

Original Article

“ANTIDIABETIC ACTIVITY OF SEED OF *EUGENIA JAMBOLANA* IN STREPTOZOTOCIN INDUCED DIABETIC MALE ALBINO RAT: AN APOPTOTIC AND GENOMIC APPROACH”

KISHALAY JANA¹, ABHINANDAN GHOSH¹, KAUSIK CHATTERJEE¹, DEBIDAS GHOSH^{1*}

Dept. of Bio Medical Laboratory Science and Management, Vidyasagar University, Midnapore 721102.

Email: debidas_ghosh@yahoo.co.in

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ABSTRACT

Objective: The study aimed to investigate the antidiabetic effect of ethyl acetate fraction of seed of *Eugenia jambolana* (*E. jambolana*) at genomic level in streptozotocin (STZ) induced diabetic male albino rat.

Methods: Diabetic rats were treated with said fraction at the dose of 200 mg/Kg of body weight/day for 35 days. Potential antidiabetic mechanisms were investigated with blood glucose (short duration and long duration model), serum insulin, haemoglobin A_{1c} (HbA_{1c}), ISEL (In-Situ End Labelling) study of pancreatic tissue and quantitative RT-PCR study of hepatic hexokinase-I (Hex-I), Bax and Bcl-2 gene expression.

Results: Results showed a significant antihyperglycemic action of the said fraction in both short and long duration treatment schedule. Serum insulin and HbA_{1c} levels were also recovered in treated group in compare to the untreated diabetic group (p<0.05). ISEL study focused the regeneration of pancreatic beta cells in treated group. It was also observed that the correction in expression in Bax, Bcl-2 and Hex-I gene in hepatic tissue after the treatment of the fraction in the diabetic rat. The antidiabetic activity of the fraction was compared with glibenclamide, a standard antidiabetic drug.

Conclusion: The findings provide information about the antihyperglycemic activity of this fraction through gene regulation.

Keywords: Bax, Bcl-2, Diabetes, *E. jambolana*, Hexokinase-I.

INTRODUCTION

It is known that diabetes is possibly the world's fastest growing metabolic disease [1]. Chronic hyperglycemia is the most well-known symptoms of Non-Insulin Dependent Diabetes Mellitus (NIDDM), which is important for development of diabetic complications [2]. There are several reasons for enhancement of blood glucose level in diabetic people. Refuse in insulin secretion, defect in glucose transport system (GLUT1, GLUT4) [3], and improvement of hydrolytic activity of intestinal brush border membrane are three important causes of hyperglycemia in diabetic people [4]. Several synthetic drugs are used for the treatment of diabetes but no one is without side effects and hence there is need for alternative improvement of oral hypoglycemic agents [5-6]. Hypoglycemic effects of different herbal plants are recognized to their ability to restore the function of pancreatic β cell by causing an increase in insulin secretion or slow down the intestinal absorption of glucose [7-8]. Many herbal plant species having antihyperglycemic activity are available in worldwide [9]. Most of the plants contain terpenoids, flavonoids, carotenoids, glycosides, alkaloids, those are frequently used as antidiabetic agents [10].

E. jambolana has been widely used in Indian traditional medicine for remedy of various ailments. Different parts of *E. jambolana* such as kernel, leaves and septum have a significant antihyperglycemic effect. Antihyperglycemic activity of ethyl acetate fraction of seed of *E. jambolana* has been reported previously by us [11-14], but its molecular mechanism for antidiabetic activity has not yet been described clearly. Hence, the present study was conducted to explore the molecular mechanism of action of ethyl acetate fraction of seeds of *E. jambolana* in STZ-induced diabetes rat.

MATERIALS AND METHODS

Bioactivity-guided ethyl acetate fractionation of hydro methanolic (2:3) extract of seed of *E. jambolana*.

For preparation of ethyl acetate fraction, the seeds of *E. jambolana* were collected in rainy season (July–August) from the local market at Midnapore town, West Bengal, India. The specimen were authenticated by the taxonomist in the Dept. of Botany and Forestry,

Vidyasagar University, Midnapore, where the voucher specimen was preserved having Ref. No.- BMLSM-10/06. The preparation of crude extract using hydro-methanol (2:3) as solvent mixture followed by its fractionation using ethyl acetate was performed according to our previously reported method [12].

Selection of animal and animal care

The matured normoglycemic wistar strain male albino rats, having three months of age, weighing about 120 \pm 10 g and fasting blood glucose (FBG) levels were 80-90 mg/dl, were used for this experiment. Rats were housed at an ambient temperature of 25 \pm 2°C with 12 h light: 12 h dark cycle. Rats were provided with protein rich standard feed and water ad libitum. The principle of animal care and directions was maintained throughout the experiment which has been given by our Institutional Ethical Committee (IEC) which is in conformity with the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA).

Induction of diabetes mellitus

Rats were made diabetic by a single intramuscular injection of STZ (Sigma Aldrich, USA) at a dose of 40 mg/kg of body weight. STZ was dissolved in 0.1M citrate buffer (pH 4.5). After 7 days of injection, diabetes was confirmed by determining the FBG level. The rats with FBG levels of 300-350 mg/dl were only considered for the experiment [15].

Design

The rats were divided into four groups having six animals in each group.

Control group

Rats of this group received the single intramuscular injection of the citrate buffer (1 ml/kg body weight) and this group was subjected to oral administration of 1 ml distilled water /kg body weight/day for 35 days at a gap of 7 days after citrate buffer injection.

Diabetic group

Rats were made diabetic by a single intramuscular injection of STZ at a dose of 40 mg/kg body weight in citrate buffer. After 7 days of

STZ injection, distilled water was administered orally for 35 days to diabetic rat at a dose of 1 ml/kg body weight per day after 7 days of STZ injection.

Ethyl acetate fraction treated diabetic group

After 7 days of STZ injection, diabetic rats of this group were treated with ethyl acetate fraction of seed of *E. jabolana* at the dose of 200 mg/2 ml distilled water/kg of body weight/day for 35 days at fasting state.

Glibenclamide treated diabetic group

After 7 days of STZ injection, diabetic rats of this group were treated with glibenclamide at the dose of 20 mg/kg body weight/day for 35 days.

Single dose of ethyl acetate fraction was administered every day orally at morning (10.00 a. m.) Using intragastric tube for 35 days. Blood glucose levels were estimated in short and long duration models. On 36th day of extract treatment (42nd day of STZ-injection) all animals were sacrificed by cervical decapitation. For the measurement of serum insulin level, blood was collected from each rat and serum was separated by centrifugation at 3000 rpm for 10 min. For ISEL study and mRNA expressions of Hex -I, Bax and Bcl-2 genes, pancreas and liver were dissected out from each animal.

Screening of antihyperglycemic activity

Short-duration experimental model

Blood samples were obtained from the tail vein and FBG levels were estimated using a glucometer (Bayer's Ascensia Entrust, Bayer, Germany), after 1, 3, 5, 8 and 24 hours following oral administration of a single dose (200 mg/2 ml distilled water/kg of body weight/rat) of ethyl acetate fraction or glibenclamide sample to rats.

Long-duration experimental model

The levels of FBG in all experimental rats were measured using a glucometer (Bayer's Ascensia Entrust, Bayer, Germany) at the end of 1, 2, 3, 4 and 5 weeks of treatments using blood samples which have been obtained from tail vein.

Assessment of glycated hemoglobin level

Glycated hemoglobin level was measured as per standard protocol [16].

Quantification of serum insulin level

The level of serum insulin was measured by enzyme-linked immunosorbent assay (ELISA) kit for rat (Boehringer Mannheim Diagnostic, Mannheim, Germany). Level of insulin was expressed in μ IU/ml.

ISEL study

ISEL histochemistry of pancreatic tissue was performed using TACS TdT-DAB in situ apoptosis detection kit (Trevigen Inc., Gaithersburg, MD, U. S. A.) [17].

RNA extraction and cDNA synthesis

Animals were sacrificed and hepatic tissue was dissected out from the animals and the tissue was immediately allowed to freeze in liquid nitrogen. Then the frozen tissue was pulverized and re suspended in lysis buffer (Roche Diagnostics, Mannheim, Germany), RNA was extracted according to the manufacturers protocol.

Residual DNA was removed by treatment with 5 units of DNase I (Roche Diagnostics, Mannheim, Germany) for 45 min at 37°C followed by inactivation for 10 min at 65°C. Then 2 μ g of RNA was reversed transcribed with Superscript II reverse transcriptase. The cDNA was diluted five-fold prior to PCR amplification.

Real-time quantitative PCR

Real-time PCR was performed in the Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) using the SYBR Green I Master mix (Roche Diagnostics). Final mixture of PCR (final volume 20 μ l) contained 10 μ l SYBR Green I mix, 5 μ l of cDNA (1:5 dilution) and 0.5 μ M of each primer and 4 μ l PCR grade distilled water. PCR amplification was initiated with a 10-min pre incubation step at 95°C, followed by a 35 cycles of denaturation at 95°C for 10 s, annealing as described in table 2 and elongation at 72°C for 10 s. After amplification, the melting curves were determined in a three-segment cycle of 95°C for 5 s, 65°C for 15 s and 97°C for 0 s. All PCR reactions were performed in triplicates. Primer specificity was assessed through melting curve analysis. Primers (rat specific) were designed for the gene of interest, i. e. Hex-I, Bax, Bcl-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference, the sequences of which are detailed in table 1 [18].

Statistical analysis

All data were presented as mean \pm SEM, for six rats in each group. Comparison between groups or between time points was made by one-way analysis of variance (ANOVA) followed by Multiple Comparison two-tail 't'-test to analyze the significance study. Differences were considered significant when p-values were less than 0.05 (p<0.05). All statistical analysis were performed using SPSS (SPSS Inc, Chicago, USA).

RESULTS

Acute effect of ethyl acetate fraction on FBG levels

Table-2, showed the antihyperglycemic activity of ethyl acetate fraction of seed of *E. jabolana* at different time intervals after single dose of oral administration of plant fraction or standard drug, glibenclamide. FBG levels were decreased gradually from 3h to 8h after administration with ethyl acetate fraction or glibenclamide, but thereafter an elevation in FBG levels were noted upto 24th hour. The most reduction of FBG level was noted in treated groups at 8h. The percentage of recovery in fraction treated group was 31%, where glibenclamide treated group showed 35% recovery in FBG levels at 8h, when compared with initial time (p<0.05) of treatment. On the other hand, when comparison was made with diabetic group the percentage of recovery of blood glucose level at 8h was 37% in fraction treated group and 38% in glibenclamide treated group.

Chronic effect of ethyl acetate fraction on FBG levels

A significant elevation was noted on FBG levels in STZ-induced diabetic rats in compare to the control group. Significant recovery of FBG level was noted in fraction treated group in respect to untreated diabetic group. After daily administration of ethyl acetate fraction or standard drug glibenclamide, at the end of 2, 3, 4 and 5 weeks, the percentage of recovery of FBG levels were noted at 27%, 57%, 70% and 73% respectively, when compared to the untreated diabetic group (p<0.05). But in case of glibenclamide treated group the percentage of recovery were noted at 26%, 59%, 72% and 74% in respect to untreated diabetic group (p<0.05) (Table 3).

Table 1: Primer-sequence and specific conditions used for PCR amplification of candidate genes

Gene	Primer Sequence	Annealing temperature (°C)	No. of Cycles	Amplification size (bp)
Hex-I	S 5'-GACCAAGTCAAAAAGATTGA-3'	62	35	256
	AS 5'-TCTTCTCGTGGTTCACCTGC-3'			
Bax	S 5'-AGACAGGGCCTTTTGTGTTAC-3'	58	35	482
	AS 5'-GAGGACTCCAGCCACAAGAT-3'			
Bcl-2	S 5'-CCGGGAGAACAGGGTATGAT-3'	60	35	689
	AS 5'-CAGGTATGCACCCAGAGTGA-3'			
GAPDH	S 5'-ACCACAGTCCATGCCATCAC-3'	58	35	452
	AS 5'-TCCACCACCCTGTTGCTGTA-3'			

GAPDH-glyceraldehyde 3-phosphate dehydrogenase, S- Sense, AS- Antisense, bp- base pair

Table 2: Acute effect of ethyl acetate fraction of seed of *E. jambolana* on FBG levels in STZ-induced diabetic rat.

Groups	FBG level (mg/dL)					
	Time (h) after a single dose of fraction administration					
	0	1	3	5	8	24
Control	69.2 ± 2.3	72 ± 3.5	74.4 ± 3.3	71.2 ± 6.4	64.8 ± 2.8	75.2 ± 4.5
Diabetic	334.8 ± 6.3*	335 ± 8.6*	345.2 ± 7.1*	340.2 ± 3.9*	333.8 ± 6.9*	346.0 ± 9.9*
Diabetic +Fraction treated	315.4 ± 13.8**	286.8 ± 8.5**	267.0 ± 6.9**	242.4 ± 9.1**	211.6 ± 7.76**	248.6 ± 9.3**
Diabetic+ Glibenclamide treated	318.7 ± 10.2**	279.2 ± 7***	258.1 ± 8.3***	226.5 ± 7.8***	208.4 ± 7***	252.8 ± 7.8**

Data expressed as Mean ± SEM, (n = 6), ANOVA followed by multiple comparisons two tail 't' test. Values with different stars as superscripts (*, **, ***) in each column differ from each other significantly, p <0.05.

Table 3: Chronic effect of ethyl acetate fraction on FBG levels in control and different experimental groups.

Groups	FBG level (mg / dL)					
	1 st day	8 th day	15 th day	22 nd day	29 th day	35 th day
Control	84.83±4.8	86.83±3.3	81.83±4.7	80.5±4.1	86.32±4.7	88.32±4.1
Diabetic	88.66±4.7	347.6±8.2*	353.33±4.1*	345.83±4.2*	341.33±4.6*	346.87±8.2*
Diabetic +Fraction Treated	84.16±6.5	345.33±6.4*	246.0±4.6**	142.81±4.9**	118.11±3.6**	102.0±3.8**
Glibenclamide treated	86.66±4.6	340.66±5.0*	241.5±3.6**	132.33±3.2***	98.8±4.1***	89.97±4.9**

Data expressed as Mean ± SEM, (n = 6), ANOVA followed by multiple comparison two tail 't' test. Values with different stars as superscripts (*, **, ***) in each column differ from each other significantly, p <0.05.

Levels of Glycated hemoglobin

Glycated hemoglobin (HbA_{1c}) level was increased significantly in diabetic group in compare to the control group. After treatment with ethyl acetate fraction of seed of *E. jambolana* or glibenclamide, the level of glycated hemoglobin was resettled towards the control group. Glycated hemoglobin level was insignificantly differ in between fraction treated and glibenclamide treated groups (Fig 1).

Effect on serum insulin

Serum insulin level was decreased in the diabetic group in respect to the control group. After administration of ethyl acetate fraction to the diabetic rats for 35days, serum level of this hormone was significantly restored towards the control level. No significant difference of this parameter was observed in between the fraction-administered and glibenclamide treated groups at 42days of experiment (Fig 1).

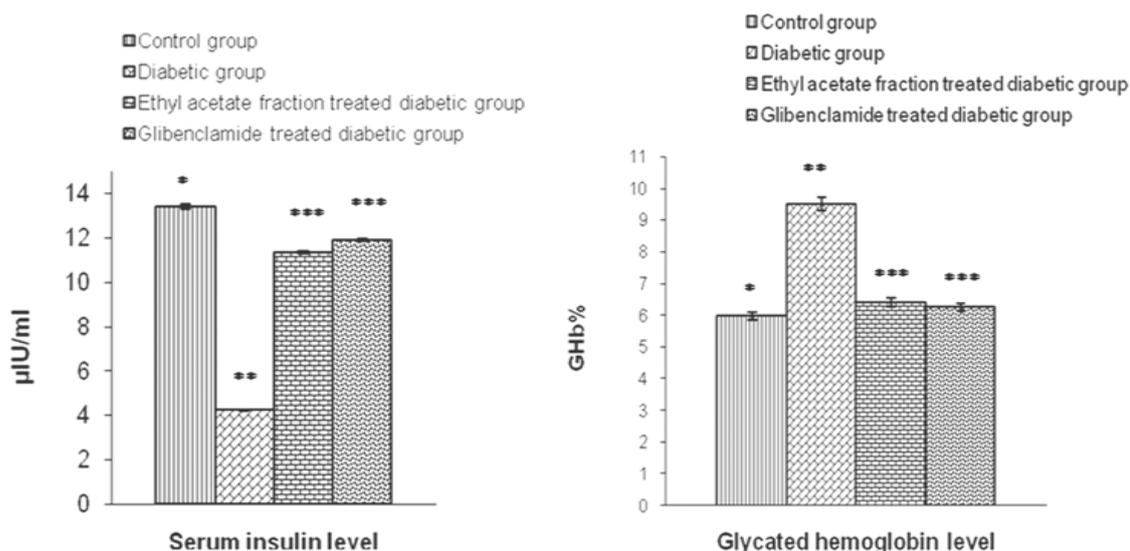


Fig. 1: Protective efficacy of ethyl acetate fraction or glibenclamide on serum insulin and glycated hemoglobin levels in streptozotocin-induced diabetic rat. Each bar represents Mean ± SEM, n=6. ANOVA followed by "Multiple Comparison two tail 't' test". Values of bar diagram with different stars (*, **, ***) differ from each other significantly at the level of p<0.05.

ISEL study of pancreas

Figure-2, shows that very few apoptotic cells were present in control group. The ISEL-positive or apoptotic cells in pancreatic islets were noted by brown colour in the tissue section (Fig 2). Moreover, qualitative analysis revealed more apoptotic cells in pancreatic tissue section in diabetic group compared with control group. After administration of fraction to the diabetic rats, count of ISEL-positive cells in the pancreas was remarkably decreased in compare to the untreated diabetic group (Fig 2).

Effect of ethyl acetate fraction of seed of *E. jambolana* on hepatic Hex-I gene expression

Down ward expression pattern of the hepatic Hex-I gene in diabetic group was noted by qRT-PCR analysis when compared with the expression pattern for the same in the control group. A significant remedial activity of ethyl acetate fraction or glibenclamide was noticed in the said gene expression in target tissue after treatment to diabetic group (Fig 3).

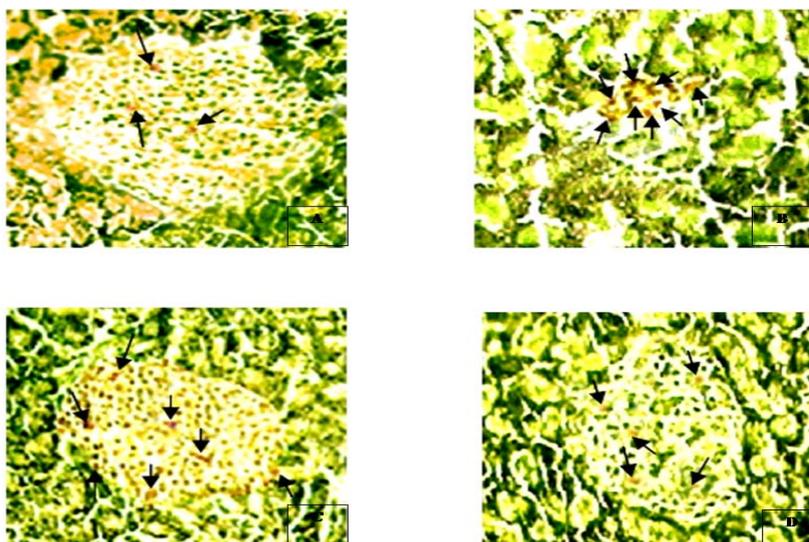


Fig. 2: Immunohistochemistry of Pancreas; ISEL Study, X 400 indicates apoptotic cells. (A) Representative microphotograph of pancreatic section showing normal density of islet cells and very few apoptotic cells in islet present in the control group identified by ISEL study. (B) Microphotograph of pancreatic section showing increase in the number of apoptotic cells in islet present in diabetic rat by ISEL study. (C) Representative microphotograph of pancreatic section from ethyl acetate fraction treated diabetic rat showing low number of apoptotic cell in islet identified by ISEL study in compare to diabetic group. (D) Microphotograph of pancreatic section from glibenclamide treated diabetic rat with low count in apoptotic cells in islet in respect to diabetic group.

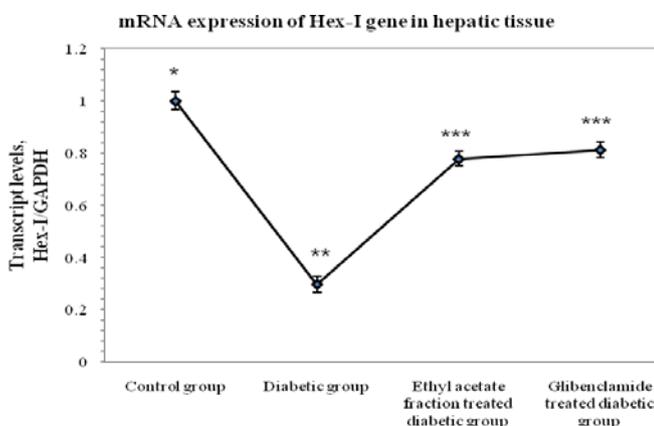


Fig. 3: Representative qRT-PCR analysis of hepatic Hex-I gene expression in rat after normalizing the result with a housekeeping gene, GAPDH. Data represents mean \pm SEM, n=6. ANOVA followed by "Multiple comparison two tail 't' test". Different stars (*, **, ***) differ from each other significantly at the level of $p < 0.05$

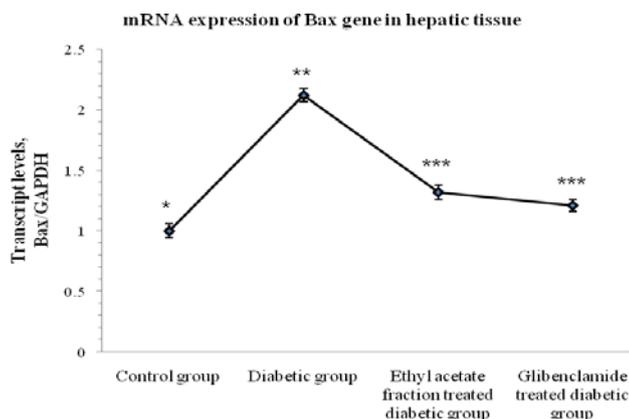


Fig. 4: Representative qRT-PCR analysis of hepatic Bax gene expression in rat after normalizing the result with housekeeping gene, GAPDH. Data represents Mean \pm SEM, n=6. ANOVA followed by "Multiple Comparison two tail 't' test". Different stars (*, **, ***) differ from each other significantly at the level of $p < 0.05$

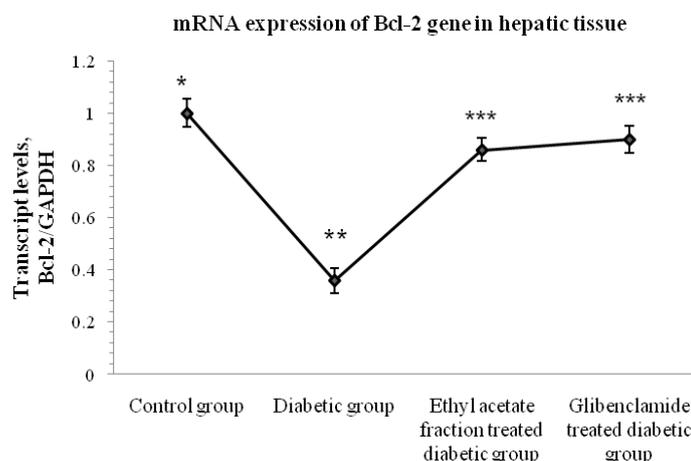


Fig. 5: Representative qRT-PCR analysis of hepatic Bcl-2 gene expression in rat after normalizing the result with housekeeping gene, GAPDH. Data represents mean \pm SEM, n=6. ANOVA followed by "Multiple Comparison two tail 't' test". Different stars (*, **, *) differ from each other significantly at the level of $p < 0.05$**

Expression of Bax, Bcl-2 genes in hepatic tissue at mRNA level

Pro-apoptotic marker, Bax was increased and anti-apoptotic marker Bcl-2, was decreased significantly ($p < 0.05$) in hepatic tissue in diabetic group with respect to the control group by qRT-PCR analysis. After the treatment of said fraction to diabetic rat for 35 days, a significant recovery ($p < 0.05$) was observed in the expression pattern of hepatic Bax and Bcl-2 genes towards the control group (Fig 4, 5).

DISCUSSION

The preamble of chemical induced type-1 diabetes mellitus in experimental rat is executed using streptozotocin that selectively destroys pancreatic β -cells is very convenient and simple to use [19]. Streptozotocin causes a massive degeneration of β -cells of the islets of Langerhans and induces hyperglycemia through several processes such as oxidation of essential-SH groups [20], inhibition in glucokinase, disturbances in intracellular calcium homeostasis or generation of free radicals [21]. The antihyperglycemic activity of ethyl acetate fraction of seed of *E. jambolana* has been reported previously by us [11-14], but its molecular mechanism has not yet been described clearly.

In this study, we found that the antihyperglycemic potency of the ethyl acetate fraction of *E. jambolana* in both short and long duration models may be due to high solubility of bioactive phytochemicals i. e. gallic acid and polyphenolic compounds present of this solvent fraction which has been published in our previous publication [12-13, 22]. The administration of this fraction at a dose level of 200 mg/Kg of body weight of rat for single time attenuates FBG level effectively up to 8 hr may be due to stimulation of pancreatic β -cells for short duration insulin release against high blood glucose level by this fraction same as activity produced by other synthetic oral drugs like glibenclamide which is responsible to increase in sensitization and stimulation of β -cells against blood glucose [23-24]. The duration of antihyperglycemic effect was up to 8 hrs, which may be due to the molecular instability or rapid plasma clearance of phytochemical.

In long term model, the ethyl acetate fraction treatment for 35 days exhibited a significant reduction in the FBG levels towards control demonstrated antihyperglycemic property of this herbal fraction. It was also supported here by the significant recovery in serum insulin level. These effects may be due to the increased release of insulin from remaining β -cells and/or regeneration of β -cells [25, 14], and restoration of insulin sensitivity [26]. This insulinotropic activity was further supported from the low level of glycated hemoglobin due to lowering of FBG level [27], as this is under the action of insulin [28].

The physiological role of hexokinase, the insulin dependent key carbohydrate metabolic enzyme of glycolysis has been well studied by others [29-30], along with us [15]. The expression pattern of Hex-

1 in total hepatic lysate of all the groups was studied through qRT-PCR techniques, which revealed the diminished expression of Hex-1 in diabetic condition in respect to the control. After 35 days of chronic treatment, expression pattern of Hex-1 was significantly improved in the diabetic rat indicated the recovery in homeostasis of carbohydrate similar with the previous findings of other [31], along with us [13], which supported the hypothesis that higher level of Hex-1 activity reflect a higher rate of glucose utilization by cells [29].

The Bax gene expression (pro-apoptotic gene) was significantly increased along with the diminution in the expression of anti-apoptotic gene i. e. Bcl-2 in STZ induced diabetic animals which is reported previously in this line [32]. So, the level of serum insulin is significantly reduced due to destruction of pancreatic β cell under the STZ toxicity. Moreover, the expression pattern of hepatic Hex-1 was significantly decreased in STZ induced diabetic animals as insulin is the positive factor in this concern [29]. After the treatment of said fraction to diabetic animals the Bax, Bcl-2 and Hex-1 gene expression pattern was significantly recovered towards the control level. From these results it may be predicted that the phytochemicals present in this fraction which elevate the serum insulin through the resists of pancreatic β cell by the regulation of gene expression of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) markers [32].

The loss of β -cells from the endocrine pancreas due to STZ-induced free radicals mediated apoptosis, as shown by an increase level of ISEL positive cells, and produces the hyperglycaemic state [33]. This circumstances enhances excess free radicals generation either by auto oxidation of glucose or enzymatic and non-enzymatic glycation of proteins with increased formation of glucose derived advanced glycosylation end products (AGEs), enhanced glucose flux through polyol pathway [34], and reduction of anti-oxidant defence. The apoptosis-induced reduction in the beta-cell volume of the pancreas also provide an explanation for the decrease of glucose tolerance in hyperglycemia and the development diabetes mellitus, which can be controlled by this fraction treatment following inhibition in absorption of dietary carbohydrates through small intestine. But after the treatment of the ethyl acetate fraction or glibenclamide to the diabetic rat, degeneration of DNA of pancreatic β -cells was inhibited which revealed from decreased ISEL positive cells in immunohistological study. The positive regulation in expression pattern of Hex-1 gene and inhibition in the breakdown of DNA towards control established its potency against diabetes may be due to its phytochemicals viz. gallic acid compounds present in it as they have major antioxidative activity with redox properties [13].

CONCLUSION

The present study demonstrated that the ethyl acetate fraction of seed of *E. jambolana* have a strong antidiabetic activity on

experimental model rat at genetic level. So, this study focuses promising evidence to the pharmaceutical industry for the development of drug by this fraction for the correction of different complications of diabetes.

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CONFLICT OF INTEREST

The authors have declared that there is no.

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