

Original Article

**DURATION DEPENDENT ANTIAPOPTOTIC EFFICACY OF *CAMELLIA SINENSIS* (GREEN TEA) ON STREPTOZOTOCIN INDUCED DIABETES LINKED TESTICULAR HYPOFUNCTION IN ALBINO RAT: GENOMIC AND FLOW CYTOMETRIC ASSESSMENT**

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ABSTRACT

**Objective:** The study has been conducted to search out the threshold duration of treatment of ethyl acetate fraction of methanolic extract of leaves of *Camellia sinensis* (L.) Kuntze at the dose of 100 mg/kg body weight for the management of diabetes-induced testicular impairment in streptozotocin-induced diabetic rat in a duration dependent fashion.

**Methods:** In this respect, the glycemic, androgenic, oxidative stress sensors, gene expression of testicular androgenic key enzymes along with apoptotic markers were evaluated in a duration dependent way (14, 28 and 56 d).

**Results:** A significant correction was noted in the levels of glycated haemoglobin (HbA<sub>1c</sub>), testicular thiobarbituric acid reactive substances (TBARS), conjugated diene (CD), sperm viability, sperm mitochondrial status, serum testosterone, and genomic expression of testicular  $\Delta 5, 3\beta$ -hydroxysteroid dehydrogenase (HSD), 17 $\beta$ -HSD, Bax, Bcl-2 after treatment for different duration with the said fraction in diabetic groups in compare to respective vehicle-treated diabetic group without any toxicity induction in general. Thin layer chromatography (TLC) study of the fraction showed two spots with retention factors (Rf) of 0.78 and 0.51.

**Conclusion:** The results showed that 28 d treatment was threshold duration of treatment for the correction of diabetes-induced testicular impairment.

**Keywords:** *Camellia sinensis*, Diabetes, Oxidative stress, Flow cytometry, Testicular dysfunction, Apoptosis

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INTRODUCTION

Diabetes mellitus is a metabolic disorder resulting from a defect in insulin secretion, insulin action, or both [1]. The pathology of diabetes mellitus is caused by Reactive Oxygen Species (ROS) that activate the non-enzymatic glycation of proteins which leads to structural and functional changes in proteins, DNA and lipid. The chronic diabetes is associated with the damage and dysfunction of various organs including testes [2] and hampered different vital functions of the reproductive system in model animal and human either directly or indirectly [3]. Many studies have been focused that streptozotocin (STZ) induced diabetes in male rat resulted diminution in the activity of sex organs, low sperm count along with a low level of testosterone [4, 5]. Spermatogenic cells at different stages of spermatogenesis are prone to routine apoptosis, and elevation in the level of apoptosis may cause male infertility [6]. The number of people with diabetes is increasing due to urbanization, altered food style, increasing prevalence of obesity, and physical inactivity [7]. Plant-derived preparations have been used as traditional remedies and in folklore medicine for the treatment of diabetes in many parts of the world [8, 9]. Traditional medicinal herbs are well accepted by the World Health Organization (WHO) expert committee to treat the diabetes mellitus due to its minimum side effect, low cost and easy availability [10, 11].

*Camellia sinensis* (Theaceae) leaves named as tea which is used in the form beverage and at present, it is claimed as a second popular beverage after water. In recent years *C. sinensis* is widely used to study its beneficial effects for the treatment and prevention of cancer, cardiovascular disease and diabetic complications [12-14]. Studies showed that administration of green tea (*C. sinensis*) to STZ induced diabetic rats promoted blood glucose reduction and antioxidant effects [15, 16]. Evidence provided through literature that green tea is useful for the management of diabetes mellitus, but no studies to date have tested the efficacy of green tea to prevent testicular dysfunctions associated with diabetes mellitus.

From our previous study, an ethyl-acetate fraction of methanolic extract of green tea at the dose of 100 mg/kg body weight has been selected as potent dose and as potent solvent fraction for the management of diabetes-induced testicular hypofunction. Therefore, in this experiment; an approach has been considered to find out the threshold duration of treatment in this concern.

MATERIALS AND METHODS

Chemicals

The chemicals and solvents were purchased from Sigma-Aldrich Chemicals Private Limited, Bangalore or from Merck (KGaA, Germany) or from Crest Biosystems, Goa, India or from standard manufacturers.

Preparation of fraction from methanolic extract

The leaves of *C. sinensis* (green tea) were used in this study. The green tea was purchased from Subodh Brothers Pvt. Ltd. (Kolkata) and preserved in an airtight manner. Preparation of crude extract of leaves of *C. sinensis* using-methanol and its fractionation using different solvents as per polarity grades such as n-hexane, ethyl acetate and chloroform were performed according to our laboratory standard method [17]. These lyophilized fractions were collected, labeled and stored at 4 °C until use. Out of these, the ethyl acetate fraction was used for this study as a potent fraction as per our previous study [13].

Selection criteria of animal and animal care

Rats were procured from an authorized vendor and housed in a well-ventilated animal house. Standard environmental conditions, i.e. 22-25 °C temperature, 45% relative humidity and 12 hr.: 12 hr. light-dark cycles were maintained. Before conduction the experiment, animals were kept in the animal house for 7 d to acclimatize the laboratory conditions. The animals were provided standard rat chow (supplied by Golden Feeds, Mehrauli, New Delhi)

and water *ad libitum*. Adult 58 male rats of Wistar strain (80-90 d old) having body weight  $140 \pm 10$  g were used in this study. Forty rats made diabetic and out of these thirty-six rats fulfill the moderate diabetic criteria (fasting blood glucose level  $>250$  mg/dl but  $<350$  mg/dl) [18]. The rest animals were not considered due to the lack of a stable diabetic state. Eighteen normoglycemic healthy rats were served as a control in three respective duration dependent treatment schedule designed below. The Institutional Ethics Committee (IEC) of Vidyasagar University gave the approval prior to conduct the experiment (IEC Approval No-IEC/3/C-5/14; Dated-3/11/14). The guidelines are given by the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Govt. of India, were strictly maintained.

### Induction of diabetes mellitus

Rats were kept for 24 h fasting conditions and then single intramuscular injection of STZ (40 mg/kg body weight) was given to the rats that produce moderate diabetes. Subsequent six days were allowed for the stability of diabetes and after that, the rats were selected for the experiment subject to fulfillment of the said criteria.

### Experimental protocol

Before starting the treatment, all the rats were divided into 9 groups of 6 animals each. Except for control group, other 6 groups were made diabetic by STZ injection.

Animals were categorized as follows-

Group I (Vehicle treated control for 14 d): Rats of this group were normoglycemic i.e. having fasting blood glucose level 70-99 mg/dl and subjected to oral feeding of distilled water at the dose of 2 ml/kg body weight (2 times/rat/day) as vehicle treatment for 14 d.

Group II (Vehicle treated diabetic for 14 d): Streptozotocin-induced diabetic rats of this group were also treated with 2 ml distilled water/kg body weight (2 times/rat/day) as for 14 d.

Group III (Diabetic+*C. sinensis* treated group for 14 d): Ethyl-acetate fraction prepared from methanolic extract of *C. sinensis* (green tea) used for the treatment of the diabetic rat in this group at the dose of 100 mg/kg body weight in 2 ml of distilled water twice/day for 14 d.

Group IV (Vehicle treated control for 28 d): Rats of this group were normoglycemic and subjected to oral feeding of distilled water at the dose of 2 ml/kg body weight (2 times/rat/day) as vehicle treatment for 28 d.

Group V (Vehicle treated diabetic for 28 d): Streptozotocin-induced diabetic rats of this group were also treated with 2 ml distilled water/kg body weight (2 times/rat/day) as vehicle treatment for 28 d.

Group VI (Diabetic+*C. sinensis* treated group for 28 d): Ethyl-acetate fraction prepared from methanolic extract of *C. sinensis* (green tea) used for the treatment of the diabetic rat in this group at the dose of 100 mg/kg body weight in 2 ml of distilled water twice/day for 28 d.

Group VII (Vehicle treated control group for 56 d): Rats of this group were normoglycemic subjected to oral feeding of distilled water at the dose of 2 ml/kg body weight (2 times/rat/day) as vehicle treatment for 56 d.

Group VIII (Vehicle treated diabetic group for 56 d): Streptozotocin-induced diabetic rats of this group were also treated with 2 ml distilled water/kg body weight (2 times/rat/day) as vehicle treatment for 56 d.

Group IX (Diabetic+*C. sinensis* treated group for 56 d): Ethyl-acetate fraction prepared from methanolic extract of *C. sinensis* (green tea) used for the treatment of the diabetic rat in this group at the dose of 100 mg/kg body weight in 2 ml of distilled water twice/day for 56 d.

The first oral dose of distilled water (in case of control and diabetic group) or fraction (in case of treated diabetic group) of this said treatment regimen was given in fasting condition (at 10:00 a. m.) and for this purpose the food container from each cage was removed at 8.00 a. m. Animal feed was supplied 2 hr later after extract/vehicle treatment. A second oral dose of the same was

administered in the afternoon (at 4:00 pm) and condition for feed supply was followed as before to minimize the drug-nutrient interaction if any.

All the rats were sacrificed at fasting state using euthanasia box on 29<sup>th</sup> day of treatment. From the hepatic vein, blood was collected. Liver and testis were dissected out and stored at  $-20$  °C for the biochemical and genomic analysis.

### Measurement of glycaemic bio-sensor

Glycated hemoglobin level (HbA<sub>1c</sub>) was measured using a glucose memory test according to the standard method [19]. Glycated hemoglobin level was expressed as GHb%.

### Estimation of the oxidative stress biomarker

For the quantification of thiobarbituric acid reactive substances (TBARS) and a conjugated diene (CD) the testicular tissue was homogenized and centrifuged at  $10000 \times g$  at  $4$  °C for 5 min. The supernatant was transferred in spectrophotometer cuvette and absorbances were noted at 535 nm for the quantification of TBARS [20], and at 233 nm to measure CD [21].

### Measurement of reproductive profile

For the counting of live and dead sperm cells through Accuri C6 flow cytometer, BD Cell Viability Kit Cat No 349480 was used. Epididymal fluids were collected and mix with 1.8 ml of pre-warmed Tris buffer ( $1 \times 10^6$  cell/ml of cell suspension). Then, 2  $\mu$ l of Thiazole Orange (TO) and 2  $\mu$ l of Propidium Iodide (PI) were added and incubated at  $37$  °C for 5 min [22]. The data were acquired on flow cytometer according to the Standard Operating Procedure (SOP) of this kit.

Mitochondrial status of spermatozoa was assessed by Accuri C6 flow cytometer using JC1 kit from BD Cat No 551302 and dimethyl sulfoxide solution. Fifty microliters of epididymal fluid and five microliters of JC1 staining solution were taken and then incubated in dark at  $37$  °C for 10 min. Five ml assay buffer was added and centrifuged at 1200 rpm for 5 min, then the supernatant was discarded. Cells in 500  $\mu$ l were resuspended in assay buffer and data was acquired on flow cytometer according to supplied Standard Operating Procedure (SOP) of this kit [23].

By solid phase-conjugated assay, the level of serum testosterone was measured. The kit was purchased from Lilac Medicare (P) Ltd, Mumbai, India. The optical density was measured using the standard method [24]. The intra-assay variation was 5.2%. No inter-assay variation was considered here as all the samples were measured at a time. The cross-reaction level with androstenedione was 0.09% and dihydrotestosterone was 1.7%.

### Gene expression of pro-apoptotic, anti-apoptotic biomarkers and androgenic key enzymes

The primers of Bax, Bcl-2, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH),  $\Delta^5$ ,  $3\beta$ -HSD,  $17\beta$ -HSD and Transferrin Receptor (TFR) were procured from Sigma-Aldrich Chemicals Private Limited, Bangalore. Light Cycler 480 II (Roche Diagnostic) was used for the assessment of expression of the above genes of testicular tissues [25]. For RNA extraction, Roche Diagnostic's kit was used. Residual DNA was removed and cDNA was synthesized using a kit ("Transcriptor First Strand Synthesis Kit" Roche Diagnostics, Mannheim, Germany Product No. 04 897 030 001).

### Assessment of metabolic toxicity

For the measurement of hepatic GOT and GPT activities, kits were used supplied by Crest Bio-systems, Gitanjali, Dr. Antonio Do Rego Bagh, Alto Santacruz, Bambolim Complex (Goa, India) and the standard protocol was followed [26].

### Statistical analysis

All data were expressed as mean  $\pm$  Standard Error of Mean (SEM), (n=6). For the statistical analysis of data, 'Analysis of Variance (ANOVA) followed by multiple comparison student's two tail t-test' was performed [27]. Differences were considered significant at  $p < 0.05$ .

### Thin layer chromatography (TLC) of the fraction

Thin layer chromatographic study of an ethyl acetate fraction of *C. sinensis* was conducted by using commercially available aluminium sheets of Silica gel 60 F254 (Merck) with dichloromethane (DCM): Methanol: : 80: 20 as the solvent system. After the test, the plates were dried under a stream of hot air and then examined within a UV cabinet at short wavelength (254 nm) and long wavelength (366 nm), H<sub>2</sub>SO<sub>4</sub> charring and I<sub>2</sub> maturation [28].

## RESULTS

### Effect on glycaemic bio-sensor

Glycated hemoglobin (HbA<sub>1c</sub>) level was significantly elevated ( $p < 0.05$ ) in all the vehicle-treated diabetic groups i.e. 14, 28 or 56 d experimental schedule in respect to the corresponding vehicle-treated control groups. Administration of ethyl acetate fraction of methanolic extract of *C. sinensis* at the dose of 100 mg/kg body weight in 2 ml of distilled water two times/day for the duration of 14 or 28 or 56 d to diabetic rats, a significant diminution ( $p < 0.05$ ) in the level of this parameter was observed in respect to vehicle-treated diabetic group of respective experimental schedule. Treatment of ethyl acetate fraction for the duration of 28 or 56 d to the diabetic rats resulted in more significant recovery ( $p < 0.05$ ) in the level of glycated hemoglobin in respect to 14 d fraction treated diabetic animal towards the control. No significant difference was found in the value of the above parameter when the results were compared between the 28 or 56 d of ethyl acetate fraction treated diabetic groups which indicated that 28 d is the threshold duration of treatment in this concern (fig. 1).

### Assessment of oxidative stress biomarkers

A significant elevation ( $p < 0.05$ ) was observed in the levels of CD and TBARS in the testicular tissues in all the duration dependent diabetic groups when the comparison was made to their respective control group for the said treatment schedules. There was significant ( $p < 0.05$ ) corrections in the quantity of said parameters towards their control levels after treatment with ethyl acetate fraction for 14 or 28 or 56 d duration. Out of these, ethyl acetate fraction treatment for a period of 28 or 56 d produced rectifications that significantly ( $p < 0.05$ ) higher with rectification achieved after 14 d of fraction treatment. The levels of these parameters were found with insignificant ( $p > 0.05$ ) difference from each other for the treatment period between 28 or 56 d (fig. 2 A, B).

### Assessment of reproductive profile

In all the duration dependent vehicle-treated diabetic groups, sperm viability was decreased significantly ( $p < 0.05$ ) in respect to the vehicle-treated control group of corresponding experimental schedule. After the treatment of ethyl acetate fraction of methanolic extract of *C. sinensis* for 14 or 28 or 56 d to the diabetic animals, sperm viability was increased significantly ( $p < 0.05$ ) in compare with the corresponding vehicle treated STZ induced diabetic animals. Results also showed that administration of said fraction for the

duration of 28 or 56 d resulted in a significant elevation ( $p < 0.05$ ) of sperm viability in compare to 14 d fraction treatment. No significant variation was noted in the case of sperm viability between 28 or 56 d fraction treated diabetic groups (fig. 3 A, B).

Depolarised sperm indicated the low mitochondrial status of sperm. Percentage of sperm with the depolarised mitochondrial membrane was increased significantly ( $p < 0.05$ ) in all the duration dependent vehicle-treated diabetic groups in respect to the vehicle-treated control group of corresponding experimental schedule. Numbers of depolarised sperm were decreased significantly ( $p < 0.05$ ) in diabetic animals treated with above-said fraction for 14 or 28 or 56 d in compare with the corresponding vehicle-treated diabetic animals. It was also observed that 28 or 56 d fraction treated groups showed significant promising effect ( $p < 0.05$ ) than 14 d treated diabetic group. No significant variation in the level of this parameter was observed between 28 or 56 d fraction treated diabetic groups (fig. 4 A, B).

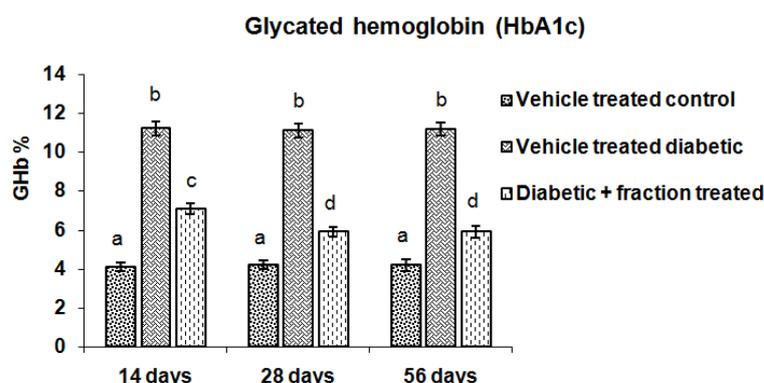
Level of serum testosterone was significantly decreased ( $p < 0.05$ ) in all the duration dependent diabetic groups in respect to the vehicle-treated control group. Treatment with above-said fraction for the duration of 14 or 28 or 56 d to diabetic rats resulted in a significant recovery ( $p < 0.05$ ) in the level of serum testosterone in respect to diabetic group. Results focused that 28 or 56 d of fraction treatment resulted in a significant correction ( $p < 0.05$ ) in compare with 14 d of fraction treated diabetic groups. There was no significant variation in the levels of this parameter between 28 or 56 d fraction treated diabetic groups (fig. 5).

### Gene expression pattern of testicular apoptotic marker

Upward and downward expression patterns were noted in case of the pro-apoptotic gene, i.e. Bax and anti-apoptotic gene, i.e. Bcl-2 in testicular tissue respectively through qRT-PCR analysis in the diabetic group when compared to the vehicle-treated control group. A significant ( $p < 0.05$ ) recovery was observed towards the control group in the expression levels of Bax and Bcl-2 genes after treatment with above-mentioned fraction for the duration of 14 or 28 or 56 d in diabetic rat. When the results of 28 or 56 d fraction treated groups compared with 14 d fraction treated group, it was observed that 28 or 56 d duration significantly effective ( $p < 0.05$ ) in this concern in compare with 14 d duration of treatment. No significant difference was noted between 28 or 56 d of fraction treatment to diabetic animals (fig. 6 A, B).

### Gene expression of androgenic key enzymes

Levels of expression of testicular  $\Delta 5$ ,  $3\beta$ -HSD and  $17\beta$ -HSD genes were decreased insignificant level ( $p < 0.05$ ) in the diabetic group in respect to the control group. After treatment with above-said fraction for the duration of 14 or 28 or 56 d to diabetic rats resulted in a significant recovery ( $p < 0.05$ ) in the transcript levels of concerned genes in respect to diabetic group. Results focused that 28 or 56 d of fraction treatment resulted in a significant correction ( $p < 0.05$ ) in compare with 14 d of fraction treated diabetic group. There was no significant variation in the levels of this parameter between 28 or 56 d fraction treated diabetic groups (fig. 7 A, B).



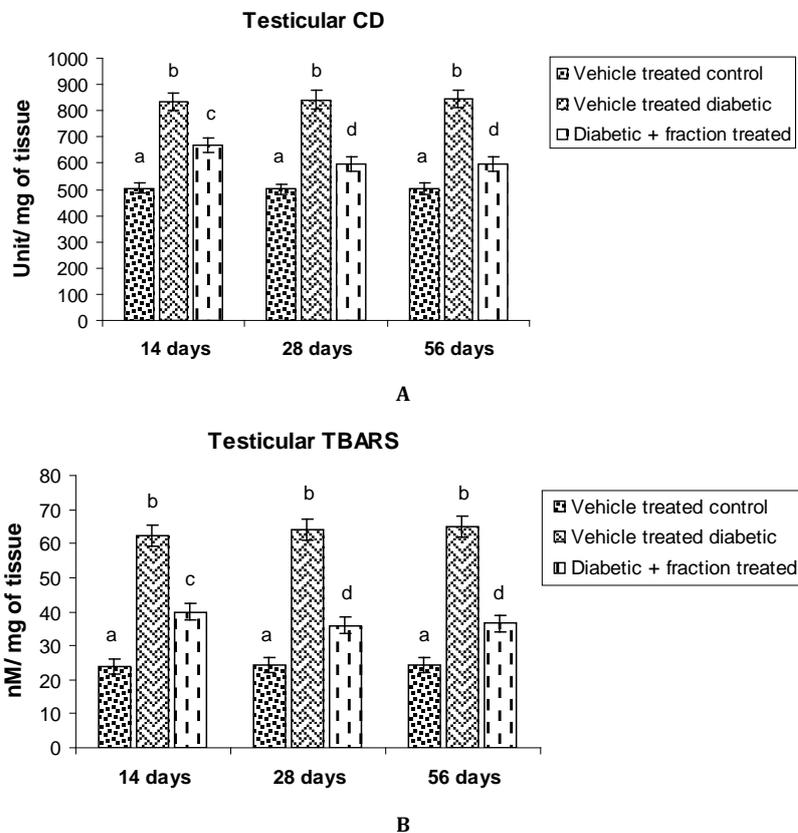
**Fig. 1: Protective effect of ethyl-acetate fraction of *C. sinensis* on glycated hemoglobin level (HbA<sub>1c</sub>) in STZ-induced diabetic male albino rat. Data represents mean $\pm$ SEM (n = 6). ANOVA followed by multiple comparison student's two tail-t-test. Bars with different superscript (a, b, c, d) differ from each other significantly,  $p < 0.05$**

**Toxicity studies**

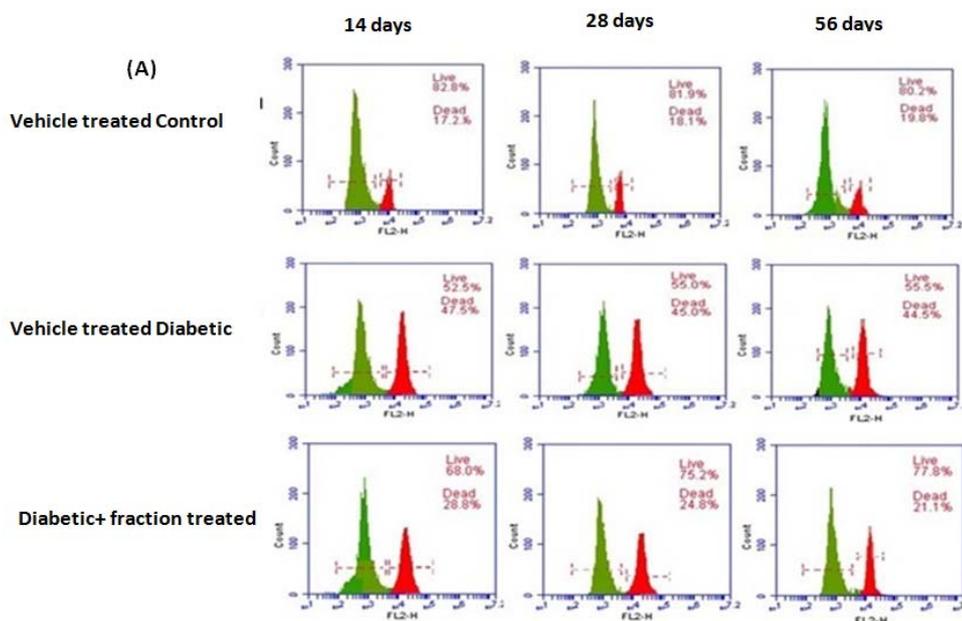
Activities of hepatic GOT, GPT were increased in all duration dependent diabetic groups when the comparison was made with the vehicle-treated control group. Above said fraction treatment for the duration of 14 or 28 or 56 d to diabetic rats can able to recover the enzyme activities towards the control at a significant level ( $p < 0.05$ ) (fig. 8 A, B).

**Thin layer chromatographic study**

Two spots were found from the analytical study of thin layer chromatogram of an ethyl acetate fraction of *C. sinensis* (fig. 9) after examined in UV cabinet at short wavelength 254 nm and long wavelength 366 nm,  $H_2SO_4$  charring and Iodine maturation. Retention factor (Rf) of these compounds were 0.78 and 0.51 and the running distance of solvent was 7 cm (fig. 9).



**Fig. 2:** Duration dependent effect of ethyl-acetate fraction of *C. sinensis* on the levels of (A) Conjugated diene (CD) and (B) TBARS in testicular tissue of an experimental animal. Bars indicate mean±SEM (n=6). ANOVA followed by multiple comparison student's two tail-t-test. Bars with different superscripts (a, b, c, d) differ from each other significantly,  $p < 0.05$



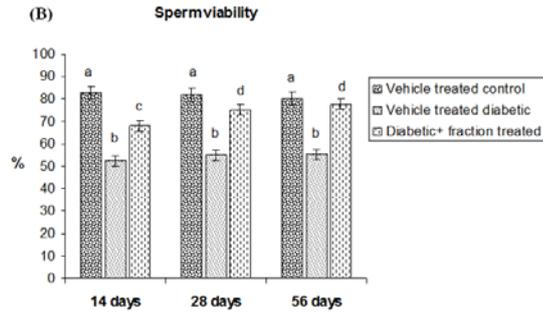


Fig. 3: Corrective effect of ethyl acetate fraction of *C. sinensis* on epididymal sperm viability. (A) Histogram of sperm viability stained with propidium iodide (PI) by flow cytometric analysis. (B) Bar diagram of viable sperms in fraction treated group. Bars indicate mean±SEM (n = 6). ANOVA followed by multiple comparison student's two-tail t-test. Bars with different superscripts (a, b, c, d) differ from each other significantly, p<0.05

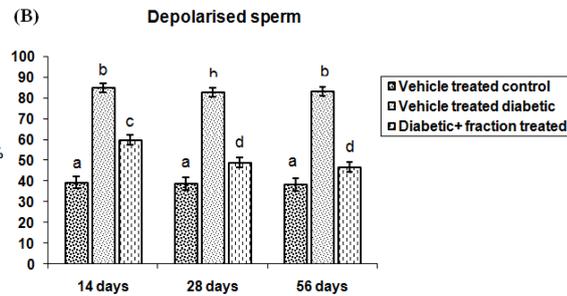
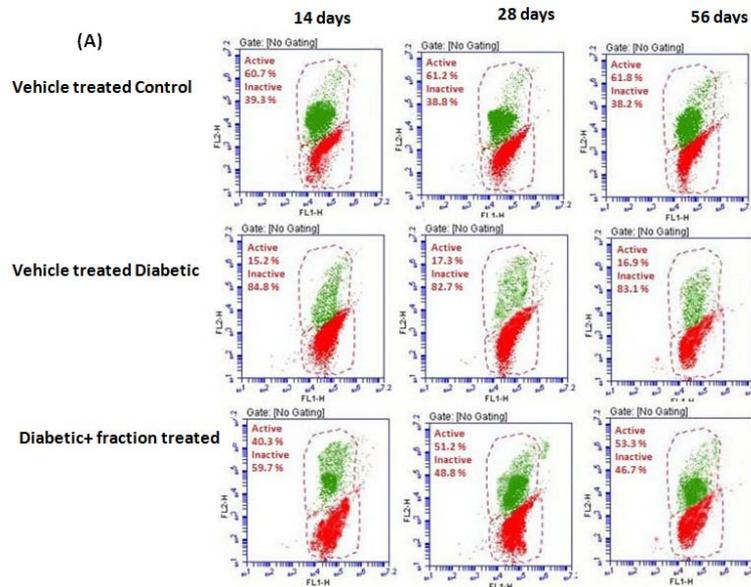


Fig. 4: Protective effect of ethyl acetate fraction of *C. sinensis* on sperm mitochondria. (A) Histogram of depolarised sperm after JC-1 staining. Units indicate fluorescence intensity (FL1 and FL2 stand for green and orange fluorescence, respectively). (B) Bar diagram of depolarised sperm. Bars indicate mean±SEM (n = 6). ANOVA followed by multiple comparison student's two tail t-test. Bars with different superscripts (a, b, c, d) differ from each other significantly, p<0.05

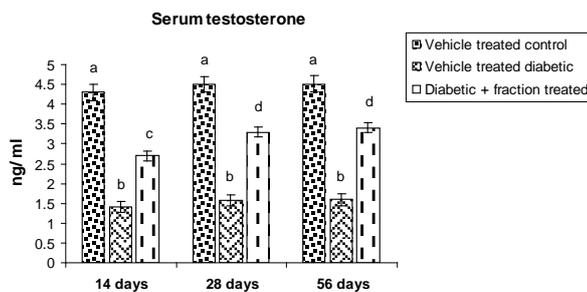


Fig. 5: Recovery in serum testosterone level after administration of ethyl acetate fraction of methanolic extract of *C. sinensis* in streptozotocin (STZ)-induced diabetic rat. Bars represent mean±SEM (n = 6). ANOVA followed by multiple comparison student's two tail t-test. Bars with different superscripts (a, b, c, d) differ from each other significantly, p<0.05

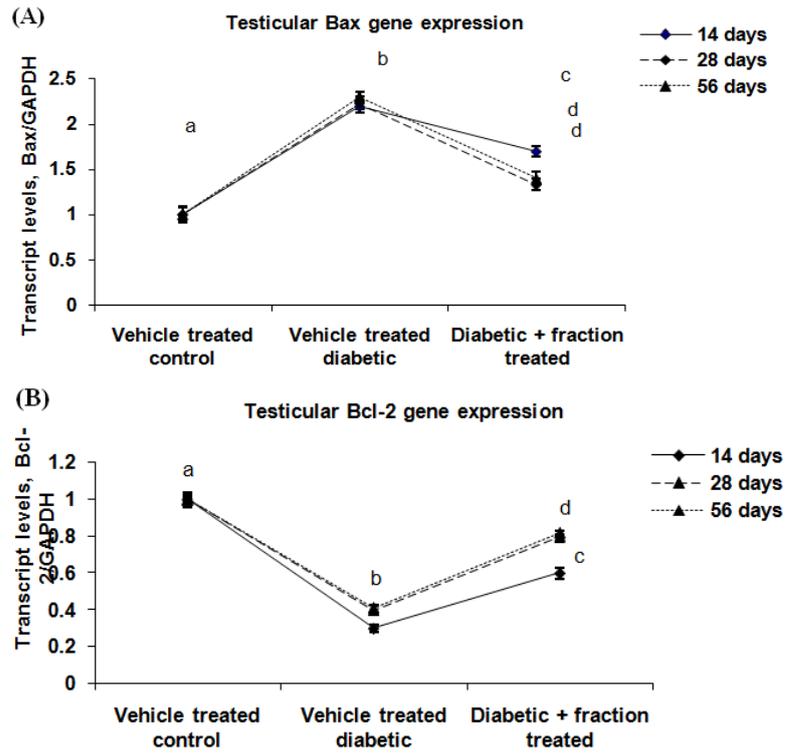


Fig. 6: Expression pattern of testicular (A) Bax gene and (B) Bcl-2 gene by qRT-PCR analysis in rat. Data represents mean±SEM (n=6). ANOVA followed by multiple comparison student's two tail 't' test. Points on the line with different superscripts (a, b, c, d) differ from each other significantly, p<0.05

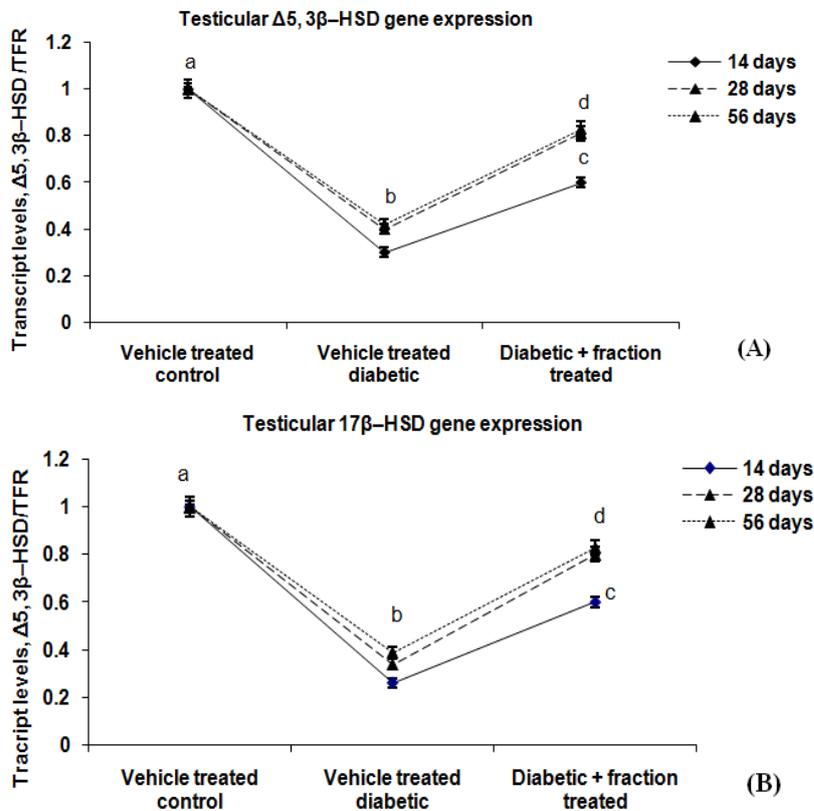


Fig. 7: Expression pattern of testicular (A) Δ5-3β-HSD gene and (B) 17β-HSD gene by qRT-PCR analysis in rat. Data represents mean±SEM (n=6). ANOVA followed by multiple comparison student's two tail 't' test. Points on the line with different superscripts (a, b, c, d) differ from each other significantly, p<0.05

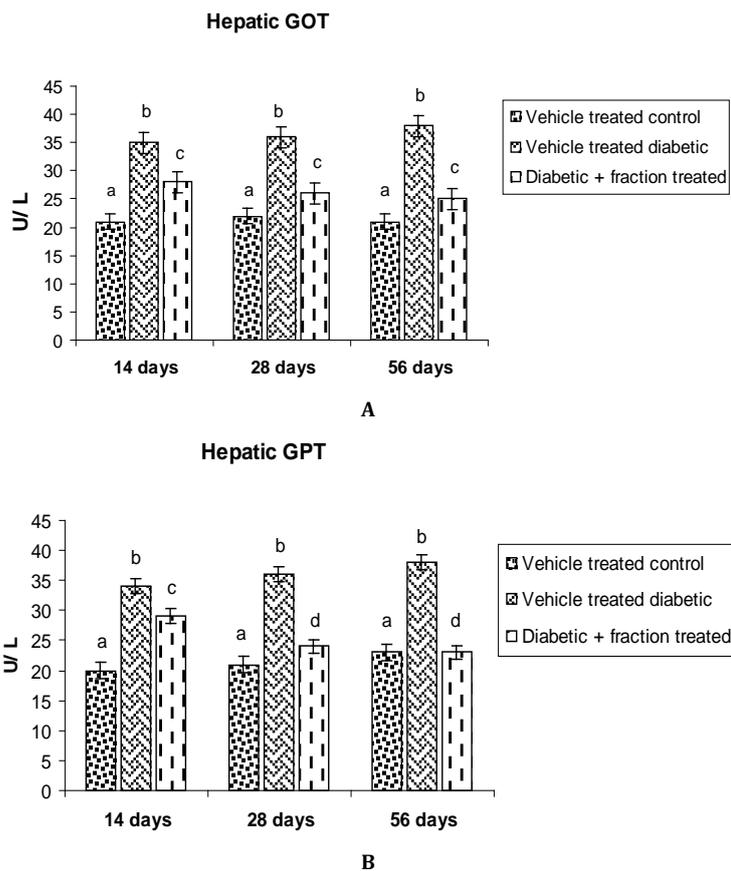


Fig. 8: A and B indicate a recovery in hepatic GOT and GPT activities after treatment with an ethyl-acetate fraction of *C. sinensis* leaves in STZ-induced diabetic male albino rat. Data represents mean±SEM (n = 6). ANOVA followed by multiple comparison student's two tail't 'test. Bars with different superscripts (a, b, c, d) differ from each other significantly, p<0.05

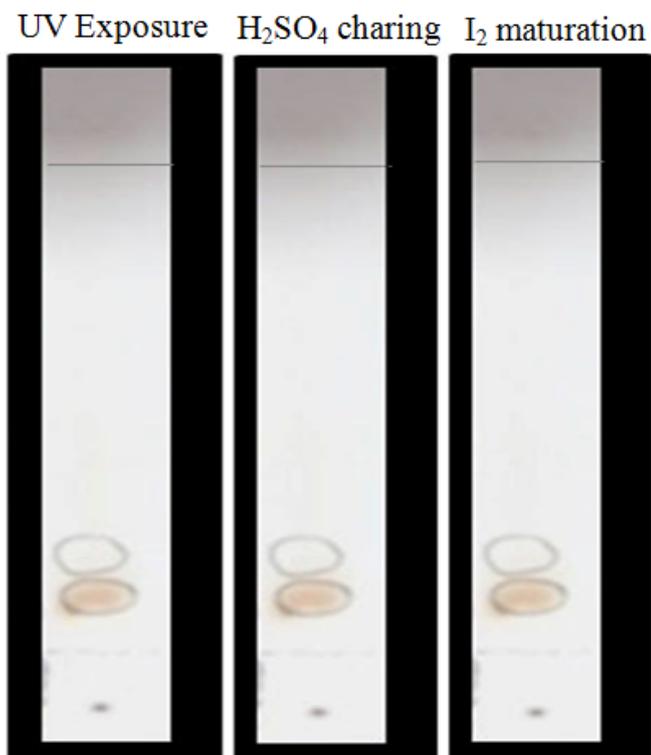


Fig. 9: TLC plates of an ethyl-acetate fraction of methanolic extract of *C. sinensis*, two spots were found in all the above cases

## DISCUSSION

Diabetes is associated with reproductive hypofunction related problems including erectile dysfunction, low semen quality and loss of libido [29, 30]. The present experiment has been conducted for the correction of testicular impairment in streptozotocin-induced diabetic rat to find out the threshold duration of the treatment with an ethyl-acetate fraction of methanolic extract of *C. sinensis* at the dose of 100 mg/kg body weight to minimize the bioburden of phyto-molecules on biological systems. The ethyl-acetate fraction at the dose of 100 mg/kg body weight has been selected as a most potent fraction out of other doses used here as the said dose showed more promising results without imposing any metabolic toxicity [13]. Elevation in the glycated hemoglobin level in all three duration dependent diabetic groups confirms the diabetic condition [31]. Flow cytometric analysis of sperm showed that the percentage of dead sperm cells and sperms with depolarised mitochondrial membrane were increased in the diabetic group that confirmed the testicular impairment in diabetic condition. Depolarised sperm indicates low mitochondrial status of sperm [32, 33]. Decreased serum testosterone level in the diabetic group confirmed the testicular hypofunction as testosterone is prime hormone for regulating testicular activities including spermatogonia [34]. Treatment with the ethyl acetate fraction for above said three duration to diabetic rats resulted in a significant recovery in the above parameters, may be due to increase in the activities of testicular androgenic key enzymes, i.e.  $\Delta^5$ ,  $3\beta$ -HSD and  $17\beta$ -HSD [34]. One possible mechanism may be the effect of the phyto-molecules (s) present in the fraction to switch on the androgenic enzymatic genes, noted here from qRT-PCR study. Another view about diabetes linked testicular hypofunction may be oxidative stress-mediated disorder [35]. Oxidative stress imposition in testicular tissue of diabetic animals was developed at different duration which has been assessed here from the significant elevation in the levels of the end products of free radical i.e. CD and TBARS in testis which may be due to high level of uncoupler protein synthesis in reproductive organs [36] which leads to increased reactive oxygen species (ROS) generation. After treatment of the different duration of ethyl acetate fraction, a significant recovery was observed in this concern. This result enlightened that this recovery by this phyto-ingredients present in ethyl acetate fraction may correct this ROS by controlling diabetes and testosterone synthesis as hyperglycaemia as well as low testosterone are associated with ROS generation [37].

To find out the apoptotic status of testicular germ cells in such conditions, the expression pattern of pro-apoptotic marker like Bax and anti-apoptotic marker like Bcl-2 genes were studied [38]. The testicular Bax gene-focused the upward nature and Bcl-2 gene showed a downward nature of expression in all duration dependent diabetic groups. Ethyl acetate fraction treatment can able to recover the expression patterns of above biomarkers in all the duration of the treatment by nutraceutical mediated switch on of gene expression which pointed out the presence of anti-apoptotic nature of the phytochemicals in the said fraction. Out of above said three duration of treatment it is established that 28 d is the threshold duration of treatment and if the duration of treatment is lengthen, there is no further correction of the concerned parameter. So, considering all the results it may be hypothesised that the active phyto-molecule(s) present in the said fraction correct the male germ cell apoptosis by decreasing Bax gene expression which results diminution in mitochondrial depolarization of sperm cell and therefore mitochondrial cyto-C mediated apoptotic pathway is corrected. Side by side ROS mediated extrinsic pathway of germ cell apoptosis is also corrected by this phyto-molecule. The said fraction has two phyto-molecules focused by TLC. The nature of the concerned phyto-molecules is beyond our knowledge at present and work is going on to explore it.

## CONCLUSION

From the results of this experiment it may be concluded that 28 d duration is the threshold duration for the management of diabetes-induced testicular hypofunction by the ethyl acetate fraction of methanolic extract of *C. sinensis* at a threshold dose of 100 mg/kg

body weight twice per day. Isolation of active constituent is under process and hopefully would be unfolded from the near future work.

## ACKNOWLEDGMENT

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## AUTHORS CONTRIBUTIONS

Barnali Das performed the research work and wrote the total manuscript. Baisakhi Biswas helped to perform the research work. Prof. Debidas Ghosh designed the experiment, supervised the entire experiment, analysed the results critically and edited the manuscript.

## CONFLICT OF INTERESTS

The authors have declared that there is no conflict of interest

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