosterone levels in diabetic animals in a dose-dependent manner. Extract also improved the histology of the testes.

Conclusion: *Azadirachta indica* phytochemicals reduced blood glucose level and improved the histology of the testis. Further study is recommended to identify the phytochemicals and mechanism of action.

Key: Diabetes, *Azadirachta indica,* Hypoglycemia, Infertility

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by persistent in blood glucose level resulting from defects in insulin secretion, insulin action or both (American Diabetes Association, 2023). Chronic diabetes is associated with numerous complications, including alterations in reproductive function. Infertility due to testosterone deficiency is one of the major health problems affecting reproductive age couples (Saad *et al*., 2011). About 40-50% of infertility in human has been associated to male factor commonly due to deficiencies in testosterone and semen quality is used as a surrogate measure of male fertility (Ahmed *et al*., 2024). People begin to show concern if conception cannot be achieved after 12 months of regular unprotected intercourse with increasing incidence rates in males (Adewoyin *et al*., 2017).

Azadirachta indica, also referred to as neem is widely recognized for its pharmacological properties, including anti-inflammatory, antioxidant, hypoglycemic, and hormone modulating effects (Subapriya *et al*., 2005; Alzohairy, 2016). The hypoglycaemic activity of Neem have been demonstrated in experimental models (Subapriya *et al*., 2005; Satyanarayana *et al.*, 2015). Its antidiabetic properties are attributed to the presence of bioactive chemicals, such as flavonoids and alkaloids (Alzohairy, 2016). Even though the hypoglycemic activity of *Azadirachta indica* is encouraging, more research is necessary to determine the effectiveness of neem leaf extract as well as its effects on the teste's biochemistry and histology.

However, limited studies have investigated its effect on testosterone levels in diabetic conditions. considering the hormonal disruptions associated with diabetes, it is imperative to explore the effect of *Azadirachta indica* leaf extract on testosterone regulation, particularly in an alloxan induced diabetic model. The objective of this research is to examine the potential antidiabetic and androgenic properties of neem leaf methanolic extract in diabetic Wistar rats produced by alloxan.

MATERIALS AND METHOD

Chemical and reagents used

Alloxan monohydrate was obtained from the Department of Pharmacology, Bingham University Karu. Glucose oxidase kit was used to measure the blood glucose levels. Others include; *Azadirachta indica* leaf, Alloxan, Metformin, methylated spirit, chloroform, formalin, methanol.

Experimental animals

Twenty-four (24) Wistar rats were obtained from the Department of Pharmacology, Bingham University Karu. The rats were kept in the Animal Care Unit of Bingham University, Nasarawa state, Nigeria. They were given a period of two weeks before the commencement of the experimental procedures to acclimatize to the new environment. Animals received standard rat diet and distilled water *ad libitum* on a daily basis under hygienic conditions.

The Animal Care Unit was well ventilated and swept every day, and the rats were housed in properly aerated plastic cages (4 per cage) alongside with wood dust as beddings. The beddings of the animals were changed every three days. They were kept under the temperature of 27 degrees Celsius under 12 hours of light and 12 hours of darkness periodically.

All experimental investigations, rat handling and treatment conforms to the guidelines of the National Institute of Health (National Institute for Health and Care Excellence, 2023). The rats were housed following the principles for animal care as recommended in Helsinki's 1964 declaration. The study protocol was approved by the Animal Ethics Committee of Bingham University, Karu, Nasarawa State.

Preparation of plant extract

Fresh Neem leaves were harvested from Neem trees preferably from healthy pesticide-free plants. It was extracted using the Maceration method with little modification. Three hundred grams of the plant powder were weighed and left to soak in distilled water. The mixture was allowed to stand for 24 hours and filtered using Whatman’s no.1 filter paper. The filtrate was then evaporated to dryness using a water bath and hot air oven at a controlled temperature of 60 degrees Celsius. (Gamde *et al,* 2023a).

Experimental design

In this study, twenty-four Wistar rats (4 rats per group) weighing 120±20 was used. The Wistar rats were left to acclimatize for two weeks and were fed with vital feed and water. Administration began and lasted for 14 days (2 weeks). The blood glucose level of the rats was measured before the commencement of the experiment and 72 hours after alloxan administration using a glucometer. The testosterone levels of the rats were measured before the commencement of the experiment using Microwell enzyme linked immunoassay (ELISA) technique (Yelwa *et al*., 2020). The rats whose blood glucose levels were greater than 200mg/dL were included in test groups II-VI (Gamde *et al*., 2023b). The rats were then grouped as follows:

Group I: Normal control group that was fed with normal feed and distilled water for 2 weeks

(no induction of diabetes).

Group II: Alloxan-induced diabetic control group that was induced with diabetes but not treated.

Group III: negative group that was induced with diabetes and treated with metformin for two weeks

Group IV: negative group that was induced with diabetes and treated daily with azadirachta indica leaf aqueous extract 200mg/kg body weight for 2 weeks.

Group V: negative group that was induced with diabetes and treated daily with azadirachta indica leaf aqueous extract 300mg/kg body weight for 2 weeks

Group VI: negative group that was induced with diabetes and treated daily with azadirachta indica leaf aqueous extract 400mg/kg body weight for 2 weeks.

After the last dose, the animal's body weights were recorded using a weighing balance. The animals were euthanized and blood samples were individually collected via cardiac puncture into plain bottles. The serum was removed from the blood; samples were labeled and stored for future analysis at 4 °C. Blood serum glucose was estimated from the serum by using a standard kit. For the determination of glucose, samples were prepared by taking 20µl of serum in small glass bottle added 1200µl of reagent present in kit mixed samples and incubated it for 10 minutes at 25°C and absorbance was read at 500 nm of the standard and samples against reagent blanks within 60 minutes (Nadir *et al,* 2013). The blood samples were spun at 2500 rpm for 10min using a centrifuge. Serum samples were assayed for levels of testosterone using the Microwell enzyme linked immunoassay (ELISA) technique (Yelwa *et al*., 2020). The testes were excised via abdominal incision and processed using the paraffin wax method. The testes were fixed in 10% buffered formalin for 24 hours. After dehydration by three changes of ethanol, clearing in xylene, and embedding in molten paraffin, 3 µm of the paraffin mass was cut into a section using a microtome (Surgcare Microtome, Model 335A USA). Cut sections were deparaffined and stained with hematoxylin and eosin (H&E) for the observation of histopathological changes (Gamde *et al.,* 2019).

Ethical approval

Appropriate ethical approval was obtained in writing from the university's ethical committee for scientific research.

Statistical analysis

The analysis of the result will be performed with the statistical packet graph pad and data will

then be expressed as mean standard error of mean. ANOVA will then be used to determine the level of significance. p value <0,05 will be taken as the significant level.

RESULT

Table 1 presents the effect of normal feed and water on the blood glucose levels of rats across different groups before and after two weeks of treatment. The glucose levels before the commencement of the study ranged from 4.1 to 4.9 mmol/L, while after two weeks, the levels remained relatively stable, showing minor fluctuations across all groups. The mean glucose level before the study was 4.53 mmol/L, which slightly decreased to 4.51 mmol/L after two weeks. This minimal change in glucose levels indicates that normal feeding and water consumption did not significantly affect the blood glucose levels of the rats during the two-week period.

The p-value of 0.9648 suggests that there is no statistically significant difference in blood glucose levels before and after two weeks of feeding and water intake. This indicates that the variations observed in glucose levels are likely due to random chance rather than any effect of the diet or water intake. Overall, the data suggests that the normal feed and water regimen had no meaningful impact on the blood glucose levels of the rats over the course of the study.

Table 1 also compares the blood glucose levels of alloxan-induced diabetic rats before alloxan administration, after alloxan induction, and following various treatments. The untreated Group II showed a substantial increase in blood glucose from 3.525 mmol/L before alloxan to 9.598 mmol/L after treatment, with a p-value of 0.538, indicating no significant change in glucose levels due to treatment. Similarly, Group III, which was treated with metformin, had an increase in glucose from 4.6 mmol/L to 14.98 mmol/L after alloxan, and then a decrease to 8.74 mmol/L after treatment. However, this change also did not reach statistical significance ($p = 0.285$), suggesting metformin had a limited effect on reducing glucose levels in this context.

Groups IV and V, treated with 200 mg/kg and 300 mg/kg body weight of *Azadirachta indica* leaf methanolic extract, respectively, showed a significant increase in glucose levels post-alloxan, followed by reductions after treatment. The p-values for these groups (0.407 and 0.408) suggest that the changes in glucose levels were not statistically significant. In contrast, Group VI, which received the highest dose of *Azadirachta indica* leaf methanolic extract (400 mg/kg body weight), showed a significant reduction in glucose levels from 18.35 mmol/L after

alloxan to 10.59 mmol/L after treatment, with a p-value of 0.0003. This result indicates that the high dose of *Azadirachta indica* leaf extract had a significant effect on lowering blood glucose levels, making it the most effective treatment among those tested.

Table 1; Blood glucose level

Parameter	**Baseline Glucose (mmol/L)**	**Alloxan-induced glucose (mmol/L)**	**Post treatment glucose (mmol/L)**	**p-value**
Group I (Control)	4.53 ± 0.43	-	-	*0.9648*
Group II	3.525 ± 0.25	8.725 ± 1.80	9.598 ± 1.98	*0.538*
Group III	4.6 ± 0.42	14.98 ± 9.20	8.74 ± 5.30	*0.285*
Group IV	4.75 ± 0.83	14.75 ± 4.43	12.18 ± 3.69	*0.407*
Group V	3.35 ± 0.62	17.75 ± 5.56	14.18 ± 5.69	*0.408*
Group VI	4.425 ± 0.36	18.35 ± 1.77	10.59 ± 1.09	*0.0003*

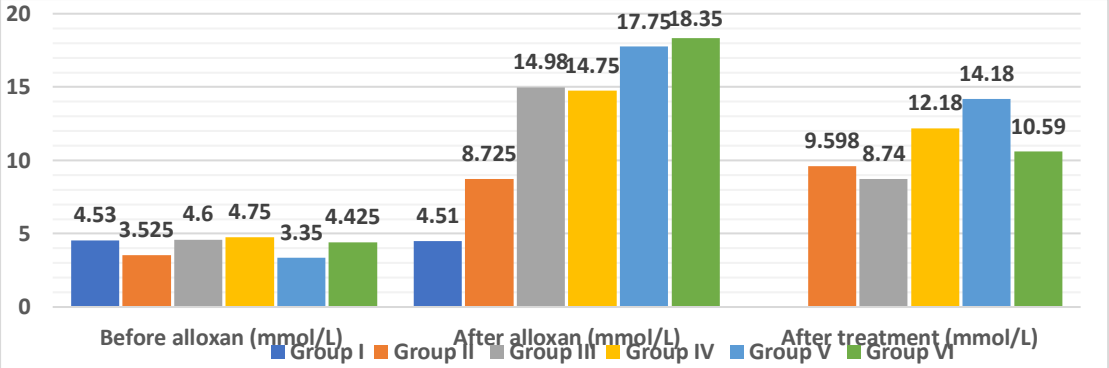

FIG. 1: BLOOD GLUCOSE LEVELS ACROSS THE DIFFERENT GROUPS

Table 2: Presents the effects of *Azadirachta indica* (Neem) leaf extract on testosterone levels in alloxan-induced diabetic Wistar rats. The control group (Group I) had a mean testosterone level of 4.3 ± 0.14, while the diabetic group (Group II) treated with alloxan showed a slight increase in testosterone levels to 4.55 ± 0.35. This suggests that alloxan-induced diabetes did not drastically lower testosterone levels in the untreated diabetic rats. The group treated with metformin (Group III) demonstrated a significant increase in testosterone levels (5.57 ± 1.77), which could be indicative of metformin's positive role in maintaining or even boosting testosterone levels in diabetic conditions. Metformin is known for improving insulin sensitivity and reducing oxidative stress, factors that may positively affect testosterone levels in diabetic rats.

Groups IV and V, which were treated with *Azadirachta indica* at different doses, showed varied results. The lower dose (200mg/kg) in Group IV caused a reduction in testosterone levels (2.85 ± 2.05), suggesting that at this dosage, Neem may have an inhibitory effect on testosterone production. However, at a higher dose (300mg/kg), the testosterone levels increased significantly to 6.45 ± 0.07, surpassing even the metformin group. This indicates a potential dose-dependent effect of Neem on testosterone levels in diabetic rats. Although the ANOVA analysis did not show statistically significant differences between the groups (F = 2.62, p = 0.160), the trend of increasing testosterone with higher doses of *Azadirachta indica* points toward its potential therapeutic role in managing hormonal imbalances in diabetes, though further research is needed to validate these findings.

Table 2: Effect of *Azadirachta indica leaf* on testosterone

Groups	**Mean ± S.D.**	**F-stat**	**p-value**
Group I (control)	4.3 ± 0.14	2.62	*0.160*
Group II (Alloxan)	4.55 ± 0.35		

Group III (Metformin)	5.57 ± 1.77
Group IV	2.85 ± 2.05
Group V	6.45 ± 0.07

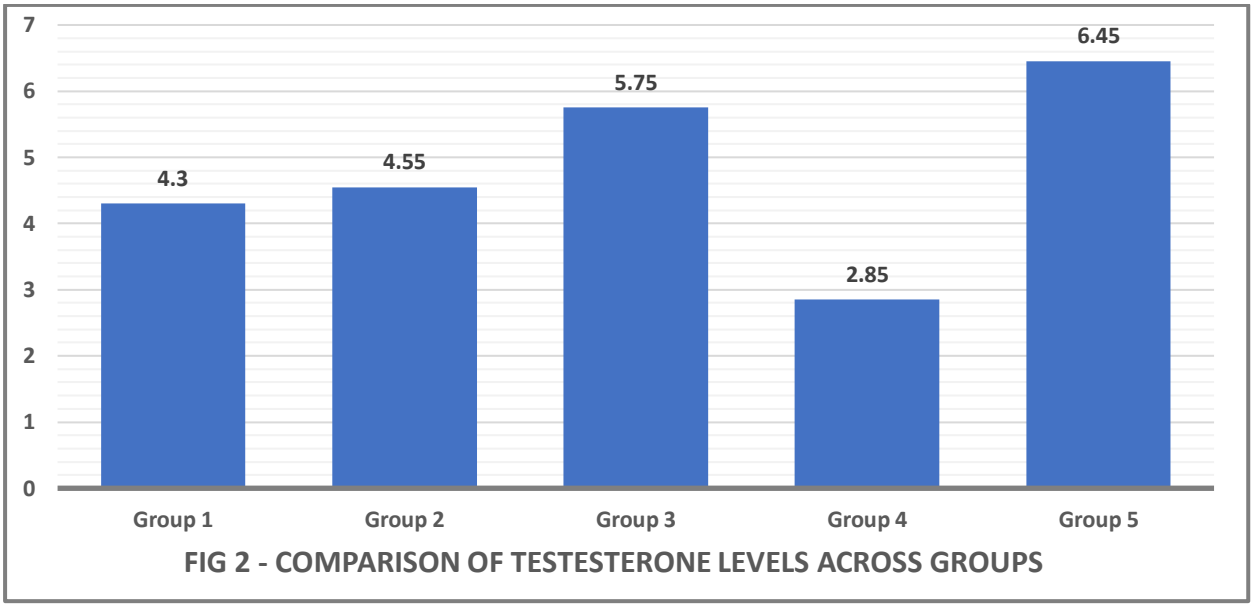

FIG 2 - COMPARISON OF TESTESTERONE LEVELS ACROSS GROUPS

Histopathological analysis

Oral administration of extract concentration led to a significant improvement in the testicular parenchyma in a dose dependent manner when compared with control (Fig. 1, 2, 3, 4, &5). This is further proved by the up regulation of the Testosterone levels which are part of the apoptotic pathway and prevent tissue damage.

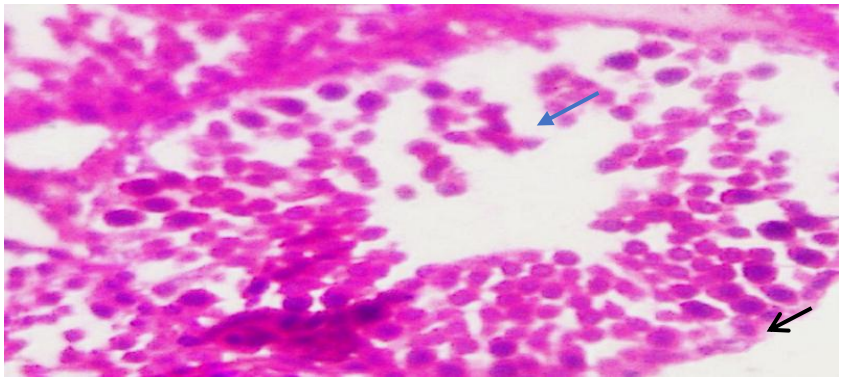

Fig. 1: Showed normal testicular cell with typical spermatogonia (black arrow) and giant spermatid (blue arrow) (H&E. X Mag. 400).

Normal control group that was fed with fed and distilled water for two weeks (no induction of diabetes). this displayed normal testicular cells with clearly identifiable spermatogonia (black arrow) and giant spermatids (blue arrow), indicating typical spermatogenic activity. the hematoxylin and eosin stain highlights the structural integrity of cells, viewed under x400 magnification.

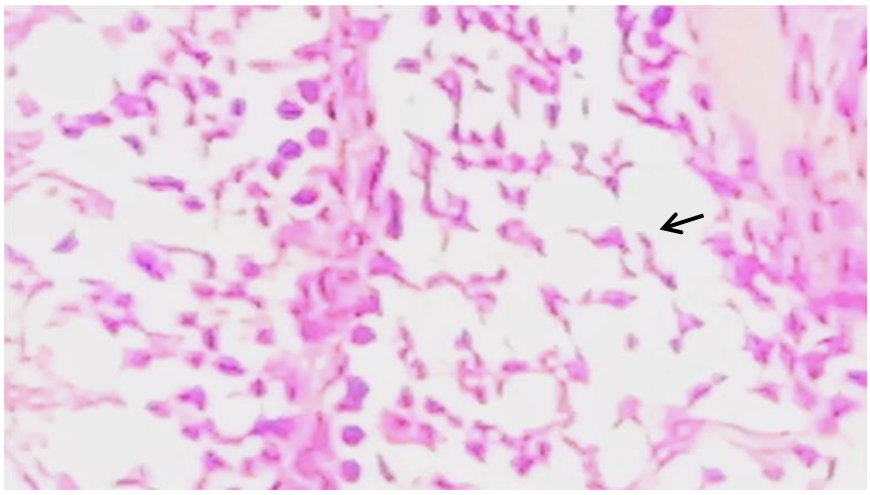

Fig. 2: Showed diabetic animal demonstrated disorganized and vacuolated seminiferous tubules (black arrow) (H&E. X Mag. 400).

Alloxan-induced diabetic control group that was induced with diabetes but not treated for two weeks. this illustrates testicular tissue showing disorganized and vacuolated seminiferous tubules (black arrow). this structural disruption suggests impaired spermatogenesis, likely caused by metabolic disturbances associated with diabetes. the vacuolation within the seminiferous tubules may indicate degeneration or apoptosis of germ cells, further emphasizing the adverse effects of hyperglycemia on testicular architecture.

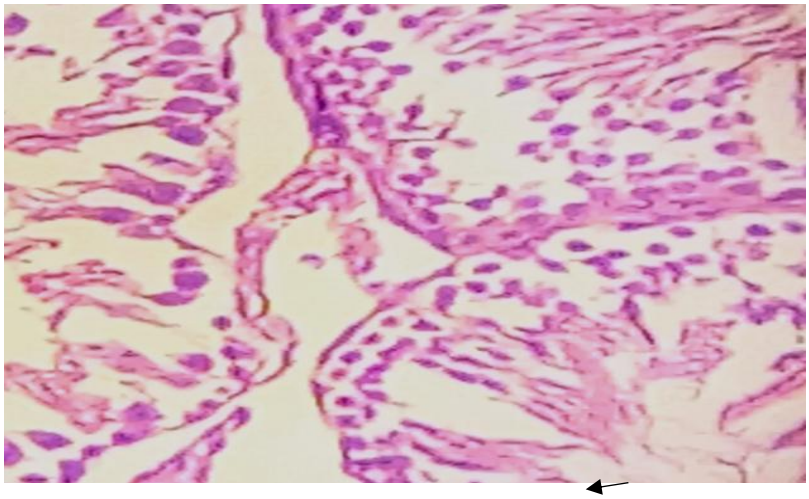

Fig. 3: Showed diabetic animal treated with metformin demonstrated a significant improvement in the seminiferous tubule (black arrow) compared with control (H&E. X Mag. 400).

Negative group that was induced with diabetes and treated with60mg/kg bodyweight of metformin via oral gavage daily for 2 weeks. the seminiferous tubules (black arrows) show significant structural improvement compared to untreated diabetic controls. the treatment appears to have mitigated the disorganization and vacuolation previously observed, indicating enhanced spermatogenic activity and restoration of testicular integrity. this suggests that metformin may have protective or restorative effects on testicular tissue.

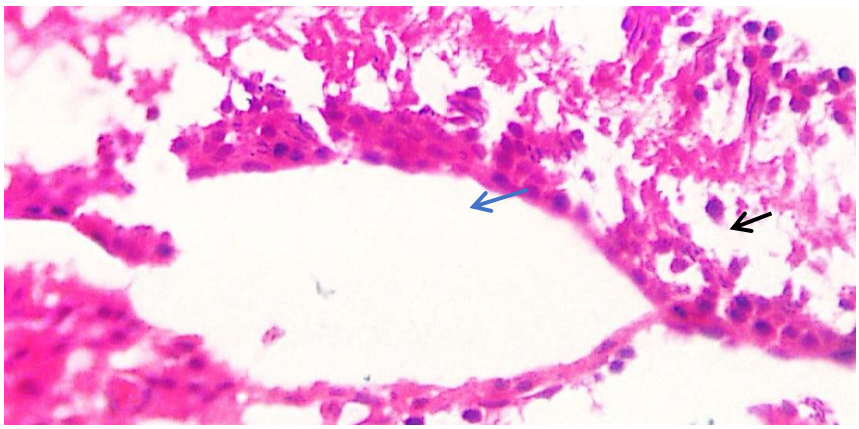

Fig. 4: Showed diabetic animal treated with 200 mg/kg extract demonstrated improved spermatogonia (blue arrow) and seminiferous tubules (black arrow) compared to diabetic control (H&E. X Mag. 400).

Negative group that was induced with diabetes and treated daily with 200mg/kg bodyweight of azadirachta indica via oral gavage for 2 weeks. this shows improved spermatogonia (blue arrow) and better structured seminiferous tubules (black arrow) compared to untreated diabetic controls.

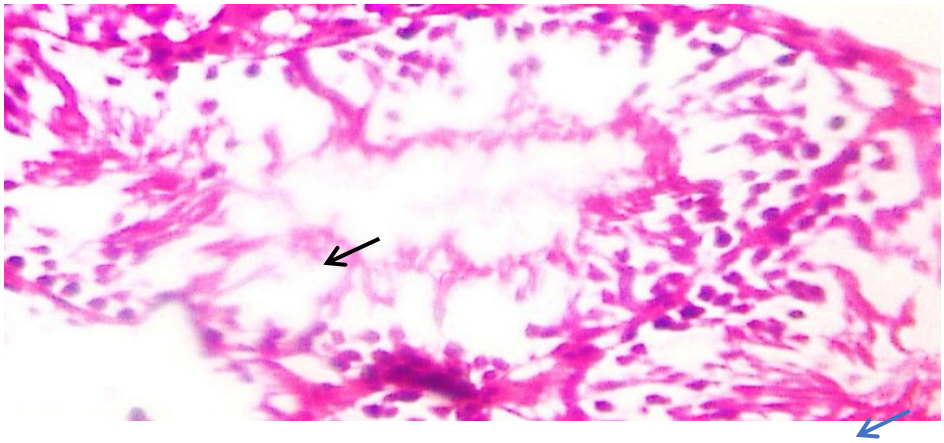

Fig. 5: Showed diabetic animal treated with 300 mg/kg extract demonstrated more improved spermatogonia (blue arrow) and seminiferous tubules (black arrow) compared to control (H&E. X Mag. 400).

Diabetic group treated with 300mg/kg of extract had greater improvement in spermatogonia (blue arrow) and seminiferous tubules (black arrow) compared to control group, indicating a stronger restorative effect on testicular structure and function at higher dose.

DISCUSSION

Alloxan is known to destroy pancreatic beta cells, leading to a reduction in insulin production and subsequently higher blood glucose levels (Cheekati *et al*., 2017; Ighodaro *et al*., 2018). From our data, diabetic animals treated with 400 mg/kg extract revealed a significant decrease in the blood glucose level ($p=0.0003$). Previous studies have demonstrated the hypoglycemic effect of *Azadirachta indica* which enhance insulin secretion and improve glucose utilization (Al-Awar *et al*., 2018; Saleem *et al*., 2018). Furthermore, diabetic animals treated with 200 and 300 mg/kg Neem extract also showed decrease in the blood glucose. However, the differences were not statistically significant ($p<0.005$). The lack of statistical significance in these groups suggests that the lower doses of the extract (200 and 300 mg/kg) might not be potent enough to induce a strong therapeutic effect in diabetic model. This result aligns with the study reported by (Iyare *et al*., 2014) where *Azadirachta indica* leaf extract at lower dose showed trends of glucose reduction, though the changes were not statistically significant.

Moreover, the partial reduction of glucose at lower doses of the extract (200 and 300 mg/kg) aligns with the known mechanism of metformin, which also reduces hepatic glucose production and increases insulin sensitivity (Rena *et al*., 2017). This finding is in contrast with that of (Yasin *et al*., 2022; Almuttairi, 2023) where it was reported that metformin significantly lowered glucose levels. Metformin's limited effectiveness here might be due to the severity of the induced diabetes, the dosage used, or the short duration of treatment.

In this study, diabetic animals further illustrate testicular tissues showing disorganized and

vacuolated seminiferous tubules. This structural disruption suggests impaired spermatogenesis, likely caused by metabolic disturbances associated with diabetes. The vacuolation within the seminiferous tubules may indicate degeneration or apoptosis of germ cells, further emphasizing the adverse effects of hyperglycemia on testicular architecture. Similarly, metabolic disorders (Cho, 2018) and conventional hormonal disorders (DeFronzo, 2015) are the most talked-about pathological conditions that associate with male infertility. However, the Neem extract showed ameliorative effects on testicular injury, improving antioxidant levels and testosterone production (El-Beltagy *et al*., 2020). Testosterone is an essential hormone in the male reproductive health, influencing whole processes from muscle mass to sexual functions (Ahmed *et al*., 2024). In diabetes, testosterone levels are often affected due to oxidative stress, insulin resistance, and damage to the endocrine system (Gamde *et al*., 2023b). At 200mg/kg, testosterone levels decreased significantly (2.85 ± 2.05), suggesting an inhibitory effect at this dosage. This could be linked to the anti-androgenic properties of certain phytochemicals in Neem, which have been observed by previous report (Raji *et al*., 2003). However, at a higher dose of 300mg/kg, testosterone levels increased dramatically (6.45 ± 0.07), surpassing even the metformin-treated group. This suggests that at higher doses, Neem may have a stimulatory effect on testosterone production, possibly through enhancing the hypothalamic-pituitary-gonadal axis.

CONCLUSION

This study demonstrates that *Azadirachta indica* leaf extract has a dose dependent effect on testosterone levels in alloxan-induced diabetic Wistar rats, with higher doses showing a potential for increasing testosterone production. While metformin was effective in boosting testosterone levels, Neem's dose-dependent response suggests it could be a natural alternative for managing testosterone in diabetic conditions. However, further research with larger sample sizes and longer treatment durations is needed to confirm these findings and better understand Neem's therapeutic potential in endocrine disorders.

Conflict of interest: None

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