

# Diabetes-induced Testicular Disorders Vis-a-vis Germ Cell Apoptosis in Albino Rat: Remedial Effect of Hexane Fraction of Root of *Musa paradisiaca* and Leaf of *Coccinia indica*

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The present study was designed to investigate diabetes induced germ cell apoptosis in testis and its protection by treatment with hexane fraction of hydro-methanolic extract of root of *Musa paradisiaca* and leaf of *Coccinia indica* in composite manner. Testicular oxidative stress injury was reflected by diminution in the activities of antioxidant enzymes such as catalase, peroxidase, superoxide dismutase and glutathione-s-transferase along with elevation in levels of conjugated diene and thiobarbituric acid reactive substances in diabetic condition. Serum testosterone and insulin levels were decreased in association with elevation in apoptosis of pancreatic islet cells in diabetic rats. Giant cells number along with elevation in the number of apoptotic cells were noted in seminiferous tubules in diabetic model animals. Significant improvements in the levels of blood glucose, serum insulin and testosterone and testicular oxidative stress parameters were noted versus control after treatment of said fraction at a dose of 2 mg (1 : 3)/0.2 ml olive oil per 100 g body weight per day for 45 days to diabetic rats. Numbers of giant cells in seminiferous tubules, apoptotic germ cells and apoptotic pancreatic islet cells were decreased significantly in fraction treated diabetic group versus control. From UV-spectroscopic and TLC studies in connection with phytochemical screening of the said fraction, phenol, flavonoid and alkaloid types of compound were found. From these results it may be concluded that the active ingredient(s) present in hexane fraction of root of *Musa paradisiaca* and leaf of *Coccinia indica* have the potential to correct diabetes-induced testicular germ cell apoptosis.

**Key words** — diabetes, apoptosis, giant cell, *Musa paradisiaca*, *Coccinia indica*

## INTRODUCTION

Sexual dysfunction is frequently associated with diabetes in males.<sup>1)</sup> Diabetes mellitus affects various functions of the reproductive system in model animals and human either directly or indirectly.<sup>2)</sup> Streptozotocin (STZ)-induced diabetes in male rats resulted in atrophy of sex organs, changes in histoarchitecture of ventral prostate,<sup>3)</sup> diminution in sperm count,<sup>3)</sup> and low levels of plasma gonadotrophins<sup>4)</sup> and testosterone.<sup>5)</sup> Such diabetes-induced testicular impairment is due to generation of oxidative

stress.<sup>6,7)</sup>

Apoptosis is a rapid physiologically programmed cell death characterized by nuclear and cytoplasmic condensation followed by cell break up into membrane-bound fragments that ultimately form structurally intact organelles known as “apoptotic bodies.”<sup>8)</sup> These apoptotic bodies are phagocytosed by neighboring cells and rapidly degraded so that there is no inflammation in the adjacent tissue. Apoptotic cell death is regulated through activation of apoptotic signal pathways, including the B-cell lymphoma 2 (Bcl-2) family protein and caspases. It is well accepted that Bcl-2 and B-cell lymphoma-extra large (Bcl-XL) are anti-apoptotic factors whereas Bcl-2-associated X protein (Bax) and Bcl-2-associated death promoter (Bad) are pro-apoptotic factors.<sup>9,10)</sup> Released Bax increases mitochondrial permeability and helps to

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release cytochrome-C from mitochondria resulting in activation of caspase-3 responsible for induction of apoptosis.<sup>11)</sup> Germ cell apoptosis at stage VII–VIII is known to be associated with testosterone levels<sup>12)</sup> since these stages of rat seminiferous epithelial cycle are mostly androgen dependent stages.<sup>12)</sup>

Many medicinal plants are used for the treatment of diabetes throughout the world. Plant drugs or herbal formulations<sup>13, 14)</sup> are considered less toxic and associated with fewer side effects than synthetic drugs.<sup>15)</sup> Diabetes-induced male reproductive disorders are corrected by polyherbal formulation as reported by us.<sup>16, 17)</sup> Based on World Health Organization (WHO) recommendations, hypoglycemic agents of plant origin used in traditional medicine are important.<sup>18)</sup> In the traditional system of Indian medicine, plant formulations and combined extracts of plants are used as drugs of choice rather than individual ingredients and many of these have shown promising effects.<sup>19)</sup>

*Musa paradisiaca* (*M. paradisiaca*) L. is a tree-like herb belonging to the “Musaceae” family and is distributed throughout India and Malaysia. Different parts of the plant have medicinal values.<sup>20)</sup> *Coccoloba indica* (*C. indica*, Wight & Arn) is a member of “Cucurbitaceae” family and distributed widely all over India. Its medicinal importance has been observed earlier by others.<sup>21)</sup>

## MATERIALS AND METHODS

**Plant Materials and Extract Preparation**— In the month of June, the roots of *M. paradisiaca* and leaves of *C. indica* were collected from local area and the materials were identified by taxonomist of Botany Department, Vidyasagar University, Midnapore, India. The voucher specimens having HPCH No-7, 8 were preserved in the Department of Botany, Vidyasagar University. In an incubator, fresh leaves of *C. indica* and small pieces of fresh root of *M. paradisiaca* were dried at 40°C for 2 days, crushed separately in an electric grinder then powdered as per the method published earlier by us.<sup>22)</sup> For the preparation of hydro-methanolic extract, 100 g of the prepared powder was suspended in 500 ml of hydro-methanol (2 : 3) mixture and kept in an incubator at 37°C for 36 hr. The slurry was stirred intermittently for 2 hr and left overnight. The slurry was dried by low pressure, residue collected and 20–25 g of hydro-methanolic extract of each plant part prepared. These extracts were again dis-

solved in 250 ml of hydro-methanol (2 : 3). In a separating funnel 250 ml of hexane was added with the hydro-methanolic extract. Hexane fraction was collected and dried in reduced pressure. Aliquots of 1.2 g of hexane fraction of *C. indica* and 800 mg of *M. paradisiaca* were collected from the said amount. When required, individual fractions were dissolved in olive oil at 1 : 3 ratio and treated to the diabetic rats.

**Animal Selection and Care**— Twenty-four Wistar strain, mature, normoglycemic (70–75 mg/dl), pathogen-free rats of 3 months of age weighing 150 ± 10 g were selected for this experiment. They were acclimatized to laboratory condition for 2 weeks prior to experimentation. Animals were housed three rats per cage in a temperature-controlled room (22 ± 2°C) with 12 : 12 hr light: dark cycle at a humidity of 50 ± 10%. They were provided with standard food and water *ad libitum*. The principles of Laboratory Animal Care were followed throughout the duration of experiment as per guideline of National Institute of Health Instructions given by our Institutional Ethical Committee.<sup>23)</sup>

**Induction of Diabetes**— Eighteen rats were fasted for 24 hr before injection of STZ. Rats were subjected to injection of STZ at a dose of 4 mg/0.1 ml citrate buffer per 100 g body weight per rat. Twenty-four hours after STZ injection type 1 diabetic state developed in 15 rats having blood glucose level > 250 mg/dl but < 350 mg/dl. These diabetic rats remained without further treatment of STZ for 7 days to stabilize the diabetic condition. Twelve stable diabetic rats were selected on day 8 and the fraction treatment was started from that day in the morning at 08:00. Two hours after the fraction treatment next feed was supplied to animals at 10:00. Rats were fasted for 10 hr for measurement of fasting blood glucose (FBG) level. For this reason food containers from each cage were removed at 22:00. This protocol was followed as per our previous protocol.<sup>24)</sup>

### Animal Treatment

**Group I**—*Control Group*: Non-diabetic rats of this group were subjected to oral intubation of 0.2 ml olive oil/100 g body weight per rat per day for 45 days.

**Group II**—*Diabetic Group*: Oral administration of olive oil was performed for 45 days to diabetic rats at a dose of 0.2 ml/100 g body weight per rat per day.

**Group III—Composite Hexane Fraction-administered Group:** Hexane fraction of hydro-methanolic extract of root of *M. paradisiaca* and leaf of *C. indica* in composite manner was treated to diabetic rats by gavage at a dose of 2 mg (1 : 3)/0.2 ml olive oil per 100 g body weight per rat per day for 45 days.

After completion of the 45-day treatment schedule, all animals were sacrificed under light ether anesthesia by decapitation on day 46. Blood was collected from dorsal aorta. Serum was separated by centrifugation at 3000 *g* for 5 min and stored at  $-20^{\circ}\text{C}$  in a refrigerator for assessment of serum insulin and testosterone levels. Pancreas and testes were collected. One testis was stored at  $-20^{\circ}\text{C}$  for assessment of activities of catalase (CAT), peroxidase (POD), glutathione-s-transferase (G-S-T) and superoxide dismutase (SOD) along with quantification of levels of conjugated diene (CD) and thio-barbituric acid reactive substance (TBARS). Epididymis was dissected out and stored at  $37^{\circ}\text{C}$  in normal saline for assessment of epididymal sperm count and sperm viability from cauda part of epididymis. Mid part of pancreas and contralateral testis of each animal were taken and fixed in neutral formalin and subjected to paraffin embedding followed by section cutting in microtome and staining for microscopic examination in accordance with routine laboratory procedures. Histological examinations of seminiferous tubules were carried out on hematoxylin-eosin and *in situ* end labeling (ISEL)-stained sections.

**Testing of Fasting Blood Glucose Level**— At the time of grouping of the animals, FBG level was measured. After 15 days, FBG levels were further recorded by single touch glucometer after collecting blood from tail vein from all animals of each group.

**Serum Insulin and Testosterone Levels**— Serum insulin level was measured by enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim Diagnostic, Mannheim, Germany).<sup>25)</sup> The insulin level in serum was expressed in  $\mu\text{IU/ml}$ .

Serum testosterone level was measured by solid phase-conjugated assay testosterone kit supplied by Lilac Medicare (P) Ltd, Mumbai, India.<sup>26)</sup>

**Sperm Viability**— Sperm viability was measured by eosin-nigrosin stain.<sup>27)</sup> One drop of epididymal fluid was fixed with two drops of 1% eosin Y and after 30 s three drops of 10% nigrosin were added. A smear of stained sperm was made by plac-

ing a drop of mixture on a clean glass slide and dried in air before examination by phase contrast microscope. Pink stained headed sperms were counted as dead sperms and unstained sperms as live sperms.

**Biochemical Assay**— The testis was homogenized at a 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 *g* at  $4^{\circ}\text{C}$  for 5 min individually from all groups. Each supernatant was used for estimation of the following biochemical parameters. The activity of CAT in testis was measured biochemically by Beers and Sizer method.<sup>28)</sup> Testicular POD activity was measured according to an acceptable standard method.<sup>29)</sup> G-S-T activity in testis was measured spectrophotometrically according to the method of Habig *et al.*<sup>30)</sup> SOD activity in testis was measured according to the procedure described by Marklund and Marklund.<sup>31)</sup> Lipid peroxidation was estimated from the concentration of TBARS and CD. Testicular TBARS was quantified by the method of Okhawa *et al.*<sup>32)</sup> and quantification of CD was performed by a standard method of Slater.<sup>33)</sup>

**ISEL Study**— ISEL histochemistry was performed using TACS TdT-DAB In Situ Apoptosis Detection Kit (Trevigen Inc., Gaithersburg, MD, U.S.A.).<sup>34)</sup> Paraffin sections were deparaffinized in xylene, dehydrated through graded alcohol, and washed with phosphate buffered saline (PBS). The sections were treated with 0.05% protein kinase K for 5 min and blocked with 0.3% hydrogen peroxide in methanol for 10 min. After washing in PBS, sections were incubated with equilibration buffer followed by terminal deoxynucleotidyl transferase (TdT) enzyme in a humidified chamber at  $37^{\circ}\text{C}$ . Sections were labeled with digoxigenin peroxidase and visualized with diaminobenzidine (DAB) substrate. Sections were counterstained with methyl green, dehydrated in graded alcohol and cleaned to quantify the incidence of apoptosis; seminiferous tubules containing three or more apoptotic cells by tunnel stain were calculated.<sup>35)</sup>

**Quantification of Germ Cell at Stage VII and Giant Cell in Testicular Section**— The hematoxylin-eosin-stained slides were scanned under light microscope. Quantification of different generations of germ cells at stage VII was performed according to the method of Leblond and Clermont.<sup>36)</sup> The cells present in this stage are spermatogonia-A (SgA), preleptotone spermatocytes (pLSc), mid-pachytene spermatocyte (mPSc), step 7 spermatids (7Sd) and step 19 spermatids

(19Sd). The different nuclei of the germ cells (except 19Sd, which cannot be precisely counted) were counted at 20 round tubular cross-sections in each rat. All the nuclear counts of germ cells were corrected for differences in nuclear diameter by the formulae of Abercrombie.<sup>37)</sup> True count = (crude count  $\times$  section thickness)/(section thickness + diameter of germ cell), and tubular shrinkage by the Sertoli cell correction factor<sup>38)</sup> were considered.

**Preliminary Phytochemical Analysis**— The presence of tannins, alkaloids, flavonoids, saponins and terpenoids was determined by simple and standard qualitative and quantitative methods.<sup>39–41)</sup> The simple quantitative analysis of the extract was based on the intensity of the color change. Briefly described, the qualitative phytochemical analysis of hexane fraction of hydro-methanolic extract of root of *M. paradisiaca* and leaf of *C. indica* was performed as follows:

**Tannins:** Plant material of 200 mg was dissolved in 10 ml of methanol water then filtered. A volume of 2 ml of filtrate was pipetted into a test tube after which 2 ml of 15% FeCl<sub>3</sub> was added. Color change was observed. Blue-black presence indicated the presence of tannins.

**Alkaloids:** Two hundred milligrams of the plant material was extracted with 200 ml of methanol for 20 min on a water bath then filtered. A volume of 2 ml of the cold water extract was taken in different tubes; 6 drops of different alkaloids reagents were added, namely: Dragendorff's or Mayer's or Wagners's reagent in the tube. Presence and colors of any precipitate were noted. Creamish, brownish-red, precipitate, or orange precipitate indicated the presence of respective alkaloids.

**Flavonoids:** In a test tube 200 mg of the hexane fraction was boiled in 10 ml of absolute ethanol for 10 min. The solution was allowed to cool then filtered. Filtrate at a volume of 2 ml was taken, and concentrated hydrochloric acid and magnesium ribbon were added. Pink-tomato red color indicated the presence of flavonoids.

**Saponins:** Frothing test. Water extract was obtained by boiling on water bath. The extract was transferred into a test tube and shaken vigorously then was left to stand for 10 min and the result noted. Frothing persistence indicated the presence of saponins.

**Terpenoids:** Vanillin (4-hydroxy-3-methoxy benzaldehyde) 0.8 g was dissolved in 40 ml glacial acetic acid and 2 ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added. This reagent was sprayed on the plate, and then heat was applied at 110°C for 3–5 min. This method used to detect terpenoids.<sup>42)</sup>

**Statistical Analysis**— Analysis of variance (ANOVA) followed by multiple comparison two-tail 't'-test was used for statistical analysis of collected data.<sup>43)</sup> Differences were considered significant at  $p < 0.05$ .

## RESULTS

### FBG Level

FBG level was initially elevated in STZ-induced diabetic rats compared with control animals. However the level of this parameter was significantly recovered towards control level after administration of hexane fraction of hydro-methanolic extract of root of *M. paradisiaca* and leaf of *C. indica* in composite manner for 15 or 30 days to the diabetic rats. Moreover, there was no significant difference of FBG between fraction-treated group and control group at 45 days (Table 1).

### Serum Insulin and Serum Testosterone

Serum insulin and testosterone levels were initially decreased in the diabetic group versus control group. After administration of fraction to the diabetic rats for 45 days, serum levels of these two hormones were significantly restored to control levels. No significant difference of these parameters was observed between the fraction-administered group and control group at 45 days (Fig. 1).

**Table 1.** Recovery of FBG Level after Treatment with Fraction

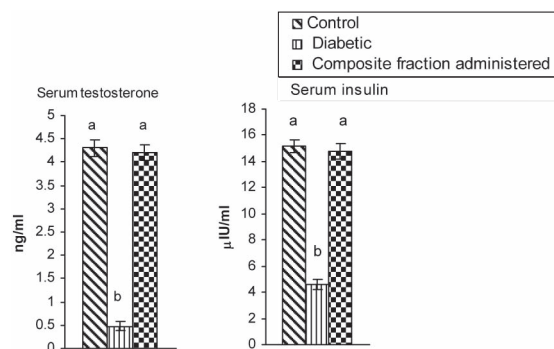
Group	Blood glucose level (mg/dl)			
	0 days	15 days	30 days	45 days
Control	74.3 $\pm$ 5.6 <sup>(a)</sup>	72.6 $\pm$ 5.1 <sup>(a)</sup>	74.4 $\pm$ 5.3 <sup>(a)</sup>	73.54 $\pm$ 4.9 <sup>(a)</sup>
Diabetic	322.3 $\pm$ 5.4 <sup>(b)</sup>	337.2 $\pm$ 5.2 <sup>(b)</sup>	341.5 $\pm$ 5.5 <sup>(b)</sup>	345.54 $\pm$ 5.3 <sup>(b)</sup>
Composite fraction administered	329.7 $\pm$ 5.1 <sup>(b)</sup>	163.0 $\pm$ 5.4 <sup>(c)</sup>	78.5 $\pm$ 5.7 <sup>(c)</sup>	74.41 $\pm$ 5.1 <sup>(a)</sup>

Data are expressed as mean  $\pm$  S.E.;  $n = 6$ . ANOVA followed by multiple comparison two-tail *t*-test. Values with different superscripts (a, b, c) in each vertical column are significantly different, ( $p < 0.05$ ).

**Table 2.** Effect of Fraction on Sperm Count and Viability and Number of Different Generations of Germ Cells at Stage VII

Group	Sperm count (10 <sup>6</sup> /ml epididymal fluid)	Sperm viability (%)	ASg	pLSc	mPSc	7Sd
Control	21.45 ± 0.4 <sup>a</sup>	77.46 ± 3.54 <sup>a</sup>	0.56 ± 0.06 <sup>a</sup>	18.43 ± 0.35 <sup>a</sup>	21.43 ± 0.56 <sup>a</sup>	62.37 ± 2.6 <sup>a</sup>
Diabetic	4.04 ± 0.3 <sup>b</sup>	34.74 ± 4.03 <sup>b</sup>	0.32 ± 0.04	9.24 ± 0.42 <sup>b</sup>	12.41 ± 0.57 <sup>b</sup>	31.9 ± 1.9 <sup>b</sup>
Hexane fraction administered	14.43 ± 0.5 <sup>c</sup>	64.31 ± 3.47 <sup>c</sup>	0.44 ± 0.06 <sup>c</sup>	13.93 ± 0.36 <sup>c</sup>	17.56 ± 0.42 <sup>c</sup>	43.6 ± 2.1 <sup>c</sup>

Data are expressed as mean ± S.E.;  $n = 8$ . ANOVA followed by multiple comparison two-tail  $t$ -test. Values with different superscripts (a, b, c) in each vertical column are significantly different, ( $p < 0.05$ ).



**Fig. 1.** Recovery of Serum Testosterone and Insulin Levels after Treatment of Hexane Fraction of Hydro-methanolic Extract of Root of *M. paradisiaca* and Leaf of *C. indica* in Composite Manner to STZ-induced Diabetic Male Albino Rats

Data are expressed as mean ± S.E.;  $n = 6$ . ANOVA followed by multiple comparison two-tail  $t$ -test. Bars with different superscripts (a, b) are significantly different, ( $p < 0.05$ ).

### Sperm Count and Sperm Viability

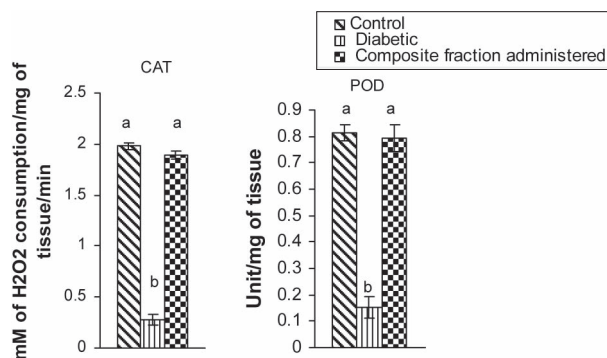
Sperm count and sperm viability were initially decreased in the diabetic group compared with control group. Administration of fraction resulted significant recovery in the levels of these parameters towards control group (Table 2).

### Testicular CAT and POD Activities

Activities of testicular CAT and POD were initially decreased in the diabetic group compared with control group. After treatment with fraction to the diabetic rats, the activities of these enzymes in testis were increased and resettled to control level (Fig. 2).

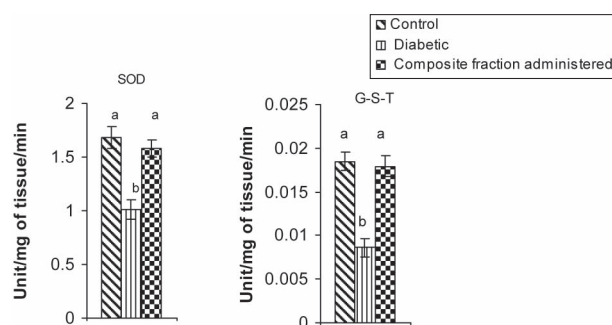
### Testicular SOD and G-S-T Activities

In comparison with the control group the activities of SOD and G-S-T were at first significantly diminished in the diabetic group. Significant restoration in the activities of these enzymes in testis was noted reaching control level after administration of fraction to the diabetic rats (Fig. 3).



**Fig. 2.** Protective Effect of Hexane Fraction of Hydro-methanolic Extract of Root of *M. paradisiaca* and Leaf of *C. indica* in Composite Manner on Testicular CAT and POD Activities in STZ-induced Diabetic Male Albino Rats

Data are expressed as mean ± S.E.;  $n = 6$ . ANOVA followed by multiple comparison two-tail  $t$ -test. Bars with different superscripts (a, b) are significantly different, ( $p < 0.05$ ).

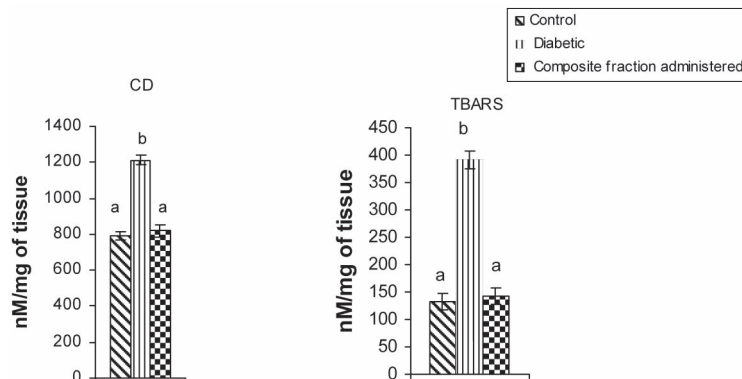


**Fig. 3.** Analysis of Testicular SOD and G-S-T Activities after Treatment of Hexane Fraction of Hydro-methanolic Extract of Root of *M. paradisiaca* and Leaf of *C. indica* in Composite Manner in STZ-induced Diabetic Rats

Data are expressed as mean ± S.E.,  $n = 6$ . ANOVA followed by multiple comparison two-tail  $t$ -test. Bars with different superscripts (a, b) are significantly different, ( $p < 0.05$ ).

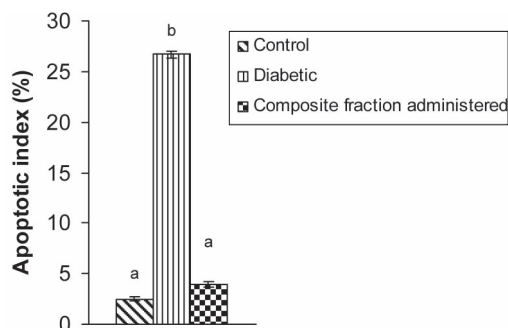
### Testicular CD and TBARS

Levels of testicular CD and TBARS were initially elevated in diabetic group compared with control group. After administration of fraction to the diabetic rats the levels of these parameters were sig-



**Fig. 4.** Effect of Hexane Fraction of Hydro-methanolic Extract of Root of *M. paradisiaca* and Leaf of *C. indica* in Composite Manner on Levels of Testicular CD and TBARS in STZ-induced Diabetic Male Albino Rats

Data are expressed as mean  $\pm$  S.E.,  $n = 6$ . ANOVA followed by multiple comparison two-tail  $t$ -test. Bars with different superscripts (a, b) are significantly different, ( $p < 0.05$ ).



**Fig. 5.** Changes of Apoptotic Germ Cell Percentage in STZ-induced Diabetic Rats

ANOVA followed by multiple comparison two-tail  $t$ -test. Bars with different superscripts (a, b) are significantly different, ( $p < 0.05$ ).

nificantly resettled to control level (Fig. 4).

### Histology of Pancreas

In STZ-induced diabetic group islet cell number and diameter of islets were at first decreased in comparison with control as shown in Fig. 6A–6C. After administration of fraction however the diameter of islets and the islet cell numbers were recovered towards control level (Fig. 6A–6C).

### Quantitative Study of Sperm at Stage VII and Giant Cells in Seminiferous Tubules

Control group animals exhibited normal histomorphological structure of testis. A dense lining of germ cells was noted in the periphery as well as middle part of testis at stage VII of seminiferous epithelial cycle with normal diameter. In STZ-induced diabetic group, however germ cells numbers at stage VII of seminiferous epithelial cell cycle as well as diameter of seminiferous tubule were decreased in

comparison with control. Number of germ cells at stage VII of seminiferous epithelial cycle and the diameter of testis were resettled towards control level after the treatment with fraction to the diabetic rats (Table 2, Fig. 7A–7C).

In the control group no giant cells were found in seminiferous tubular structure. However these cells were noted in the diabetic group. After treatment of fraction the number of giant cells decreased in the seminiferous tubules to the same level as control (Fig. 8A–8C).

### ISEL in Pancreas

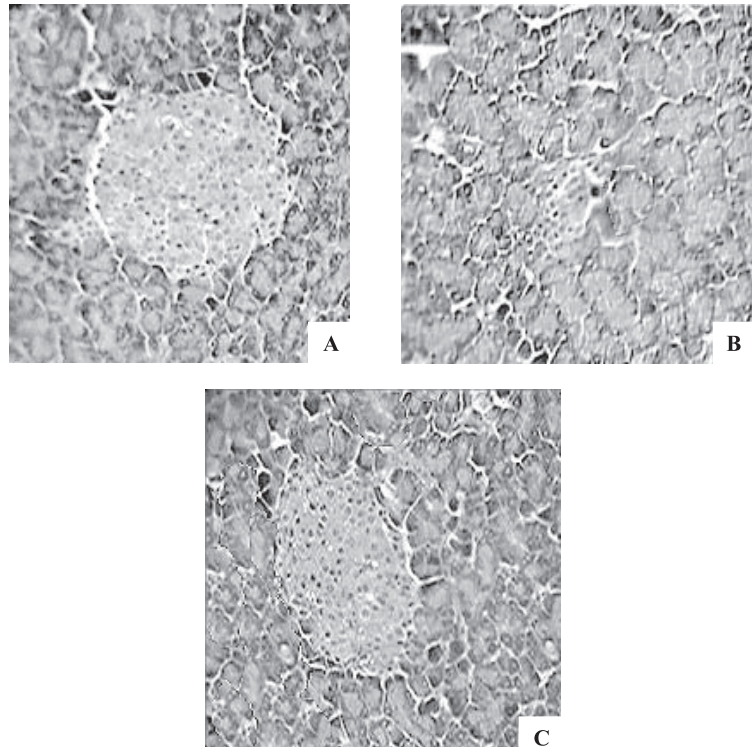
In the control group very few apoptotic cells were present. ISEL-positive or apoptotic cells in pancreatic islets were noted by brown color in plates (Fig. 9A–9C). Moreover qualitative analysis revealed more apoptotic cells in diabetic group compared with control group. After administration of fraction to the diabetic rats, ISEL-positive cells in pancreas were remarkably decreased (Fig. 9A–9C).

### ISEL in Testis

In the control group only minimal numbers of ISEL-positive cells were present as indicated by brown staining. In STZ-induced diabetic group the numbers of ISEL-positive cells were initially elevated but reduced following treatment with fraction (Figs. 5 and 10A–10C).

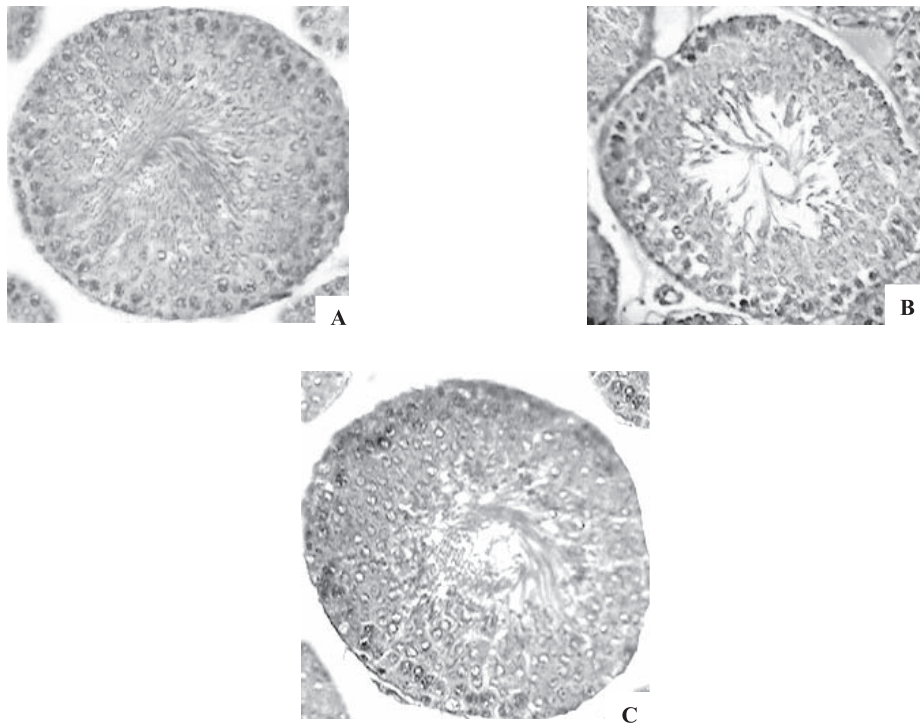
### Qualitative Test for Phytochemicals

Phytochemical screening revealed that whereas flavonoid and terpinoid compounds were present in hexane fraction of hydro-methanolic extract of leaf of *C. indica* flavonoid, alkaloid and terpinoids were



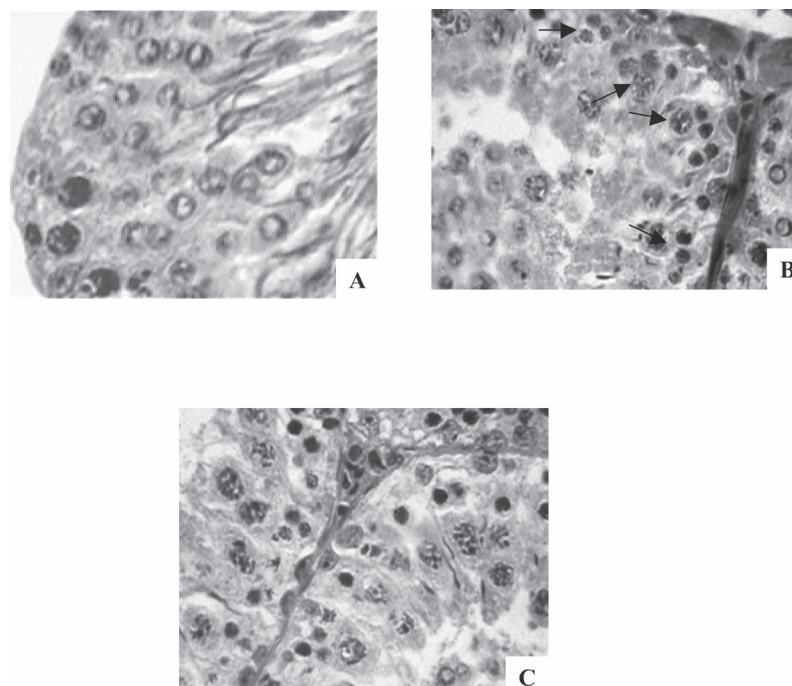
**Fig. 6.** Histology of Pancreas, X 400 (Hematoxylin-eosin Stain)

A) Representative sample of pancreatic tissue of control rat focusing on cell density in islets of Langerhans. B) Diminution in area of islets with low cell density in representative pancreatic islet tissue sample in STZ-induced diabetic rat. C) Representative pancreatic tissue sample showing remarkable recovery of cell density along with area of islet of Langerhans after administration of hexane fraction of hydro-methanolic extract of *C. indica* and *M. paradisiaca* in composite manner in STZ-induced diabetic rats.



**Fig. 7.** Histology of Testis, X 400 (Hematoxylin-eosin Stain)

A) Representative sample of testicular section at stage VII showing normal density of different generations of germ cells in control group. B) Representative sample of testicular section showing diminution in number of different generations of germ cells at stage VII of diabetic group. C) Representative sample of testicular section at stage VII showing a recovery in the number of different generations in germ cells of fraction-administered group.



**Fig. 8.** Histology of Testis X 400 (Hematoxylin-eosin Stain)

A) Representative sample of testicular section of control group. No giant cells are present. B) Representative sample of testicular section of diabetic group showing giant cells. → giant cell. C) Representative sample of testicular section of the hexane fraction-administered group showing decreased numbers of giant cells in seminiferous tubules.

**Table 3.** Screening of Phyto Constituents from Hexane Fraction of *C. indica* and *M. paradisiaca*

Phytochemicals	<i>C. indica</i>	<i>M. paradisiaca</i>
Volatile oil or essential oil	–	–
Flavonoids	+	+
Alkaloid	–	+
Saponin	–	–
Tanins	–	–
Terpenoids	+	+

present in hexane fraction of hydro-methanolic extract of *M. paradisiaca* (Table 3).

#### UV spectrophotometric Study

UV spectrophotometric study revealed that hexane fraction of *C. indica* and *M. paradisiaca* contain total phenolic compounds and flavonoid in different proportions (Table 4).

#### Thin Layer Chromatographic Study

*C. indica*: From the results of thin layer chromatography three spots were found in 100% chloroform solvent of hexane fraction of *C. indica* (Fig. 11A) after maturation in iodine chamber and after H<sub>2</sub>SO<sub>4</sub> charring. Retention factor (R<sub>f</sub>) values

**Table 4.** Quantification of Phenols and Flavonoids from Hexane Fractions of Root of *M. paradisiaca* and Leaf of *C. indica*

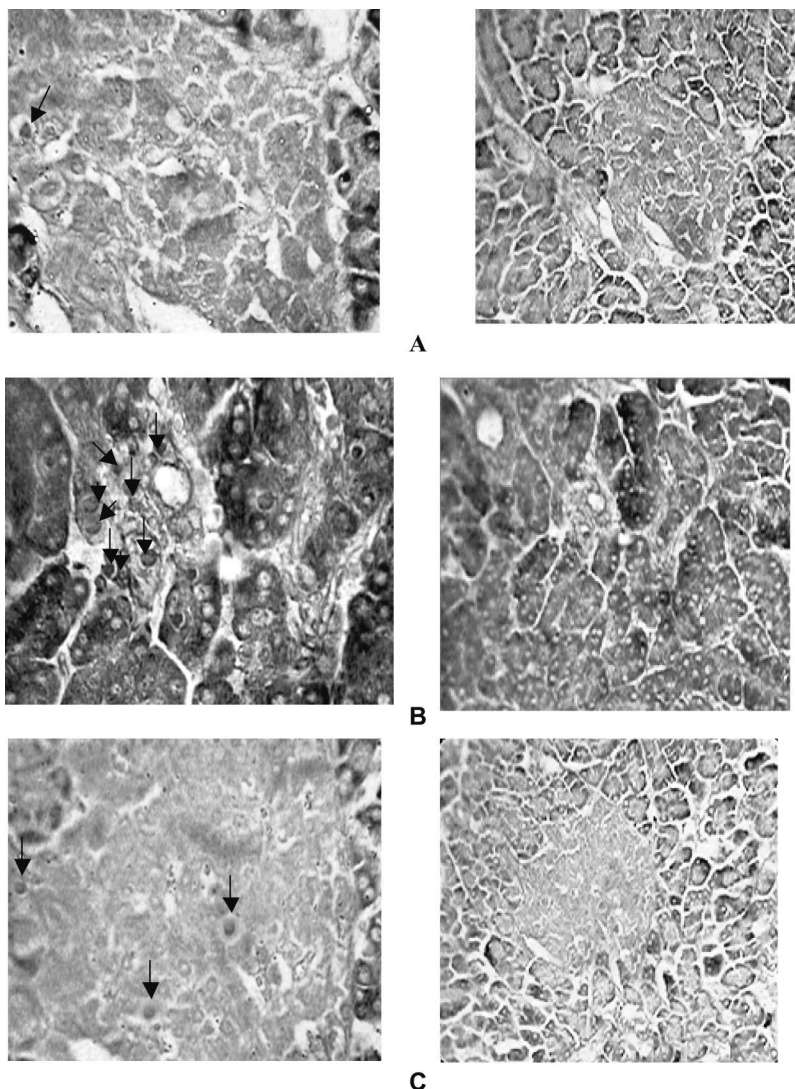
Fraction	Phenol or phenolic compound (µg/mg of fraction)	Flavonoids (µg/mg of fraction)
<i>M. paradisiaca</i>	16.5	50.25
<i>C. indica</i>	15.3	40.16

of these compounds were 0.72, 0.64, and 0.21 and the running distance of solvent was 7.2 cm.

Literature survey revealed that two major compounds are present in hexane fraction of *C. indica*. Such as tri-tria contane heptaposane and β-sitosterol.

*M. paradisiaca*: Six spots were found in hexane fraction of *M. paradisiaca* from TLC plate in 100% chloroform solvent after H<sub>2</sub>SO<sub>4</sub> charring. R<sub>f</sub> values of these compounds were 0.93, 0.81, 0.68, 0.58, 0.49, and 0.25, respectively. Running distance of solvent was 7.2 cm. One orange spot was noted in Dragendorff's reagent having R<sub>f</sub> as value of 0.81; this spot also showed pink color fluorescence on 254-nm UV ray exposure. Therefore it was confirmed that alkaloid-type compounds are present





**Fig. 9.** Immunohistochemistry of Pancreas, ISEL Study, X 400

A) Representative sample of pancreatic section showing normal density of islet cells and very few apoptotic cells present in the control group by ISEL study. → apoptotic cells. B) Representative sample of pancreatic section showing increase in the number of apoptotic cells present in diabetic group by ISEL study. → apoptotic cells. C) Representative sample of testicular section showing recovery in fraction-administered group. Apoptotic cell number also decreased by ISEL study compared with diabetic group. → apoptotic cells.

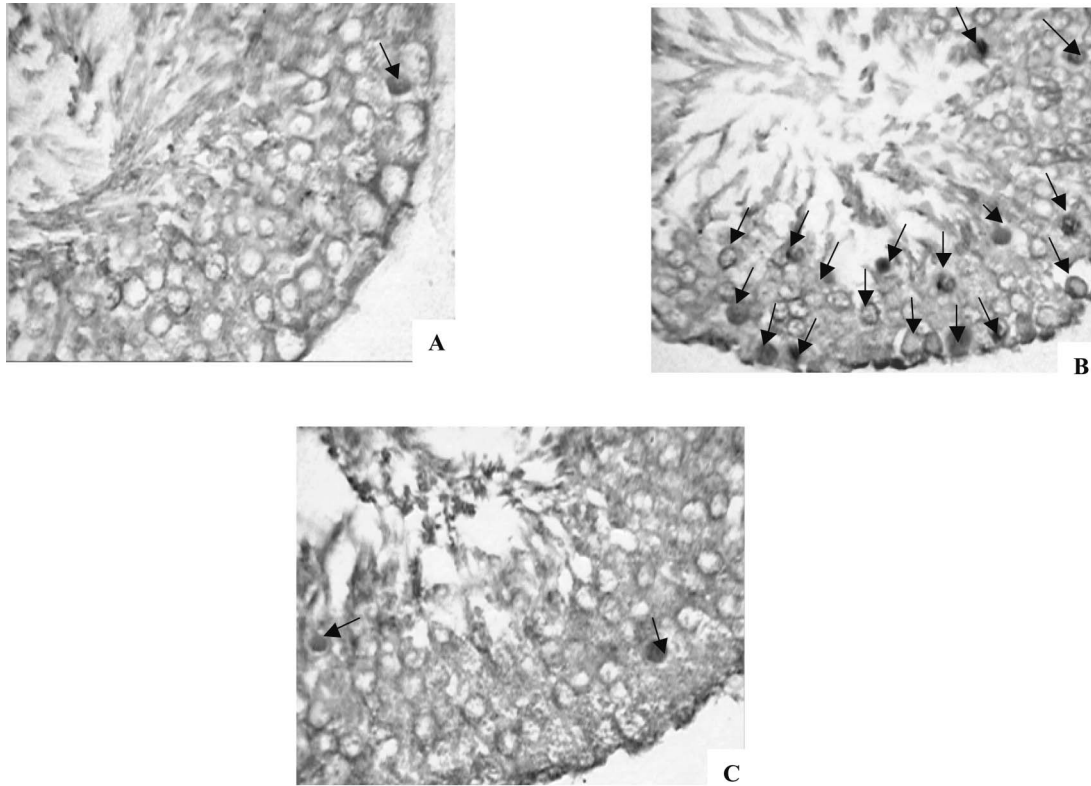
with as Rf value of 0.81. Two compounds were found in iodine maturation having Rf values 0.82 and 0.25 (Fig. 11B).

## DISCUSSION

Diabetes mellitus is a rapidly increasing metabolic disease worldwide and as knowledge of multifactorial/heterogenous nature of diabetes increases so there is need for more challenging and appropriate therapies. Impaired glucose metabolism leads to oxidative stress and protein glycation that in turn lead to free radical generation. The diabetic

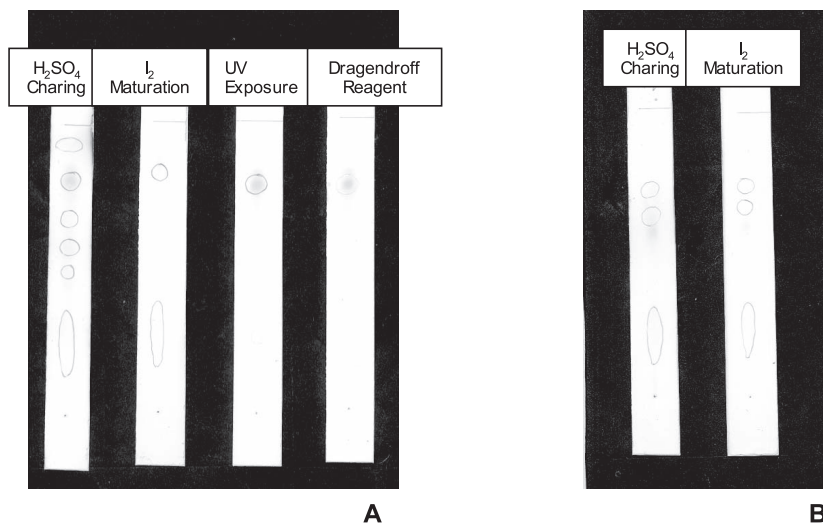
condition suppresses reproductive function resulting in male and in female infertility.<sup>44,45)</sup>

STZ-induced diabetes in male rats results in low sperm counts.<sup>46)</sup> This may be due to inhibition in spermatogenesis as reflected in this study by quantification of different generations of germ cells at stage VII of spermatogenic cycle. Another possible cause of low sperm count may be testicular oxidative stress in diabetic state as reported earlier.<sup>47)</sup> Sperm motility and viability and serum testosterone level were also diminished significantly. This result was supported by different groups of workers.<sup>48,49)</sup> Impairment of these reproductive parameters may be due to diabetes-induced oxidative stress genera-



**Fig. 10.** Immunohistochemistry of Testis, ISEL Study, X 400

A) Representative sample of testicular section showing normal density of germ cells and apoptotic germ cells present in control group. B) Representative sample of testicular section showing increased numbers of apoptotic germ cells in the diabetic group. C) Representative sample of testicular section showing recovery in the number of apoptotic germ cells after administration of hexane fraction of *M. paradisiaca* and *C. indica* in composite manner. Apoptotic cells were decreased in number. → apoptotic germ cell.



**Fig. 11.** TLC Plate of *M. paradisiaca* and *C. indica*

A) TLC plate of hexane fraction of *M. paradisiaca* focusing on six compounds after  $H_2SO_4$  charring. Two compounds were found after iodine maturation. One UV-positive and one Dragendorff's-positive compound were present in the same spot. B) TLC plate of hexane fraction of *C. indica* showing three compounds after iodine maturation and  $H_2SO_4$  charring. No UV-sensitive and Dragendorff's-positive compounds were found.

tion;<sup>50,51</sup>) indeed we observed diminished of activities of CAT, POD, SOD and G-S-T along with elevated levels of CD and TBARS in testis which are

the sensitive biomarkers of oxidative injury.<sup>52)</sup>

Testicular inhibition in diabetes was confirmed by elevation of germ cell apoptosis in seminiferous

tubule studied by ISEL and this finding is also consistent with the results of others.<sup>53)</sup> Germ cell apoptosis in stage VII and VIII is known associated with serum testosterone levels.<sup>54)</sup> Impairment of Leydig cells in STZ-induced diabetes is considered associated with insulin-mediated androgen synthesis.

Several group of workers<sup>55–57)</sup> proposed that glucose-insulin axis acts as tuning system for controlling activity of pituitary-testicular axis in connection with diabetes-induced male infertility. The results of the present experiments focused on inhibitory effect of low insulin in diabetes on testicular steroidogenesis and gamatogenesis and were consistent with our previous publications.<sup>16, 17)</sup>

There are several reports suggestive that multinucleated germ cells or giant cells appear in seminiferous tubules linked with germ cell apoptosis.<sup>58)</sup> Formation of giant germ cells is the initial stage of degeneration followed by nuclear fragmentation, irrespective of the stage of division or differentiation. Germ cell apoptosis noted in diabetic condition may be one of the causes of generation of giant cells in seminiferous epithelium. Moreover, giant cells produced in seminiferous tubules as trigger of apoptosis in germ cell are supported by evidence that these cells induce apoptosis in other systems.<sup>59)</sup> Another way for testicular inhibition in diabetes may be oxidative stress induction as proposed by our previous publications.<sup>16, 17)</sup>

Traditional plant remedies have been used for centuries. In our study recovery of testicular apoptosis may be due to correction of oxidative injury in testis induced by STZ after treatment with hexane fraction of hydro-methanolic extract of root of *M. paradisiaca* and leaf of *C. indica* in composite manner.<sup>60)</sup> Antioxidative effect of the hexane fraction has been supported here by assessment of the activities of testicular antioxidant enzymes as well as the quantity of end products of free radicals which are sensitive indicators for oxidative stress evaluation.<sup>61)</sup> The antioxidant activity of hexane fraction may be due to the presence of phytoingredients such as flavonoid and alkaloid which are established natural antioxidants.<sup>62, 63)</sup> This antioxidative activity of hexane fraction of *M. paradisiaca* and *C. indica* is also supported by the results of our previous polyherbal formulation comprising crude extract of *M. paradisiaca* and *C. indica*.<sup>22)</sup> Diminution in the number of giant cells in testis after treatment with composite hexane fraction also supports the antioxidative function of this fraction.<sup>64)</sup>

Composite hexane fraction of *M. paradisiaca*

and *C. indica* can also recover testicular dysfunction including germ cell apoptosis in STZ-induced diabetes as focused by our assessment of quantification of germ cells at stage VII and diminution in the number of apoptotic cells in seminiferous tubules, which are important biosensors.<sup>65)</sup> This recovery may be due to correction of testicular androgenesis<sup>66)</sup> as well as by diminution in the number of apoptotic cells in islets. The composite hexane fraction of these two plants parts has the property to regenerate pancreatic  $\beta$ -cells; thus insulin levels also increase and stimulate testicular androgenesis.

In conclusion hexane fraction of hydro-methanolic extract of *M. paradisiaca* and *C. indica* contains active ingredient(s) with the capacity to correct diabetes-induced testicular dysfunction by regeneration of pancreatic  $\beta$  cells and/or recovery of oxidative stress injury. The results of future gene expression experiments in this line will explore the molecular aspect of the action of phytoingredient(s) in this context.

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