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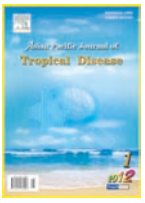

Document heading

Diabetes induced testicular dysfunction amelioration by ethyl acetate fraction of hydromethanolic extract of root of *Musa paradisiaca* L. in streptozotocin-induced diabetic rat

Kausik Chatterjee[1], Kazi Monjur Ali[1], Debasis De[1], Tushar Kanti Bera[1], Kishalay Jana[1], Soumyajit Maiti[1], Abhinandan Ghosh[1], Ramapati Samanta[2], Debidas Ghosh[1*]

[1]*Andrology, Endocrinology and Molecular Medicine Laboratory, Biomedical laboratory Science and Management (UGC Innovative Programme Funded Department), Vidyasagar University, West Bengal, India*
[2]*Dept. of Biotechnology, Indian Institute of Technology (IIT, Kharagpore), West Bengal, India*

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ABSTRACT

Objective: To investigate the diabetic therapeutic potentiality and antioxidative efficacy of ethyl acetate fraction of hydro-methanol (40:60) extract of root of *Musa paradisiaca* Lam. (Musaceae) in streptozotocin-induced diabetic rat. **Methods:** Streptozotocin-induced diabetic state was confirmed by decreased serum insulin level and carbohydrate metabolomics i.e. increased fasting blood glucose level, glycated hemoglobin level and diminished glycogen contents in liver and skeletal muscle. Reproductive homeostasis alteration in diabetes was evaluated by reproductive organo-somatic indices, sperm count, motility and histological analysis of testicular seminiferous tubule along with levels of serum testosterone, testicular cholesterol and seminal vesicular fructose assessment. Oxidative stress in primary and accessory sex organs, and in sperm pellet was assessed by measuring antioxidant enzyme activities along with quantification of free radicals products. Testicular pro-apoptotic Bax-α mRNA expression pattern was studied semi-quantitatively by PCR technique. Reverse phase HPLC fingerprinting was performed using methanol and acetonitrile as mobile phase. **Results:** Oral administration of ethyl acetate fraction at a dose of 20 mg/0.5 mL of distilled water/100 gm body weight twice daily to the diabetic rats for 28 days significantly recovered organo-somatic indices, protected reproductive activities, corrected oxidative stress markers and pro-apoptotic mRNA expression pattern, which were deviated in diabetes mellitus from control level without any type of toxicity. HPLC fingerprinting shows five completely resolved peaks at λ max 254 nm and 342 nm. **Conclusions:** It has a promising antihyperglycaemic and antioxidative activity for curing diabetes induced reproductive disorders in streptozotocin-induced diabetic rat.

1. Introduction

Diabetes mellitus, a syndrome, is an endocrine and chronic metabolic disorders characterized with hyperglycemia resulting from malfunction in insulin secretion and/or insulin action both causing by impaired regulation of carbohydrate, lipids and protein homeostasis[1]. Despite progress in the management of diabetes by synthetic drugs, the search for improved, safe and natural antidiabetic agent is still ongoing, which has also been recommended by World Health Organization[2]. Worldwide use of natural plant products as traditional medical treatment of different non-communicable diseases is increasing rapidly. For screening out immense potentiality against diabetes mellitus and antioxidant from the wide varieties of unexplored folklore medical plants used by tribal practitioners, they are being used either in the form of single plant extract or fraction or in composite manner to discover standard drug[3]. *Musa paradisiaca* (*M. paradisiaca*) Lam. (Musaceae) is a tree like herb distributed throughout India and Malaysia. Different parts of this plant are claimed to have medicinal values including anti diabetic activity[4,5]. We have also reported about the anti diabetic efficacy of the root of *M. paradisiaca* [6]. Diabetes mellitus effects detrimentally male sexual profile by endocrine alteration of spermatogenesis, finally

*Corresponding author: Debidas Ghosh, Professor & Head Department of Bio-Medical Laboratory Science & Management (UGC Innovative Programme Funded Department), Vidyasagar University, West-Bengal, India.
Tel: 092326990993
Fax: 03222275329; 03222275297
Email: debidas_ghosh@yahoo.co.in

leads to disruption in reproductive homeostasis by imposing oxidative stress induced infertility with poor semen quality and significant increase in degenerated germ cells at various stages of spermatogenesis along with decreased levels of serum gonadotrophins and testosterone[7,8,9,10]. A positive relationship between low levels of fasting insulin induced decreased free testosterone and increase in Reactive Oxygen Species (ROS) generation[11,12], directly associated with impaired spermatozoal fructose metabolism[13], in diabetic men has been established. In diabetic infertile pathological situations ROS play an important role as it cross membranes, diffuse away from the site of generation and induce apoptosis by signal transduction as second messenger to stimulate protein kinase cascades coupled apoptosis through mitochondrial intrinsic pathway, involves pro apoptotic Bcl-2 associated X (Bax) protein[14,15]. It leads to release of cytochrome C into the cytosol by changing the mitochondrial membrane permeability[16]. Cytochrome C forms complex with the apoptotic protease activating factor-1 which activates caspase-9 and downstream caspases cascade leads to apoptosis [17]. Previous studies proved that high glucose concentration induced oxidative stress which triggers Bax-mediated caspases activation and progression of apoptotic cell death [18]. The interdependent relationship between increased levels of fasting blood glucose, glycated hemoglobin and over expression of Bax mRNA that magnifies cell death by endothelial dysfunction in diabetes has been established [19].

The present study was designed to investigate the antihyperglycaemic effects of ethyl acetate fraction of hydromethanolic extract of *M. paradisiaca* using hyperglycemic rat model and to confirm its potentiality for managing diabetes mellitus induced oxidative stress and apoptotic related testicular and germ cell damage along with its chromatographY dependent bioactive compound purification.

2. Materials and methods

2.1. Plant material preparation

Fresh root of *M. paradisiaca* was collected from rural areas of Paschim Midnapur District, West Bengal, India in the month of May-July. Preliminary identification of the plant was made by a taxonomist in the Botany Department, Vidyasagar University, Midnapur, India. A voucher specimen (HPCH No. 7) was deposited in the Dept. of Botany, Vidyasagar Univerity, West Bengal, India. After collection, plant part was first separated and washed thoroughly with tap water and deionized water. The root was finally dried in an incubator completely at 37 ^{0}C. About 6.2 Kg of dried roots were collected from 11.6 Kg of fresh roots. Dried plant parts were pulverized in industrial electrical grinder, taken in 20 lit percolator and maceration (250 mL of solvent was used for 50 gm of plant part) was carried out with hydro-methanol solvent, H_2O: MeOH :: 40:60, v/v. with an intermittent stirring for the first 2 hr and left for 36 hr at 37 ^{0}C to avoid any degradation or deactivation of the active component(s). The extract was collected on the second day and again freshly prepared hydro-methanol solvent with same ratio was added to the extraction chamber and the slurry was stirred again with glass rod following the previous process, followed up for the third day also and the final extract was collected on the fourth day. The extract was then filtered first through cotton filter and then by No. 1 Whatman filters paper in a form of slurry. The hydro-methanol filtrate was evaporated under reduced pressure by Rotavapour instrument (HAHN-SHIN HS-2000NS, Hahn-Shin Scientific Co., Korea) at 38 ℃ for complete removal of methanol. Finally, plain aqueous filtrate was lyophilized on benchtop K Lyophilizer to produce 810 gm brown colour powder. The lyophilized extract powder was then subjected to polarity grade solvent fractionation with 2 lit, 5 lit and 7 lit of laboratory grade n-hexane, chloroform and ethyl acetate solvent and dried under partial vacuum at 38 ^{0}C to finally have their respective fractions of 9.07 gm, 17.70 gm and 20.20 gm, collected as solvent free residues into amber colored glass containers. The ethyl acetate fraction was suspended in distilled water and administered to diabetic albino rats.

2.2. Animals and experimental design

Normoglycemic Wistar male albino rats of two months of age and weighing about 130 ±10 g were used in these experiments. The animals were housed at an ambient temperature of 25 ±2℃ under 12 h : 12 h light-dark cycle and acclimated to these conditions for 15 days before use in experimental trials. All animals had free access to standard rat food and water *ad libitum*. The principles of laboratory animal care and instruction given by our "Institutional Ethical Committee" were followed throughout the experiments[20].

18 hours fasting rats were subjected to a single intramuscular injection of streptozotocin (STZ) (Sigma Chemical Co., USA) at a dose of 3.5 mg/0.1 mL of citrate buffer/100 g body weight/rat (pH-4.5) as standardized by the previous work in our laboratory [21]. Diabetic condition was confirmed in fasting rats from blood glucose level more than 250 mg/dL determined after 72 h interval and then on the 7th day after day of injection to investigate the stability of the diabetic condition. Diabetes was induced in eighteen rats in this way.

Initial body weight of all the twenty four rats were recorded and divided into following four equal groups. The duration of experiment was 28 days.

Group I (Control group) received a single intramuscular injection of citrate buffer (0.1 mL/100 g body weight/rat).

Group II (Diabetic group) was made diabetic by a single intramuscular injection of STZ at a dose of 3.5 mg/0.1 mL

citrate buffer/100 g body weight.

Group III (Ethyl acetate fraction treated group) diabetic rats were fed with ethyl acetate fraction by gavage at a dose of 20 mg/0.5 mL distilled water/100 g body weight for twice a day at fasting state on and from 7th day for 28 days.

Group IV (Glibenclamide treated group) diabetic rats were fed with standard drug glibenclamide by gavage at a dose of 6.0 mg/0.5 mL distilled water /100 g body weight for twice a day at fasting state on and from 7th day for 28 days.

Group I and Group II were subjected to feeding of 0.5 mL distilled water /100 g body weight/twice a day for 28 days at the time of Ethyl acetate fraction and glibenclamide treatment to the animals of Groups III and IV to keep all the animals under same experimental conditions.

Starting from the day of fraction or standard drug administration to diabetic rats, fasting blood glucose level in all the groups was measured by single touch glucometer in every two days. On the 29th day of the experiment (considering the day of treatment of ethyl acetate fraction and glibenclamide as the 1st day), all the animals were sacrificed by decapitation after recording the final body weight and organs weight. Blood was collected from dorsal aorta by syringe and the serum was separated by centrifugation at 3000 r/min for 5 min for insulin, testosterone and metabolic toxicity parameters assessment. Packed cell pellet was used for glycated hemoglobin (HbA1c) measurement.

2.3. Hyperglycemic profile measurement

2.3.1. Testing of fasting blood glucose level

Fasting blood glucose (FBG) level was measured using the single touch glucometer by collecting blood from tip of the tail of all experimental and control animals in all groups at the initial time of experiment and every two days interval throughout the experiment [21].

2.3.2. Assay of serum insulin by ELISA

Serum insulin level was measured using solid phase-conjugated sandwich ELISA kit for rat (EZRMI-13K, Millipore, USA) [22]. The optical density of standard and unknown samples was measured against blank using a 480 nm selective filter and a 650 nm differentiating filter. No inter-assay variation was occurred as all samples were assayed at the same time.

2.3.3. Assessment of glycated hemoglobin level

Glycated hemoglobin (HbA1c) was measured according to standard protocol [21].

2.4. Antioxidative enzyme activities profile Assessment

2.4.1. Biochemical estimation of catalase, peroxidase, superoxide dismutase (SOD) and glutathione-s-transferase (GST) activities

The activities of catalase, peroxidase, SOD and GST of the testis, cauda epididymis and sperm pellet were measured biochemically following standard laboratory protocol [21].

2.4.2. Estimation of end products of lipid peroxidation (CD and TBARS)

The sample tissues were homogenized separately at the tissue concentration of 50 mg/ mL in 0.1 M of ice-cold phosphate buffer (pH: 7.4) and the homogenates were centrifuged at 10000 r/min at 4 ℃ for 5 min separately. Each supernatant was used for the spectrophotometerical quantification of CD and TBARS following standard method [21].

2.5. Measurement of reproductive profile

2.5.1. Epididymal sperm count and sperm motility assessment

Spermatozoa were collected from an equal length of the cauda of the excised epididymis of each rat and microscopic examination performed as per standard protocol [23]. The numbers of motile spermatozoa in a 100 μL suspension counted under the microscope after placing it on a glass slide and covering it with a cover slip [24]. The result was expressed as % after counting 100 sperm in each field.

2.5.2. Quantification of seminal vesicular fructose level

For fructose quantification, seminal vesicular homogenate was prepared at a tissue conc. of 50 mg/mL. The supernatant was deproteinized by adding 50 μL of zinc sulphate and 50 μL of sodium hydroxide to make a total dilution of seminal plasma 1:16, followed by centrifugation at 2500 r/min for 15 min. For fructose measurement 200 μL of this clear supernatant was used. The optical density of standard and sample were measured against blank at 470 nm. The concentration of fructose was obtained by plotting the value in standard curve and the value expressed in the unit of μM/ mL of seminal plasma [25].

2.5.3. Estimation of testicular cholesterol level

Testicular cholesterol was estimated using the kit supplied by Angstrom Biotech Pvt. Ltd. (Vododara, India) following spectrophotometric method [26].

2.6. Testicular Bax-α mRNA expression pattern by PCR study

Total RNA was extracted from rat testis with TRI-Reagent (Ambion, USA). Purity of extracted RNA was estimated by 260/280 nm absorbance ratio. cDNA was synthesized from 2 μg of total RNA using the as per instructions of Omniscript RT kit (Qiagen, Germany). The composition of cDNA master mix for cDNA preparation was 10x RT Buffer 2 μL, 5 mM dNTP mix 2 μL, 10 μM Oligo dT primer 2 μL, 10 units RNase inhibitor 1 μL, Omniscript RT 1 μL, Template RNA 2 μg and DEPC water to make the total volume of 20 μL and cDNA extraction was completed within 60 min at 37 ℃[27]. After the end of cDNA production, the reverse transcription mix was diluted with Promega nuclease free water (Promega Corporation, USA). For PCR, target Bax-α gene was co-amplified with a housekeeping gene β-actin (used as an

internal control) by PCR master mix with a composition of 2 μL reverse transcription mix, 36.5 μL nuclease free water, 5 μL of 10X Taq buffer (2.5 mM MgCl2), 5 μL of 10X dNTP mix, 0.5 μL of 5U/μL Taq DNA polymerase, 2 μL target primers (7.5 μM of each primers; R & D Systems, RDP-43, GenBank Accession Number: U49729, for rat) and 0.5 M β-actin primer. The PCR reactions were carried out with profile - denaturation at 95 ^{0}C for 4 min; 30 cycles at 94 ℃ for 45 seconds, 62 ℃ for 1 min and 72 ℃ for 1 min, and final extension at 72 ℃ for 10 min. At the end co amplified target and housekeeping PCR products were resolved on 1.5% agarose gel, exposed on translimunator and Densitometric documentation performed with the help of a gel documentation system (UVP. Inc.) Every sample was analysed in triplicate.

2.7. Histological study

The testicular tissue was dissected out and slices were prepared from the same region of all the groups and fixed in Bouin's fixative. These were subjected to paraffin embedding followed by section cutting in microtome and heamatoxylin-eosin staining for microscopic examination in accordance with laboratory procedures. Histological examinations were carried out on stained sections as per our laboratory established standard method [21].

2.8. Biochemical estimation of SGOT and SGPT

Metabolic toxicity bio-markers serum GOT and GPT activities were measured following the instructions of specific supplied kits (Span Diagnostics Ltd., Surat, India). The activities of these enzymes were expressed as relative units [21].

2.9. Acute toxicity study

Healthy adult Wistar albino rats of either sex, starved overnight, were divided into four groups containing six rats each and were orally fed with the ethyl acetate fraction of hydromethanolic extract of *M. paradisiaca* in increasing dose levels of 50, 100 and 300 mg/100 g body weight. The rats were kept under supervision continuously for 2 h for behavioral, neurological and autonomic profiles and after a period of 24 and 72 h for any lethality or death [28].

2.10. Statistical analysis

All experimental trials were replicated three times. An analysis of variance (ANOVA) followed by multiple comparison two tail t test was used to compare in between the groups[21]. Differences were considered significant at $P<0.05$.

2.11. HPLC fingerprinting purification of ethyl acetate fraction

A reversed phase high performance liquid chromatography (HPLC) was used for the purification of ethyl acetate fraction of hydromethanolic (40:60) extract of root of *Musa paradisiaca* on an Agilent 1100 series HPLC system equipped with two isocratic pumps and Diode Array Detector (DAD). The column used for separation was a 'ZORBAX SB-C18 (Agilent technologies)' column (Stable Bond Analytical - 4.6 X 20 mm, 5μ particle size) [29]. A sample of (mg/ mL) 50 μL solution was injected into HPLC C-18 column through a guard column by using the sample injector. The 50 μL loop was used in sample injector. The method for elution of the ethyl acetate fraction from the C-18 column was methanol (CH_3OH) and acetonitrile (CH_3CN) used as mobile phase solvent system. The gradient used was isocratic mode at 5% CH_3OH & 95% CH_3CN for 2 min increased linearly to 40% CH_3OH & 60% CH_3CN over 30 min and isocratic at 50% CH_3OH & 50% CH_3CN for 30 min. The flow rate was 1 mL/min throughout the separation. The diode array detection system was allowed for separation of the compounds in the sample. Earlier the sample was scanned was scanned by the spectrophotometer. It was observed that two maximum wave lengths which were λ max 254 nm and 342 nm. Therefore the HPLC column elutes were monitored by their UV absorbances at λ max 254 nm and 342 nm. Data from the system was collected and evaluated using Agilent online Instrument I software.

3. Results

Body weight and reproductive organo-somatic indices of the diabetic animals were decreased significantly in comparison with the control group. Ethyl acetate fraction of hydromethanolic (40:60) extract of root of *Musa paradisiaca* or glibenclamide treatment to the diabetic rat twice/day for 28 days resulted in a significant recovery of above parameters though not to the level of the control group (Table 1).

Fasting blood glucose level greater than 250 mg/dL was noted significant in untreated diabetic animals when compared with non-diabetic control rats (Figure 1). Treatment with ethyl acetate fraction or glibenclamide to diabetic animals for 28 days resulted in a significant lowering of fasting blood glucose level (Table 2).

Serum insulin level and glycogen contents in hepatic and skeletal muscle were decreased in the untreated diabetic group in comparison with the control group of animals. Level of glycated hemoglobin in total blood of untreated diabetic rats was also found with higher values than the control. The administration of the fraction or glibenclamide to diabetic animals resulted significant recovery in the serum insulin and glycated hemoglobin levels along with glycogen content towards the control levels (Table 3).

Semen quality monitoring reproductive biomarkers e.g. sperm count and sperm motility were decreased significantly in untreated diabetic rats in compare to control rats, which furthermore increased significantly in ethyl acetate fraction or glibenclamide treated diabetic rats towards the control

Table 1

Corrections in body weights and reproductive organo-somatic indices and neutralization of elevated toxicity biomarkers serum GOT and GPT activities towards the control level after treatment with ethyl acetate fraction of hydromethanol extract of *M. paradisiaca* or glibenclamide in streptozotocin-induced diabetic rats.

Groups	Body Weight (gm)		Testiculo-somatic index	Epididymis-somatic index	Seminal vesiculo-somatic index	SGPT(IU/L)	SGOT(IU/L)
	Initial	Final					
Group I (Control group)	123±1.5 [a]	131±2.4[b]	3.16±0.14[a]	0.637±0.024[a]	0.432±0.02 [a]	33±1.46 [a]	13±1.57[a]
Group II (Diabetic group)	121±1.1 [a]	115±1.4[c]	0.99±0.04[b]	0.373±0.016[b]	0.168±0.01[b]	107±4.77[b]	57±2.50[b]
Group III(Ethyl acetate fraction treated group)	122±2.1 [a]	128±1.9[b]	2.07±0.08[c]	0.554±0.025[c]	0.351±0.03[c]	53.2±2.33[c]	34±1.52[c]
Group IV(Glibenclamide treated group)	123±2.3 [a]	129±2.2[b]	2.11±0.09[c]	0.549±0.025[c]	0.346±0.02d	47.3±2.11d	29±1.29[c]

Data are expressed as mean ± SEM, n=6. ANOVA followed by multiple comparison two tail t est. Values with different superscripts each other significantly (P<0.05).

Table 2

Correction in the mean fasting blood glucose level after treatment with ethyl acetate fraction or glibenclamide in streptozotocin-induced diabetic rats.

Groups	Mean Fasting Blood Glucose Level (mg/dL)											
	Day 0(STZ-injection)	Day 1	Day 7(treatment Started)	Day 10	Day 13	Day 16	Day 19	Day 22	Day 25	Day 28	Day 31	Day 35Group I
(Control group)	68±3.04[a]	65±2.90[a]	66±2.90[a]	64±2.86[a]	61±2.72[a]	63±2.82[a]	65±2.89[a]	61±2.72[a]	64±2.87[a]	62±2.69[a]	64±2.79[a]	63±2.81[a]
Group II (Diabetic group)	66±2.89[a]	259±11.49[b]	264±11.8[b]	263±11.72[b]	263±12.01[b]	259±11.7[b]	262±11.72[b]	265±11.78[b]	259±11.44[b]	263±11.79[b]	266±11.90[b]	263±11.76[b]
Group III (Ethyl acetate fraction treated group)	65±2. 91[a]	233±10.39[c]	262 ±11.68[b]	249±4.12[d]	216±3.06[e]	183±4.11[f]	143±3.40[h]	102±4.57[i]	86±3.79[j]	79±3.40[k]	69±3.08[a]	68±3.09[a]
Group IV (Glibenclamide treated group)	67±2.89[a]	231±10.48[c]	261±11.86[b]	246±6.85[d]	219±4.90[e]	195±7.64[g]	147±4.02[h]	106±4.74[i]	90±4.16[j]	77±3.25[k]	71±3.17[a]	69±3.09[a]

Data are expressed as mean ± SEM, n=6. ANOVA followed by multiple comparison two tail t test. Values with different superscripts differ from each other significantly (P<0.05).

Table 3

Protective efficacies of ethyl acetate fraction of hydromethanol extract of *M. paradisiaca* or glibenclamide on biomarkers of hyperglycaemia and serum testosterone level in streptozotocin-induced diabetic rats.

Group	Glycated hemoglobin level (GHb%)	Glycogen (μg of glucose/mg of tissue)		Serum insulin level (ng/mL)	Serum testosterone level(ng/dl)
		Liver	Muscle		
Group I(Control group)	2.34 ±0.11[a]	9.30±0.45[a]	6.60±0.33[a]	3.29 ± 0.10[a]	1.29 ± 0.06[a]
Group II (Diabetic group)	4.78 ±0.21[b]	4.30±0.20[b]	3.28±0.13[b]	0.31 ± 0.02[b]	0.09 ± 0.003[b]
Group III(Ethyl acetate fraction treated group)	3.21±0.14[c]	6.45±0.29[c]	4.80±0.19[c]	1.70 ± 0.06[c]	0.74 ± 0.03[c]
Group IV(Glibenclamide treated group)	3.09±0.12[c]	6.92±0.31[c]	4.60±0.21[c]	1.39 ± 0.06[d]	0.53 ± 0.02[d]

Data are expressed as mean ± SEM, n=6. ANOVA followed by multiple comparison two tail t test. Values with different superscripts differ from each other significantly (P<0.05).

levels (Table 4).

Decreased level of serum testosterone along with increased testicular cholesterol and seminal vesicular fructose levels were found in diabetic group in respect to the control group, which were rectified towards the control after treatment with ethyl acetate fraction or glibenclamide (Table 4).

Catalase, peroxidase, superoxide dismutase and glutathione-s-transferase activities in testis, cauda epididymis and sperm pellet were decreased significantly in diabetic group in respect to control group. After the treatment with this herbal formulation or glibenclamide to STZ-treated diabetic rats, the levels of these parameters were restored towards the control levels (Figure 1). Levels of end products of the lipid peroxidation i.e. conjugated diene and thiobarbituric acid reacting substance in the said reproductive tissues and sperm pellet were increased

Table 4

Amelioration in the reproductive biosensors after treatment with ethyl acetate fraction of hydromethanol extract of *M. paradisiaca* or glibenclamide treatment in streptozotocin-induced diabetic rats.

Group	Sperm count (Million/ mL epididymal fluid)	Sperm Motility (%)	Seminiferous tubular diameter (mm), X 400	Seminal plasma fructose level (μ M/mg)	Testicular Cholesterol level(mg/dl)
Group I(Control group)	18.14±0.8[a]	82.31 ±3.66[a]	76.33±3.41[a]	2.68±0.12[a]	22.18±1.61[a]
Group II(Diabetic group)	6.01 ±0.3[b]	27.92 ±1.25[b]	51.28±2.27[b]	4.61±0.23[b]	44.32±2.17[b]
Group III(Ethyl acetate fraction treated group)	13.99±0.6[c]	66.11 ±2.96[c]	62.91±2.79[c]	3.25±0.15[c]	28.22±1.74[a]
Group IV(Glibenclamide treated group)	14.41±0.5[c]	61.74 ±2.67[c]	61.79±2.64[c]	3.63±0.15[d]	34.13±1.61[a]

Data are expressed as mean ± SEM, n=6. ANOVA followed by multiple comparison two tail t test. Values with different superscripts differ from each other significantly (P<0.05).

significantly in diabetic group when compared to the control group. There was a significant recovery in the levels of the above parameters in target tissues after treatment with ethyl acetate fraction or glibenclamide to the diabetic group animals (Figure 1).

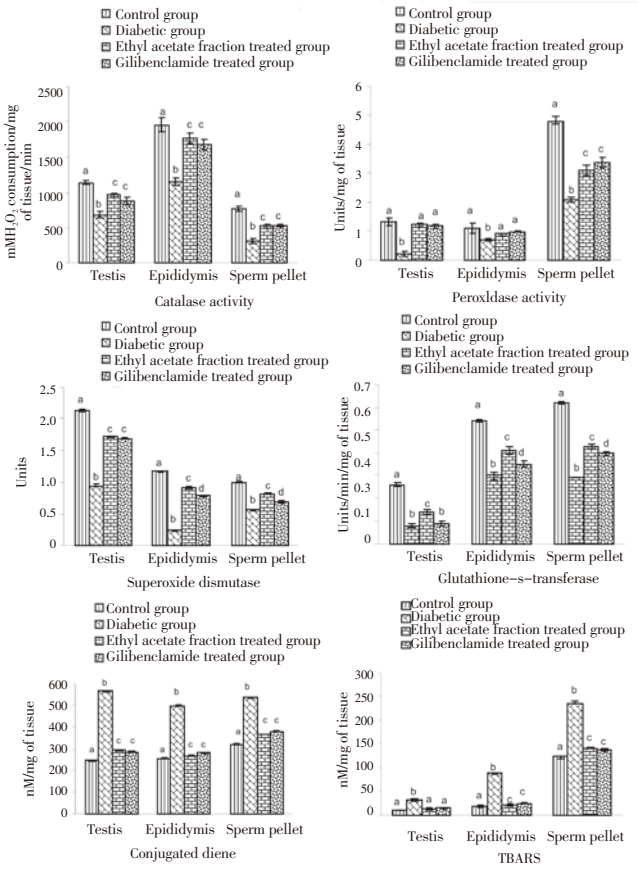

Figure 1. Effect of ethyl acetate fraction or glibenclamide on antioxidant enzymes activities and amendment of free radical generated end products, CD and TBARS levels in testis, epididymis and sperm pellet in streptozotocin-induced diabetic rats. Each bar represents mean ± SEM, n=6 for each group. ANOVA followed by multiple comparison two tail t test. Values of bar diagram with different superscripts (a,b,c,d) differ from each other significantly at the level of P<0.05.

PCR-denositometric analysis of the testicular apoptosis biomarker Bax-α mRNA up regulated expression also indicated increased level of testicular apoptosis in diabetic rat when compared with the expression pattern for the same in the control group. A significant remedial activity of ethyl acetate fraction or glibenclamide noticed in the mRNA expression of above pro-apoptotic marker in target tissue after treatment to diabetic group animals (Figure 2).

The diminution in the seminiferous tubular diameter and degeneration in germ cell layers in seminiferous tubule were observed in the diabetic rat in respect to the control group rat which was corrected in qualitative aspect after ethyl acetate fraction formulation or glibenclamide treatment to diabetic rat (Table 4, Figure 4).

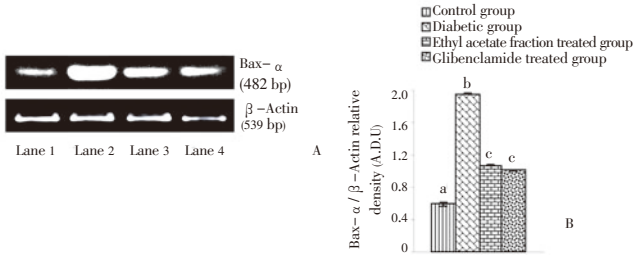

Figure 2. PCR-denositometric analysis of the testicular apoptosis biomarker.

A: Representative PCR analysis of Bax-α mRNA expression in experimental rat testes (Lane 1: control rat; Lane 2: Untreated diabetic rat; Lane 3: Ethyl acetate fraction treated rat; Lane 4: Glibenclamide treated rat) and its semiquantitative densitometric analysis after normalizing the result with housekeeping gene, β-Actin.

B: Each bar represents mean ± SEM, n=6 for each group. ANOVA followed by multiple comparison two tail t test. Values of bar diagram with different superscripts (a, b, c) differ from each other significantly at the level of P<0.05 at each time period. A.D.U. - arbitrary densitometric units.

Activities of serum GOT and GPT were increased in diabetic group compared to the control group. A significant attenuation of the enzyme activities towards the control level were found after treatment with ethyl acetate fraction or glibenclamide (Table 1).

Ethyl acetate fraction of hydromethanolic extract of *M. paradisiaca* at its maximum dose level of 300 mg/100 gm of body weight did not produce any significant changes in the autonomic, behavioral or neurological alteration. Acute toxicity studies revealed the non-toxic nature of the Ethyl acetate fraction of hydromethanolic extract of *M. paradisiaca*.

The reversed phase HPLC chromatogram of the sample

at λ max 254 nm has been shown in Figure 3 with five well resolved peaks. These were 3.11 mins, 5.35 mins, 15.2 mins, 29.9 min & 30.9 min. Therefore five compounds were separated and purified. HPLC Chromatogram of the same sample at λ max 342 nm has been shown in Figure 3, which also shows five well resolved peaks at 3.10 min, 5.34 min, 15.2 min, 29.9 min and 30.9 min.

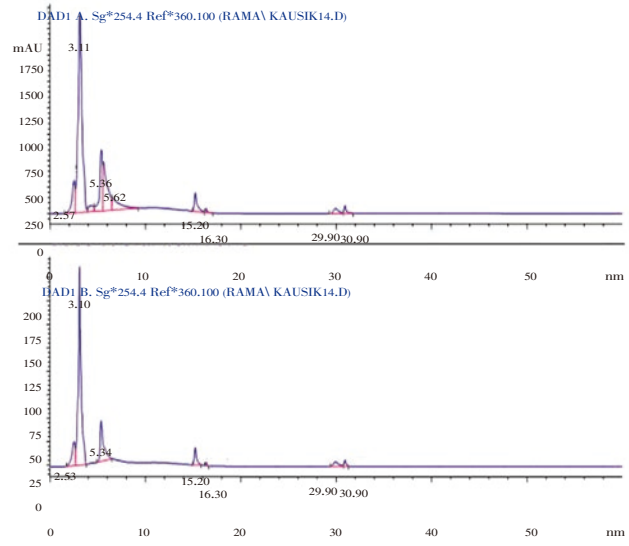

Figure 3. RP-HPLC Chromatogram of sample ethyl acetate fraction of hydro-methanol extract of *M. paradisiaca*. Chromatographic conditions: mobile phase, methanol-acetonitrile; UV detection at 254 and 342 nm (λ max) with five well resolved peaks at 3.11 min, 5.35 min, 15.2 min, 29.9 min and 30.9 min.

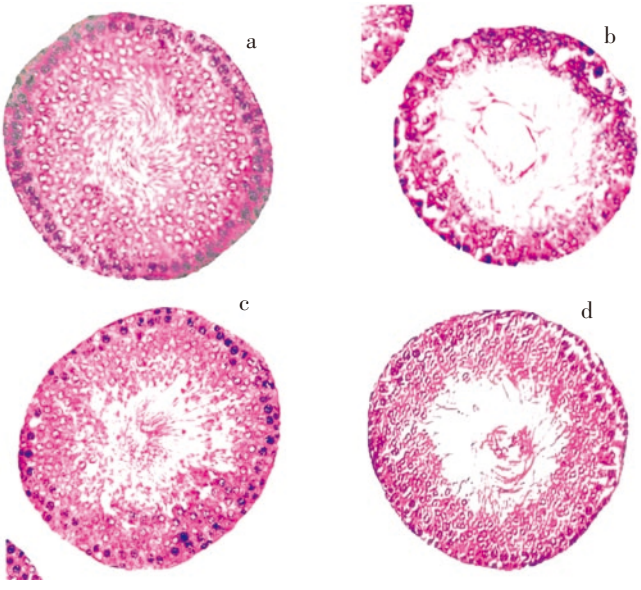

Figure 4. Histology of testis, 400 X (Haematoxylin-Eosin Stain).
a: Representative microphotograph of testicular section at stage VII showing normal arrangements of different generation of germ cells in seminiferous tubule of control group; b: Representative microphotograph of testicular section showing diminution in the number and disarrangements in different generation of germ cells at stage VII of diabetic group; c: Representative microphotograph of testicular cross section at stage VII showing recovery in the number of different generation of germ cells. d: Representative sample of testicular section at stage VII showing recovery in the number of different generation of germ cells of glibenclamide treated group.

4. Discussion

Streptozotocin causes selective destruction of insulin secreting pancreatic β-cells by reactive oxygen species dependent oxidative damage [30] resulting insulin dependent diabetes mellitus.

In streptozotocin induced diabetic rat, the elevation in levels of fasting blood glucose and glycated hemoglobin along with diminution in liver and skeletal muscle glycogen levels due to low levels of serum insulin as per present findings and in parallel with our previous reports[21,31].

The present study shows streptozotocin-induced diabetes resulted in significant diminution in testiculo-somatic, epididymo-somatic and seminal vesiculo-somatic indices along with atrophy of seminiferous tubule, which may be due to apoptotic cell death caused by low serum testosterone level as it is the prime regulator of normal growth of these organs [32].

Deviation of sperm parameters i.e. sperm count and motility, which were decreased in diabetes may be due to inhibition in spermatogenesis or may be due to oxidative free radical deposition in sperm and diminution in the activities of antioxidant enzymes in sperm pellets. The oxidative stress induction in sperm in diabetic state may be due to high level of uncoupler protein synthesis in sperm or may be due to hypoxic state condition in testis, epididymis as diabetes results glycated hemoglobin formation that interfere oxygen delivery at target tissues[33].

This has been proved here by the study of catalase, peroxidase, superoxide dismutase and glutathione-s-transferase in testis, cauda epididymis and sperm pellet along with the levels of end products of free radicals like TBARS and CD may be due to low serum testosterone that has been reflected here[12].

Level of seminal plasma fructose level, main energy source for sperm viability and motility, has been increased in diabetes may be due to the diminution of the sperm count that may interfere in fructose utilization because of oxidative stress is also in the same line of previous works [34].

After treatment with ethyl acetate fraction or glibenclamide the recovery of above organo-somatic indices, correction in the sperm count and motility along with the arrangement of different stages of spermatogenesis towards control was noted. Testicular oxidative stress imposed in STZ induced diabetic state was recovered towards the control level reflected from the recovery of different anti oxidant enzymes activity and low levels of end products of free radicals. Androgenesis process was also corrected, supported by upliftment in the level of serum testosterone and lowering of testicular cholesterol level [12]. The lowering level of seminal vesicular fructose after ethyl acetate fraction treatment shows the correction in fructose synthesis and its utilization by sperm reflected by increased motility.

Our present finding from densitometric analysis of testicular proapoptotic Bax-α mRNA up-regulated

expression strongly indicates the involvement of the intrinsic pathway in the diabetes-induced testicular germ cell apoptosis which has been corrected after treatment with herbal formulation or glibenclamide. The up regulated expression of the pro apoptotic marker gene may be due to diabetes induced oxidative stress which is consistent with the previous observation [18].

From the results it may be predicted that in diabetes the pituitary-testicular axis is deviated towards the negative side and its tuning system is altered so that reproductive system is affected drastically that may lead to infertility. The dose of this ethyl acetate fraction is comparatively less than our previously used hydro-methanolic extract [6] and has a promising effect on glycemic as well as testicular disorders.

This ethyl acetate fraction has no toxicity which has been indicated here from the improved body weight as well as correction in serum GOT and GPT activities seems to be its ability to enhance glucose utilization and reduce hepato-renal dysfunction as these are the indicators of general toxicity [21]. In respect to maximum non-fatal doses studied revealed the non-toxic nature of this fraction of this plant. There was no lethality or any toxic reactions found at any of the doses selected until the end of the study period. According to toxicity classification [35], the ethyl acetate fraction of hydro methanol extract of *M. paradisiaca* is non toxic.

From the HPLC purification of this fraction we found five separate compound peaks which indicating the said corrective effect of anyone of the compound or summative of the five type of compound.

So, the ethyl acetate fraction of hydro methanol extract of *M. paradisiaca* may be used as an agent for the correction of testicular dysfunctions noted in diabetes.

Conflict of Interest

We declare that we do have no conflict of interest.

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