RESEARCH ARTICLE

Protective effect of hydro-methanolic extract of *Abroma augusta* leaves in diabetes-induced testicular dysfunction in albino rat

Sayandev Midya, Prabir Mondal, Parag Ranjita Bera, Mukul Kumar Giri, Chhanda Mallick Mukherjee*

ABSTRACT

Objective: To evaluate the protective efficacy of *Abroma augusta* leaf extract on testicular impairment in streptozotocin (STZ) induced diabetic rats. *Methods*: Eighteen normoglycemic male albino rats were randomly divided into three groups: control group, STZ-induced (40 mg/kg body weight) diabetic group, and hydro-methanolic extract of leaves of *A augusta* (HMAA) treated group (200 mg/kg body weight) and 42 days of treatment were followed. Fasting blood glucose (FBG) and glycated hemoglobin levels (HbA_1C) were elevated, but hepatic hexokinase activities and serum insulin levels significantly decreased in the diabetic group. Sperm count, viability, motility, testicular Δ^5, 3β-HSD, and 17β-HSD activities, and serum testosterone levels were also reduced in diabetic rats. Superoxide dismutase and catalase activities and protein expression patterns in the testis were diminished significantly. At the same time, thiobarbituric acid reactive substance and conjugated diene levels were elevated significantly in STZ-induced diabetic rats. *Results*: Fasting blood glucose and glycated hemoglobin levels were significantly decreased towards the control level after the treatment of HMAA to the diabetic group due to significant recovery of serum insulin level and restoration of pancreatic cell number and islets size. Spermatogenic markers were significantly recovered after HMAA treatment due to recovery of tunning of the pituitary-gonadal axis as well as serum testosterone level. HMAA also has potent antioxidant properties that significantly restore testicular oxidative stress markers. *Conclusion*: The HMAA has significant corrective efficacy in diabetes-induced testicular disorders.

Keywords: Diabetes, Insulin, Spermatogenesis, 17β-HSD, Testicular dysfunction.

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INTRODUCTION

Diabetes mellitus (DM) is one of the most prevalent lifestyle-related metabolic disorders.[1] About 425 million people are affected by diabetes globally.[2] Around 20 to 85% of diabetic males suffer from sexual dysfunctions[3], though the prevalence of female sexual dysfunction is slightly lower (20–80%).[4] Low sperm count,[5] hypotestosteronemia,[6] abnormal spermatogenesis,[7] reduced libido, and erectile dysfunction[8] are the main problems observed in diabetic patients as well as experimental model animals.[9] In diabetes mellitus, excessive oxidative free radicals were generated with limited antioxidant capacity.[10] As a result, oxidation and glycoxidation of protein and other molecules occur, producing an advanced glycation end (AGE) product that causes cellular damage.[11] Many antidiabetic drugs (metformin and glibenclamide) are used, but they have several side effects.[12] Insulin is expensive for low-income countries[13] and additionally, it also causes insulin resistance, fatty liver, and anorexia nervosa over time.[14] Therefore, the use of natural medicines is most demandable for the management of DM throughout the world as these are less toxic.[15]

Abroma augusta is an evergreen shrub that is found all over India in muggy and hot regions.[16] Various parts of this tree are frequently employed in the manufacture of ayurvedic medicines to treat a variety of illnesses, including uterine disorders, inflammation, rheumatic joint pain, dysmenorrhea, gonorrhea, migraines, and diabetes.[17] It is known from folk medicine reputation that leaves of this plant are used as an antidiabetic medication. Some preliminary works have been conducted on the potential of *A. augusta* leaves to prevent diabetes complications.[18] We have already established that HMAA has protective efficacy in diabetes-induced carbohydrate metabolic disorders.[16] The purpose of the present research is to investigate the antioxidant and/or androgen stimulatory effects of *A. augusta* for the management of diabetes-induced male reproductive dysfunction.

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MATERIALS AND METHODS

Plant materials Collection

A. augusta leaves (specimen voucher number VU/CM/101) were collected during May-June from different places

in Paschim Medinipur district, West Bengal, India and a taxonomist from the Botany Department of Vidyasagar University confirmed the information. Leaves were dried in the shed at 25ºC at room temperature. Extraction of leaves was done by conventional method as per our earlier report.[16]

Criteria for selecting animals and their maintenance

Rats were obtained from a CCSEA-approved supplier and housed in a well-ventilated animal house. Ethical (No-VU/IAEC/CPCSEA/8/2024, Date-19.01.2024) has been taken from the institutional animal ethical committee. This experiment included eighteen mature normoglycemic male rats with a body weight of 120 ± 10 g. Rats were randomly divided among three cages and housed in our laboratory for 15 days at 26 ± 2°C temp with a 12-h light/dark cycle and suitable humidity before the experiment for acclimatization. All the animals were supplied with a sufficient amount of standard pellet diet and unlimited access to water.

Chemicals

Streptozotocin (STZ) was bought from SRL Pvt. Ltd., India. The insulin (ELISA) kit was acquired from Boehringer Mannheim in Germany. The HbA_1C kit was taken from Span Diagnostics Ltd. Surat, India.

Preparation of *A Augusta* leaves extract

Fresh leaves of *A. augusta* were cleaned in regular distilled water, chopped into small pieces, and shed-dried at room temperature. Dried and crushed leaves (100 g) were soaked in 2 L of hydro-methanol (40:60) in a glass container in airtight conditions, shaking constantly. The filtrates are collected after 7 days, dried under the pressure of a rotary evaporator, and kept in the fridge.[16]

Initiation of diabetes mellitus in rat

Rats were made diabetic by a single intra-muscular (IM.) injection of STZ (4 mg /0.1 mL citrate buffer/100 g body weight) after 12 hours of fasting. Animals with fasting blood glucose (FBG) levels between 350 – 450 mg/dL after 48 hours of STZ injection were selected for the study.[19] Next, every 7-day intervals, the FBG levels were measured.

Experimental design

Eighteen rats were randomly divided into three groups, with six rats in each.

Group A (Control group)

Six normoglycemic and healthy rats were introduced in this group. Oral feeding of 0.5 mL distilled water was allowed by gavage per 100 g body weight per day.

Group B (Diabetic group)

A single *i.m.* injection of STZ at a dose of 4 mg/ 0.l mL citrate buffer/100 g body weight was introduced to develop diabetes. Rats were allowed to administer 0.5 mL distilled water /100 g body weight/day orally by gavage.

Group C (HMAA treated group)

Rats of this group received 20 mg of HMAA /0.5 mL distilled water/ 100 g body weight/ day through oral route by gavage. Every day, the overnight fasted rats were administered an oral dose of HMAA in the morning (at 11:00 am). After two hours of extract treatment (at 1:00 pm), the rats were given food. The feed box was cleaned in the evening (at 8:00 pm). FBG levels in all groups were checked in the morning (8:00 am) using a one-touch select™ glucometer at every 7th day interval. The final body weight and FBG levels of all rats were recorded. The animals were euthanized using carbon dioxide, carbon monoxide, and halothane. After sacrifice, blood was collected from the portal vein to assess the levels of FBG, serum insulin, testosterone, and HbA_1C. Livers were collected and stored as slices in a -80°C refrigerator to assess enzymes. Oxidative stress parameters such as CAT, SOD, CD, and TBARS were detected in the testis. The pancreas and testis were also collected and stored in formalin for histological analysis.

Measurement of FBG level

FBG level was measured at 7-day intervals throughout the study. After warming up the tail, blood was extracted from the vein using a syringe. A one-touch select™ glucometer was used to measure the FBG level.[20,21]

Measurement of glycemic bio-sensor

The glucose memory test was used to measure the HbA_1C level following the standard protocol.[22] HbA_1C level values were given as GHb%.

Serum insulin level

Levels of serum insulin were determined using a solid phase-conjugated sandwich enzyme-linked immune sorbent assay (ELISA) and expressed as µIU/mL.[23]

Assay of hexokinase activity

The assay mixture was produced with 3.7 mM glucose, 11 mM thioglycerol, 7.5 mM $MgCl_2$, and 45 mM HEPES buffer in a spectrophotometric cuvette. Phosphate buffer (0.1M) homogenized the tissues at 50 mg/mL concentration. In a cuvette, 0.9 mL of the assay mixture was taken and 0.03 mL (0.22 M) ATP was added to it and then mixed properly. After that, a supernatant of liver tissue (0.1 mL) was added, and absorbance was recorded at 340 nm. Hepatic hexokinase activities were expressed as µg of Glucose-6-phosphate formed/mg of tissue.[24]

Assay of serum testosterone

Testosterone in serum was measured using an ELISA kit from Fine Biotech Co., Ltd. Serum testosterone values were expressed as ng/mL.[25]

Count, motility, and viability of spermatozoa

Epididymal spermatozoa were collected from the cauda epididymis of each rat. The percentage of motile spermatozoa was recorded.[26] Sperm cell count was performed following

a standard procedure.[27] Sperm viability was determined via supravital staining of spermatozoa.[28]

Assessment of Δ^5, 3β-HSD and 17β-HSD activities

Δ^5, 3β-HSD activity in testis tissue was assessed following the conventional procedure. 100 mg of testis was homogenized using homogenizing buffer in 1-mL of HSD buffer and centrifuged the homogenized sample for 30 min at 9000 rpm at 4°C. In a cuvette, 500 µL supernatant part of the sample, 450 µL of redistilled water, 500 µL of TNaPP, and 20 µL of DHEA were added. After 30 seconds, 50 µL of NAD was added to that cuvette, and six readings of each sample were taken in a UV spectrophotometer at 340 nm every 30-second intervals for 3 minutes.[29]

The standard protocol has estimated 17β-HSD activity in testis tissue. A sample of testis tissue was prepared in the same way as followed for Δ^5, 3β-HSD. 450 µL of redistilled water, 500 µL of TNaPP, 20 µL of testosterone, and 500 µL of supernatant of testis were collected in a cuvette and after 30 seconds, 50 µL of NAD was added. Reading was recorded for 3 min at 340 nm at 30-second intervals.[30]

Measurement of CAT activity

According to standard procedure, the CAT activity in the testis was analyzed biochemically.[31] At first, testis tissue was homogenized by 0.05 mM Tris-HCl buffer solution (pH 7.4) at a concentration of 50 mg/mL. In a cuvette, H_2O_2 (500 µL) and distilled water (2500 µL) were taken. After adding the supernatant, absorbance was measured at 240 nm and six values were collected at 30-second intervals.

Measurement of SOD activity

SOD activity in testis tissue was assessed according to the conventional protocol.[32] 50 mM of 2.04 mL Tris buffer (pH 8.2), 20 µL of the sample, and 20 µL of pyrogallol were collected in a cuvette. The absorbance of each sample against blank was taken at every 30-second interval at 420 nm for 3 minutes.

Measurement of TBARS and CD level in testis

Levels of TBARS in testis tissue were estimated by conventional protocol by using a TBA-TCA mixture and reading was taken at 535 nm.[33] CD was quantified by the established biochemical method from a homogenized testis sample.[34] Amount of formed hydro-peroxide was estimated at 233 nm by spectrophotometric method and expressed in nM TBARS formed/mg of tissue.

Analysis of CAT and SOD protein in testis tissue through gel

100µg of testis tissue proteins were loaded into 8% PAGE gel and electrophoresed at 40mA for catalase. Then, electrophoresis gel was washed with 0.003% H_2O_2 three times. Then, the gel was incubated for 10 minutes in 0.003% H_2O_2 solution at room temp. Then washed with distilled water and stained with 2% ferric chloride (w/v, 0.6 g in 30 mL distilled water) and 2% potassium ferricyanide (w/v, 0.6 g in 30 mL distilled water). After the formation of achromatic bands, the stain was removed, and the gel was rinsed extensively with distilled water.[35]

12% native gel was used for the assessment of SOD expression. 100µg of testis tissue protein was loaded and electrophoresed at 40mA. After the electrophoresis technique, SOD native gel was stained. Then, exposed to light for 15 minutes achromatic bands of SOD were appeared.[35]

Assay of ALT and AST activities in serum

Serum ALT and AST activities were assessed according to standard protocol. The activities of these enzymes were expressed as U/L.[36]

Histological studies of pancreas and testis

According to the standard procedure, Bouin's fixative was used to fix the pancreas and testis of experimental rats, and paraffin sections were prepared.[16] Sections of tissue were cut at thicknesses of 5 µM and stained with hematoxylin and eosin.[37] Microphotography of these histological samples was taken at 400× magnification.

Statistical analysis

The results were presented as mean ± SD (n=6). ANOVA followed by a two-tailed t-test was performed to compare the mean values of different groups.[38] The significance level was expressed at $p < 0.05$.

RESULTS

Body weight and organo-somatic indices

Body weight and the organo-somatic ratio of the testis, prostate, epididymis, and seminal vesicle were significantly ($p < 0.05$) diminished in the diabetic group with respect to untreated control. Significant ($p < 0.05$) recovery of these parameters was observed in the HMAA-treated group (Table 1).

Table 1: Effect of HMAA treatment on body weight (g) and organo-somatic indices (g%) in diabetic rat.

	Control	*Diabetic*	*HMAA*
Body weight ***(g)***			
Before treatment	121.2 ± 2.90	121.4 ± 2.99	120.83 ± 2.34
After treatment	134.83 ± 2.16	99.66 ± 3.68*	130.01 ± 2.44*#
Organo-somatic index (g%)			
Testis	1.88 ± 0.04	0.40 ± 0.03*	1.62 ± 0.04*#
Epididymis	0.32 ± 0.03	0.08 ± 0.02*	0.26 ± 0.03*#
Prostate	0.17 ± 0.02	0.04 ± 0.03*	0.10 ± 0.02*#
Seminal vesicle	0.25 ± 0.03	0.07 ± 0.03*	0.19 ± 0.02*#

Data are expressed as mean ± SD, n = 6. ANOVA followed by 'Multiple comparisons Student's two-tailed t-test. All data are equally distributed. *p < 0.05 compared with the control group; #p < 0.05 compared with diabetic group.

Fasting blood glucose level

FBG level was significantly ($p < 0.05$) elevated in the diabetic rat in comparison to the untreated control group. Treatment with HMAA at the dose of 20 mg/100 g of body weight to the diabetic rats resulted in a significant ($p < 0.05$) diminution of FBG levels towards control (Table 2).

Levels of glycated hemoglobin, serum insulin, and hexokinase activities

HbA_1C level was elevated but serum insulin levels and hexokinase activities were decreased significantly ($p < 0.05$) in the diabetic group in comparison to untreated control. These three parameters were significantly ($p < 0.05$) recovered towards the control after HMAA treatment in the diabetic rats (Figure 1).

Levels of reproductive profile

Count, motility and viability of spermatozoa were diminished significantly ($p < 0.05$) in the diabetic group with respect to untreated control. These parameters were significantly ($p < 0.05$) restored in the HMAA-treated group (Figure 2).

Table 2: Changes in fasting blood glucose level after HMAA treatment in male diabetic rat.

	Control	***Diabetic***	***HMAA***
Day 0	85.50 ± 2.43	371.33 ± 2.50*	362.33 ± 1.47*#
Day 7	85.00 ± 4.60	385.66 ± 14.31*	284.83 ± 10.21*#
Day 14	87.16 ± 3.43	398.00 ± 11.57*	267.50 ± 8.53*#
Day 21	85.66 ± 2.73	402.50 ± 3.39*	215.66 ± 1.86*#
Day 28	83.66 ± 4.32	425.83 ± 11.60*	184.83 ± 2.93*#
Day 35	85.83 ± 3.25	430.66 ± 5.12*	155.33 ± 4.76*#
Day 42	85.50 ± 5.24	448.33 ± 15.81*	136.33 ± 2.25*#

Data are presented as mean ± SD, n = 6. ANOVA followed by 'Multiple comparisons Student's two-tailed t-test. Data were equally distributed. *p < 0.05 compared with the control group; #p < 0.05 compared with diabetic group.

Androgenic key enzymes and serum testosterone level

Testicular Δ^5, 3β-HSD, and 17β-HSD activities and testosterone levels were diminished significantly ($p < 0.05$) in the diabetic group compared to the untreated control. After HMAA treatment, the diabetic rats showed a significant ($p < 0.05$) recovery of these parameters toward the untreated control with respect to the diabetic group (Figure 3).

CAT and SOD activities and expression in testis

Testicular CAT and SOD activities and protein expression patterns were assayed. Both these parameters were significantly ($p < 0.05$) diminished in the diabetic group in comparison with the untreated control. CAT and SOD activities in testis tissues were significantly ($p < 0.05$) improved towards the control level in the HMAA-treated group and the band densities of these two enzymes were also improved significantly in HMAA treated group (Figure 4).

Levels of CD and TBARS in testis

Levels of CD and TBARS were significantly ($p < 0.05$) amplified in testis tissues of the diabetic group with respect to untreated control. Treatment with HMAA in diabetic rats resulted in a significant ($p < 0.05$) diminution of these two parameters towards the untreated control (Figure 5).

Levels of ALT and AST activities in serum

Levels of serum ALT and AST activities were increased significantly ($p < 0.05$) in the diabetic group when compared to the untreated control. Treatment with HMAA in diabetic animals resulted in a significant ($p < 0.05$) diminution of these enzyme activities towards the control (Figure 6).

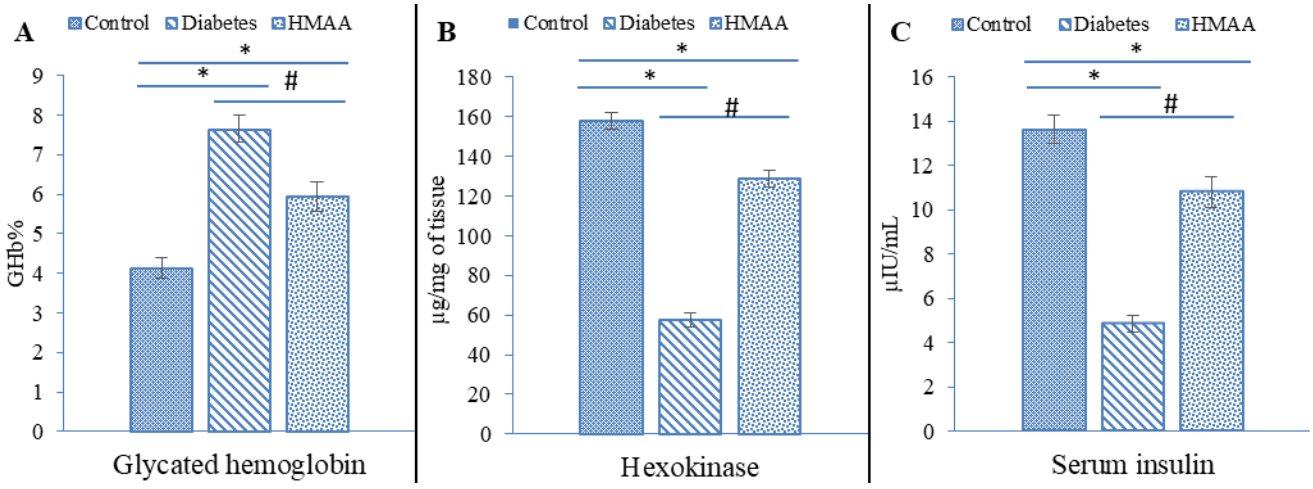


Figure 1: Recovery of (A) glycated hemoglobin, (B) hexokinase activity and (C) serum insulin level after HMAA treatment in diabetic rat. Data are presented as mean ± SD, n = 6. ANOVA followed by two-tail t-test. Data were distributed equally. *p < 0.05 compared with the control group; #p < 0.05 compared with diabetic group.

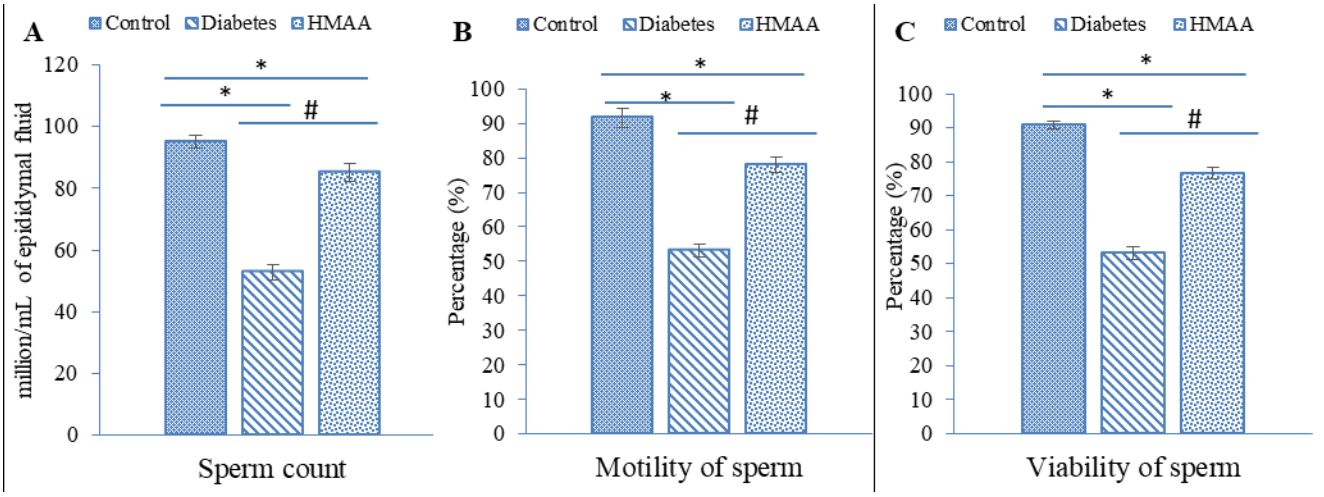


Figure 2: Effect of HMAA treatment on (A) sperm count, (B) motility and (C) viability in STZ induced diabetic rat. Data are presented as mean ± SD, n = 6. ANOVA followed by two-tail t-test. Data were equally distributed *p < 0.05 compared with the control group; #p < 0.05 compared with diabetic group.

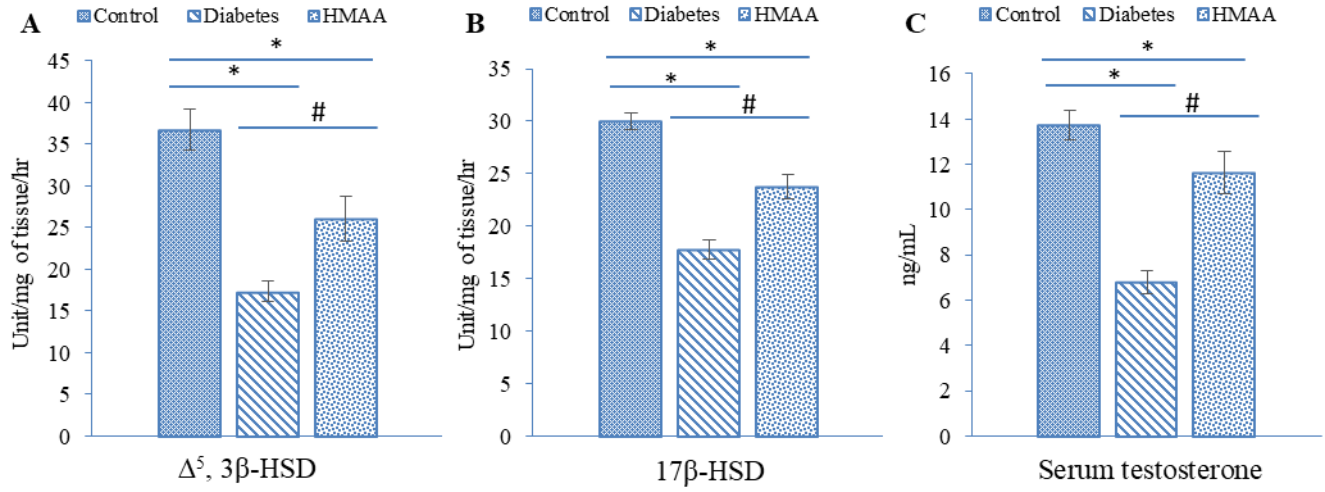


Figure 3: Protective effect of HMAA on (A) Δ^5, 3β-HSD, (B) 17β-HSD activities and (C) serum testosterone level in diabetic rat. Data are presented as mean ± SD, n = 6. ANOVA followed by two-tail t-test. Data were equally distributed. *p < 0.05 compared with the control group; #p < 0.05 compared with diabetic group.

Table 3: The effect of HMAA treatment on islet number, islet cell number and islet diameter in diabetic male albino rats.

Group	*Islet of number*	*Number of Islet cells/Islet*	*Diameter of islet (μM)*
Control	24.76 ± 0.63	183.80 ± 1.42	285.77 ± 0.68
Diabetic	7.03 ± 0.087*	76.55 ± 0.59*	127.13 ± 0.51*
HMAA	17.98 ± 0.62*#	162.78 ± 0.49*#	266.19 ± 0.88*#

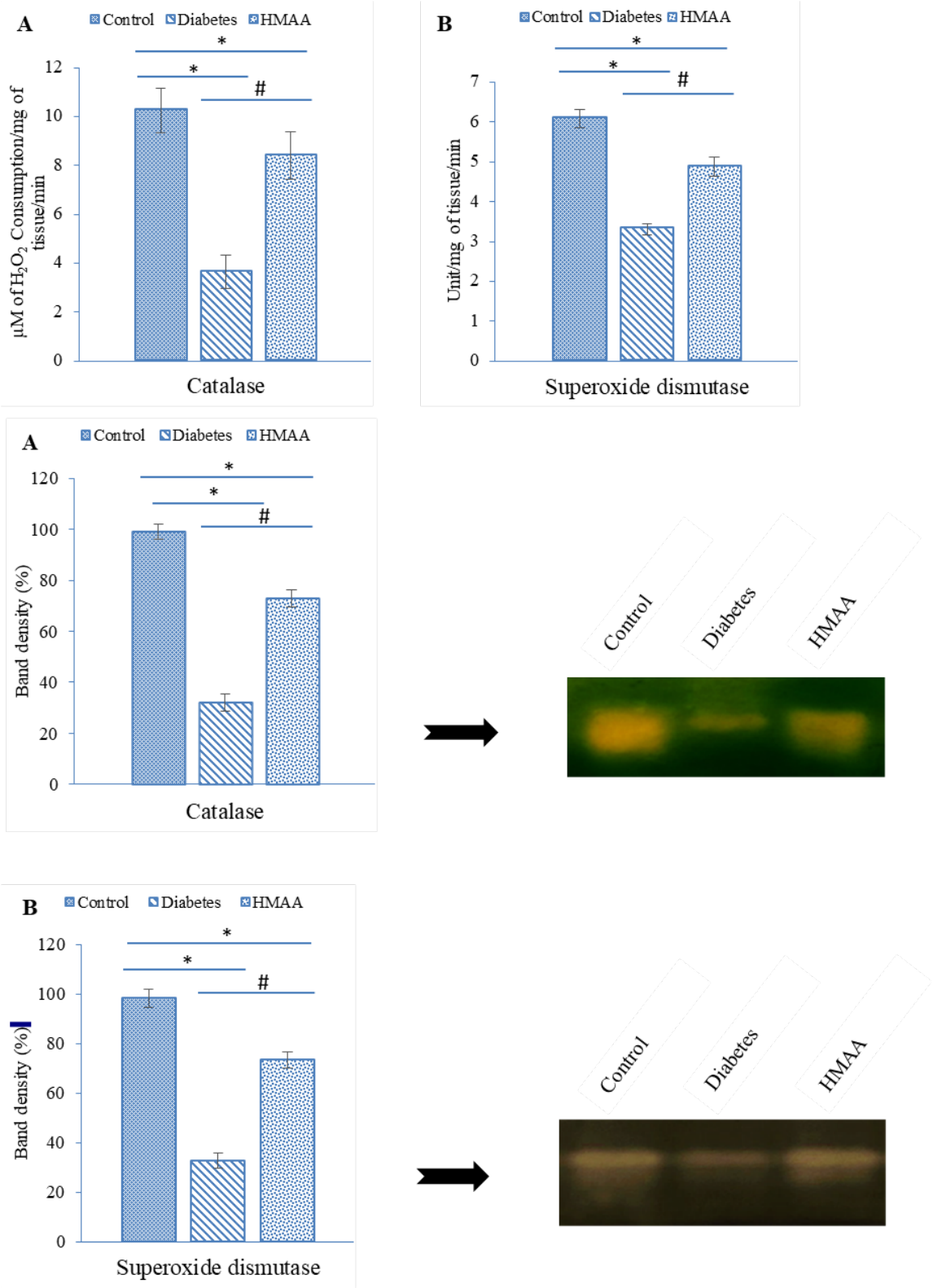
Data are presented as mean ± SD, n = 6. ANOVA followed by 'Multiple comparisons Student's two-tailed t-test. Data were equally distributed *p < 0.05 compared with the control group; #p < 0.05 compared with diabetic group.

Histological observation of the testis

Germ cell layers were damaged and seminiferous tubular diameter (STD) was reduced in the diabetic group compared to control. After the treatment of HMAA in the diabetic rats, significant ($p < 0.05$) recovery of STD and germ cell layer was observed in the control group when compared with the diabetic group (Figure 7).

Histological observation of the pancreas

In diabetic rats, pancreatic islet cell density, islet count, and diameter were lower than in control rats. The treatment of HMAA in diabetic rats resulted in significant ($p < 0.05$) improvement in islet count, islet cell number, and diameter of islets towards the untreated control (Table 3; Figure 8).



AFigure 4: Ameliorative role of HMAA on (A) catalase and (B) superoxide dismutase activities and protein expression pattern in STZ -diabetic male rat. Data are presented as mean ± SD, n = 6. ANOVA followed by two-tail t-test. Data were equally distributed. *p < 0.05 compared with the control group; #p < 0.05 compared with diabetic group.

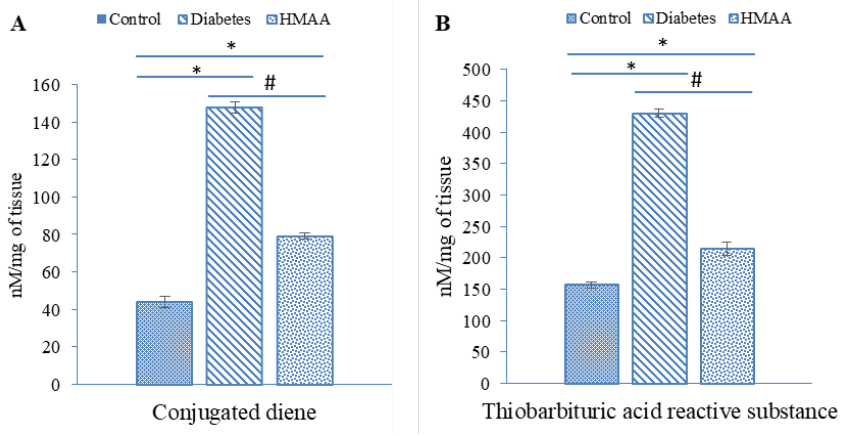


Figure 5: Recovery of (A) conjugated diene and (B) Thiobarbituric acid reactive substance levels in testis after treatment of HMAA in diabetic rat. Data are presented as mean ± SD, n = 6. ANOVA followed by two-tail t-test. Data were equally distributed. *p < 0.05 compared with the control group; #p < 0.05 compared with diabetic group.

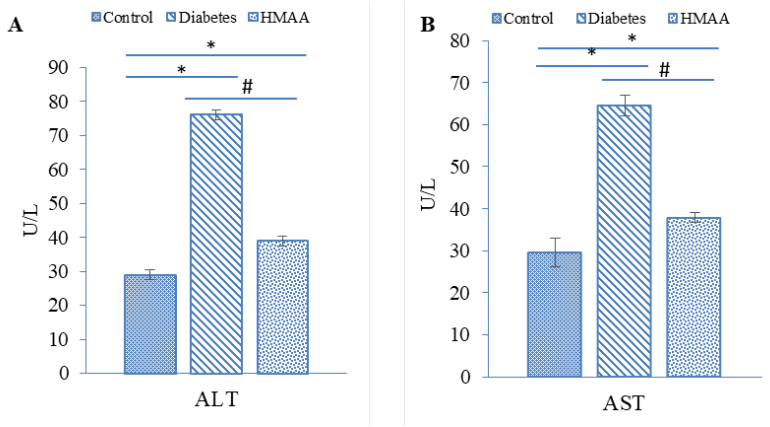


Figure 6: Recovery of serum (A) ALT and (B) AST activities after treatment of HMAA in diabetic rat. Data are presented as mean ± SD, n = 6. ANOVA followed by two-tail t-test. Data were equally distributed *p < 0.05 compared with the control group; #p < 0.05 compared with diabetic group.

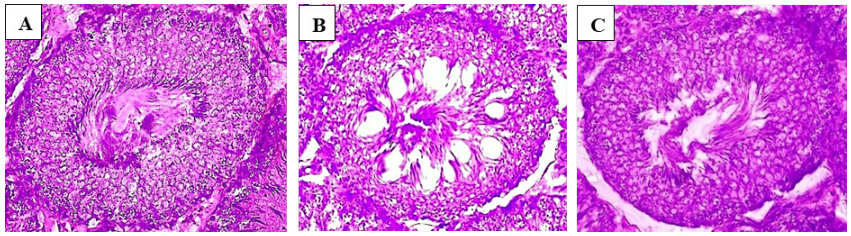


Figure 7: (A) Control group: showing spermatogenesis with normal cell density stage. (B) Diabetic group: The density of germ cells decreases, significant damage observed. (C) HMAA treated group: Normal germ cells and spermatids density observed towards control.

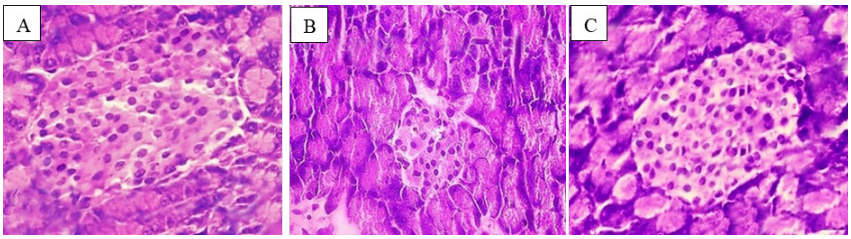


Figure 8: Pancreatic histology, x 400 (H&E stain) (A) Control group: The cell density and islet size are normal. (B) Diabetic group: The islet's size and cell density reduced significantly. (C) HMAA treated group: Cell density and islet size increase towards control.

DISCUSSION

Previous research has indicated that male sexual dysfunction is frequently associated with diabetes mellitus (DM).[39] In this background, the current investigation has been carried out to focus on the possible correction of testicular dysfunction in diabetic rats by HMAA. The STZ-induced diabetic rat model is considered standard for this purpose.[16] FBG and HbA_1C levels were elevated due to low utilization of glucose by the insulin as well as excess glycation of hemoglobin.[40] STZ significantly destroys pancreatic β cells that lower insulin production in diabetic model animals. HbA_1C is one of the important advanced glycation products in diabetes.[22] As hexokinase activity is insulin-mediated so, this enzyme decreased in diabetic rats. Treatment of HMAA extract in diabetic rats significantly recovered FBG and HbA_1C levels may be due to the recovery of insulin.[16] The hydro-methanolic extract of *A. augusta* treatment reduced the pancreatic damage. It regenerated the pancreatic islet cell, so the islet size and cell density were recovered as well, and insulin secretion was also elevated after HMAA treatment. The hexokinase activities also restored towards the control after the HMAA treatment group.

The percentage of count, motility, and viability of spermatozoa diminished significantly in diabetic rats due to inadequate testosterone production and/or secretion, which is directly related to low levels of insulin.[41] This is consistent with the previous study.[20] Accessory sex organ weight decreased as testosterone serves as the primary regulator of these organs. Testicular androgenic key enzyme activities were also significantly diminished in the diabetic group.[20] HMAA treatment showed a considerable recovery of these sensors, possibly due to the recovery of testosterone and testicular androgenic enzyme activities.[42]

Diabetes is highly connected with oxidative stress; hence testicular CAT, SOD enzyme activities, and protein expression patterns were decreased significantly, but CD and TBARS levels were significantly elevated[24] in diabetic rats. Testicular antioxidant enzyme activities and lipid peroxidation end product significantly recovered in the HMAA-treated group, and these observations are consistent with other reports.[43] So HMAA has antioxidant activities by which it can scavenge the excess free radicle generation in diabetes. Serum ALT and AST enzymes are important metabolic toxicity indicators and were increased significantly in the diabetic group; however, significant restorations of those were observed in the HMAA group.[16]

So, the hydro-methanolic extract of *A. augusta* significantly recovered the diabetes-induced testicular dysfunction through its insulin-stimulatory effect, elevation of testosterone production, and antioxidant activities without any metabolic toxicity. Further research may identify the nature of this extract's active phyto molecule(s).

CONCLUSION

A. augusta leaves showed an antihyperglycemic effect by regenerating pancreatic cells, including β-cells, which increase insulin levels and recover FBG, HbA_1C, and other metabolic markers. High insulin level stimulates testosterone production. As a result, the spermatogenic and androgenic markers are restored, and diabetes-induced testicular dysfunction is recovered. *A. augusta* leaves also have antioxidant activities, thus, the oxidative stress markers are recovered significantly. The free radical-induced damage was reduced significantly in the testis, and the testicular dysfunction recovered.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.