Anti diabetic and anti oxidative potencies study of ethyl acetate fraction of hydromethanolic (40:60) extract of seed of Eugenia jambolana Linn and its chromatographic purification

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Received on: 20-09-2011; Revised on: 15-10-2011; Accepted on: 10-12-2011

ABSTRACT

Eugenia jambolana Linn. of Myrtaceae family is widely distributed all through India and its traditional medicinal use is well recognized. Diabetic therapeutic potentiality of ethyl acetate fraction of hydromethanolic (40:60) extract of seed of E. jambolana was investigated in streptozotocin-induced diabetic rat. Diabetes state was confirmed by increased fasting blood glucose and glycated hemoglobin levels along with diminished body weight and serum insulin level. Diabetes induced carbohydrate metabolic homeostasis alteration was evaluated by increased activities of glucose-6-phosphatase, lactate dehydrogenase and decreased activities of hexokinase and glucose-6-phosphate dehydrogenase enzymes in hepatic and skeletal tissues along with low glycogen contents in liver and skeletal tissues. Oxidative stress development in diabetes was confirmed by diabetes from diminished activities of free radical scavenging enzymes and elevated levels of end products of lipid peroxidation in said tissues. Oral administration of ethyl acetate fraction at a dose of 20 mg and glibenclamide at a dose of 0.6 mg in 0.5 mL water (100 g body weight/rat for twice a day at fasting state to diabetic rats for 28 days significantly (p<0.05) resettled all the above parameters to their respective control levels. To establish safety profile of the said fraction, metabolic as well as acute toxicity studies were carried out as per established guidelines. HPTLC fingerprinting of bioactive ethyl acetate fraction was recorded with mobile phase Ethyl acetate: Methanol: Water :: 100:13.5:10.

Key words: Eugenia jambolana, Antihyperglycemic agent, Oxidative stress, HPTLC, HPLC

INTRODUCTION

Eugenia jambolana (E. jambolana) Linn. belongs to the family Myrtaceae, which is with immense medicinal values used in traditional treatment of diseases⁶. Diabetes mellitus, a multidimensional disease, characterized with chronic hyperglycaemic state caused by endocrine disorders in insulin secretion and/or insulin action both that results chronic metabolic impairment in carbohydrate, lipid and protein homeostasis.⁷ A strong relationship between diabetes mellitus and oxidative stress has been established by us⁸.⁹ supported by others¹⁰.¹¹ Despite progress in the management of diabetes and oxidative stress by synthetic drugs till now no effective medicine has been developed that offers a permanent cure for diabetes mellitus and oxidative stress has been established by others¹².¹³. From the very ancient period in rural India, various parts of E. jambolana are being used for treatment and management of diabetes and related disorders. The medicinal values of E. jambolana have been published earlier by us (...), supported findings of others (...). The present study was designed to investigate the therapeutic potentiality of the ethyl acetate fraction of hydromethanolic (40:60) extract of E. jambolana against hyperglycaemia and oxidative stress condition in experimental diabetes along with the isolation of active plant ingredients through HPTLC and HPLC mediated chromatographic purification.

MATERIALS AND METHODS

Plant material preparation

Fresh seeds of E. jambolana were collected from rural areas of Paschim Medinipur District, West Bengal, India, in the month of May-July. Preliminary identification of the plant was made by a taxonomist and a voucher specimen (IPCCH No. 6) was deposited in the Dept. of Botany, Vidyasagar University, Midnapur, West Bengal, India. After collection, plant parts were separated and washed thoroughly with tap water and then with deionized water. The seeds were dried in an incubator completely at 37 °C. About 4.8 Kg of dried seeds were collected from 6 Kg of fresh seeds and pulverized with electrical grinder. Then maceration was carried out with hydro-methanol solvent (H₂O: MeOH :: 40:60, v/v; 250 mL of solvent used for 50 gm of plant part) with an intermittent stirring for the first 2 hr and left for 36 hr at 37 °C. The extraction process was continued for 3 days following the previous process and the final extracts were collected on the fourth day. The extract was then filtered through No. 1 Whatman filter paper in a form of slurry. The hydro-methanol filtrates were evaporated under reduced pressure by Rotavapour instrument (HAHN-SHIN HS-2000NS, Korea) at 38 °C for complete removal of methanol. Finally, plain aqueous filtrates were lyophilized on benchtop K Lyophillizer to produce 890 gm lyophilized extract. The lyophilized extract was a mixture of dark brownish sticky layer and light brownish solid powder (slightly hygroscopic in nature). In a 5 L separating flask, 890 gm lyophilized extract of E. jambolana were dissolved with 2 L hydromethanolic (H₂O: MeOH :: 40:60 v/v) solution and was subjected to bio activity guided solvent fractionation. For this purpose different laboratory grade solvents [n-hexane (2 lit), chloroform (5 lit) and ethyl acetate (10 lit)] with increasing polarity were used and thin-layer chromatography (TLC) was carried out to monitor progress in fractionation. Collected separate fractions were dried under reduced pressure (10 to 200 mbar) at 40 °C using rotavapor. Hexane, chloroform and ethyl acetate fractions finally afforded 4.01 gm, 28.24 gm and 68.35 gm respectively. On the basis of previous bioactivity study carried out on male wistar rats at various dose levels, n-hexane and chloroform fractions were found inactive and thus excluded from this study. The ethyl acetate fraction was dissolved in distilled water and administered orally to experimental diabetic rats for this experiment.
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Chemicals
Streptozotocin (STZ) was obtained from Sigma, USA. All other chemicals used were of analytical grade obtained from HIMEDIA, Mumbai, India or Sigma, USA. Kits for the ELISA and various enzyme assays were purchased from Millipore, USA and Crest Biosystems, India. All the chemicals used for chromatography were of HPLC grade. Deionized water from Milli-Q water filtration system (Millipore, Bedford, USA) was used in analysis.

Selection of animal and animal care
Normoglycaemic (fasting blood glucose level 60-80 mg/dL) wistar male albino rats having body weight about 120 ± 10 g were used in these experiments. The animals were housed at an ambient temperature of 25 ± 2°C under 12 hr : 12 hr light-dark cycle and acclimated to these conditions for 15 days before use in experimental trials. All animals had free access to standard rat food and water ad libitum. The principles of laboratory animal care [12] and instruction given by our “Institutional Ethical Committee” (VU/IAEC/BioMed/08/2008-2009) were followed throughout the experiments.

Experimental design
Initially 30 normoglycaemic rats were selected for this study, out of which twenty four rats were kept under eighteen hours fasting and then subjected to diabetic by single intramuscular injection of streptozotocin (STZ) (Sigma Chemical Co., USA) at a dose of 3.5 mg/100 g body weight in citrate buffer (pH-4.5) as standardized by the previous work in our laboratory [13]. Diabetic condition was confirmed by estimation of fasting blood glucose (FBG) level after 24 hrs interval and then on the 7th day after day of injection to investigate the stability of the diabetic condition. The rats with FBG more than 250 mg/dL but less than 500 mg/dL were included for this investigation. Out of 24 rats, 20 rats were found to be diabetic and from these eighteen diabetic rats and six normoglycaemic rats were included for this study. The duration of experiment was 28 days. Initial body weight of all the twenty four rats were recorded and divided into following four equal groups:

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

Group II (Diabetic group) was made diabetic by a single intramuscular injection of STZ at a dose of 3.5 mg/100 g body weight in citrate buffer (pH-4.5) as standardized by the previous work in our laboratory [13]. The rats were made diabetic by single intramuscular injection of STZ at a dose of 3.5 mg/100 g body weight in citrate buffer (pH-4.5) as standardized by the previous work in our laboratory [13]. The rats were made diabetic by single intramuscular injection of STZ at a dose of 3.5 mg/100 g body weight in citrate buffer (pH-4.5) as standardized by the previous work in our laboratory [13]. The rats were made diabetic by single intramuscular injection of STZ at a dose of 3.5 mg/100 g body weight in citrate buffer (pH-4.5) as standardized by the previous work in our laboratory [13]. The rats were made diabetic by single intramuscular injection of STZ at a dose of 3.5 mg/100 g body weight in citrate buffer (pH-4.5) as standardized by the previous work in our laboratory [13]. The rats were made diabetic by single intramuscular injection of STZ at a dose of 3.5 mg/100 g body weight in citrate buffer (pH-4.5) as standardized by the previous work in our laboratory [13]. The rats were made diabetic by single intramuscular injection of STZ at a dose of 3.5 mg/100 g body weight in citrate buffer (pH-4.5) as standardized by the previous work in our laboratory [13]. The rats were made diabetic by single intramuscular injection of STZ at a dose of 3.5 mg/100 g body weight in citrate buffer (pH-4.5) as standardized by the previous work in our laboratory [13]. The rats were made diabetic by single intramuscular injection of STZ at a dose of 3.5 mg/100 g body weight in citrate buffer (pH-4.5) as standardized by the previous work in our laboratory [13]. The rats were made diabetic by single intramuscular injection of STZ at a dose of 3.5 mg/100 g body weight in citrate buffer (pH-4.5) as standardized by the previous work in our laboratory [13].

Group III (Ethyl acetate fraction treated group) diabetic rats were forcefully fed with ethyl acetate fraction

Group IV (Glibenclamide treated group) diabetic rats were forcefully treated with glibenclamide

and unknown samples was measured against blank using a 480 nm selective filter and a 650 nm differentiating filter. No inter-assay variation occurred as all samples were assayed at the same time.

Assessment of glycated hemoglobin and glycogen level
Glycated hemoglobin (HbA1c) along with hepatic and skeletal muscle glycogen level were measured following standard protocol [13]. Blood was collected and after serum separation the packed cell pellet was washed six times with normal saline (0.9% NaCl). The hemolysate was prepared by adding 1 mL of distilled water and 1/4th part of 0.7% thiobarbituric acid was added to 443 nm. The glycated hemoglobin level was expressed as GHb%.

Hepatic and skeletal glycogen levels were measured according to the accepted standard protocol [13]. Tissue was homogenized in hot 80 percent ethanol at a tissue concentration of 100 mg/mL, centrifuged at 8,000 g for 20 min. The residue was collected, dried over a water bath, and then extracted at 0 °C for 20 min by adding a mixture of 5 mL water and 6 mL of 52 percent ethanol. The residue was centrifuged at 8,000 g for 15 min for recovery of the supernatant. A 0.2 mL sample of the recovered supernatant was transferred in graduated test tube and made to 1 mL volume by the addition of distilled water. Graded standards were prepared using 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mL of a working standard solution and volume of all these standards were made to 1 mL using distilled water. Anthrone reagent (4 mL) was added to all test tubes and the tubes were then heated in a boiling water bath for 8 min. The solution was then cooled to room temperature, and the intensity of the green to dark green color of the solution recorded at 630 nm. Glycogen content of the sample was determined from a standard curve prepared with standard glucose solution.

Biochemical assessment of carbohydrate metabolic enzyme (hexokinase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and lactate dehydrogenase) activities in liver, skeletal and cardiac tissues
The enzyme activity was determined on the basis of reduction of NADPH coupled with hexokinase which was measured spectrophotometrically at 340 nm [14].

Glucose-6-phosphatase activity was measured according to standard protocol [14]. Tissue was homogenized in ice cold 0.1 M phosphate buffer saline (pH=7.4) at the tissue concentration of 50 mg/mL. In a calibrated centrifuge tube, 0.1 mL of 0.1 M glucose-6-phosphate solution and 0.3 mL of 0.5 M maleic acid buffer (pH=6.5) were taken and brought to 37°C in water bath for 15 min. The reaction was stopped with 1 mL of 10% trichloroacetic acid (TCA) followed by chilling in ice and centrifuged at 3000 x g for 10 min. The optical density was noted at 340 nm. The enzyme activity was expressed as mg of inorganic phosphate liberated per gm of tissue.

The glucose-6-phosphate dehydrogenase activity of hepatic, skeletal and cardiac tissues were measured spectrophotometrically [14]. One unit of enzyme activity is defined as that quantity which catalyses the reduction of 1 µM of NADP per minute. Activity of this enzyme was recorded by using glucose-6-phosphate as a substrate and absorbance was measured at 340 nm.

The L-lactate dehydrogenase enzyme activity was assessed spectrophotometrically as per the standard protocol [14]. The assay mixture was kept into a 3 mL cuvette prepared using 0.1 mL of NADH solution, 0.04 mL of pyruvate solution, 0.36 mL of phosphate buffer saline and 2.4 mL of distilled water solution. The total collected material was centrifuged at 8,000 g for 15 min. The packed cell pellet was transferred in graduated test tube and made to 1 mL volume by the addition of distilled water. The enzyme activity was expressed as units of activity per mg of protein.

Oxidative stress profile assessment
Evaluation of antioxidative enzyme viz. catalase, peroxidase, superoxide dismutase, glutathione-s-transferase activities
The catalase enzyme activities of hepatic, skeletal and cardiac tissues were measured biochemically [15]. For the evaluation of catalase activity target

Testing of fasting blood glucose level
Fasting blood glucose (FBG) level was monitored using single touch glucometer by collecting blood from tip of the tail of all experimental and control animals in all groups at the initial time of experiment and at two days interval throughout the experiment [14].

Assay of serum insulin by ELISA
Serum insulin level was measured using solid phase-conjugated sandwich ELISA kit for rat (EZRM-13K, Millipore, USA) [15]. The optical density of standard...
samples were homogenized separately in 0.05 M Tris-HCl buffer solution (pH: 7.0) at a tissue conc. of 50 mg/mL. These homogenized solutions were centrifuged at 10,000 g at 4°C for 10 min. To a spectrophotometric cuvette, 0.5 mL of 35 mM H$_2$O$_2$ and 2.5 mL of distilled water were mixed and the absorbance was measured at 240 nm. 40 µL of sample supernatant were added and the subsequent six readings were noted at 30 sec intervals.

Activities of peroxidase (Px) enzyme of target tissues were measured according to the standard method[10]. Guaiacol (20 mM) was mixed with 0.1 mL of sample. In the presence of 0.3 mL of 12.3 mM H$_2$O$_2$, the time was recorded for an increase in the absorbance by 0.1 at 436nm.

The superoxide dismutase (SOD) enzyme activities of the tissue samples were estimated by measuring the percentage inhibition of the pyrogallol auto oxidation by SOD according to the standard method [11]. The buffer was prepared by 50 mM Tris (pH-8.2). In a spectrophotometric cuvette, 2.04 mL of TRIS buffer, 20 mL of sample and 20 mL of pyrogallol were taken and the absorbance was noted in spectrophotometer at 420 nm for 3 min period. One unit of SOD was defined as the enzyme activity that inhibits the auto-oxidation of pyrogallol by 50 %.

Activities of glutathione-s-transferase (GST) enzyme in the tissue samples were measured spectrophotometrically[12] using CDNB (1-chloro-2, 4- dinitrobenzene) as a substrate. The assay mixture of 3 mL contained 0.1 mL of 1mM CDNB in ethanol, 0.1 mL of 1 mol GSH, 2.7 mL of 100 mM potassium phosphate buffer (pH: 6.5) and 0.1 mL of supernatant of the tissue homogenate. The formation of 2,4-dinitrophenylglutathione, was monitored by measuring the net increase in absorbance at 340 nm against the blank. The enzyme activity was calculated using the extinction coefficient 6.9 M/cm and expressed in unit/mg of tissue.

**Estimation of end products of lipid peroxidation (TBARS and CD)**

The sample tissues were homogenized separately at the tissue concentration of 50 mg/mL in 0.1 M of ice-cold phosphate buffer (pH: 7.4) and the homogenates were centrifuged at 10,000 g at 4°C for 5 min separately. Each supernatant was used for the estimation of TBARS and CD.

For the measurement of TBARS, the homogenate mixture of 0.5 mL was mixed with 0.5 mL of normal saline (0.9% NaCl) and 2 mL of thiorbituric acid- trichloroacetic acid (TBA-TCA) mixture (0.392 g TBA in 75 mL of 0.25 N HCl with 15 g TCA). The volume of the mixture was made up to 100 mL by 0.25 N HCl and boiled at 100°C for 10 min. The mixture was then cooled at room temperature and centrifuged at 4000 g for 10 min. The absorbance of the whole supernatant was measured spectrophotometrically at 535nm [13].

Quantification of the CD was performed by a standard method [13]. The lipids were extracted with chloroform methanol (2:1) followed by centrifugation at 1000 g for 5 min. The chloroform layer was evaporated to dryness under a stream of nitrogen. The lipid residue was dissolved in 1.5 mL of cyclohexane and the absorbance was measured at 233 nm to estimate the amount of hydro peroxide formed.

**Histological study**

Pancreas was dissected out from all the groups and fixed in Bouin’s fixative. These were subjected to paraffin embedding followed by section cutting in microtome and hematoxylin-eosin staining for microscopic examination in accordance with laboratory procedures. Histological and histometric examinations were carried out on stained sections.

**Biochemical estimation of Serum Glutamate Oxaloacetate Transaminase and Serum Glutamate Pyruvate Transaminase**

Serum GOT and GPT activities were measured as metabolic toxicity biomarkers following the instructions of specific supplied kits (Span Diagnostics Ltd., Surat, India). The activities of these enzymes were expressed as relative units [13].

**Acute toxicity study**

To establish safety profile of the said fraction, acute toxicity study was carried out as per established guidelines. Healthy separate adult normoglycaemic wistar albino rats of either sex, starved overnight, were divided into three groups containing six rats each and were orally fed with the ethyl acetate fraction of hydromethanolic extract of E. jambolana in increasing dose levels of 50, 100 and 300 mg/100 gm body weight. The rats were kept under supervision continuously for 2 h for behavioral, neurological and autonomic profiles and after a period of 24 and 72 h for any lethality or death [14].

**Statistical analysis**

All experimental trials were replicated three times. An analysis of variance (ANOVA) followed by multiple comparison two tail “t” test was used to compare in between the groups. Differences were considered significant at p<0.05 [14].

**Analysis of phytoconstituents**

**Chromatographic isolation**

Glass column chromatography [15] with silica gel Si gel (Si gel (100 -200 mesh)) as stationary phase (adsorbent) was applied for isolation of compounds present in the bioactive ethyl acetate fraction. For this purpose 25 g of ethyl acetate fraction was subjected to column chromatography and Chloroform, followed by petrol ether (CHCl3, CHCl3, CHCl3, CHCl3) and finally methanol (CH3OH) were used as eluting solvents with gradient technique. Fractions found similar on TLC (Merck silica gel 60F$_{254}$ plates) were combined together and finally dried under reduced pressure on Lábortoba-4000 at 40°C. Anti-hyperglycaemic activity of different column fractions was carried out at various dose levels (5-200 mg/kg of body weight) and finally the combined fraction No-2 (9.3 gm, w/w) containing two compounds U$_1$ and U$_2$ as main constituents was found to be anti hyperglycaemic in nature.

**Phytochemical analysis**

Phytochemical analysis of these two compounds U$_1$ and U$_2$ were carried out to establish their chemical natures (alkaloids, flavonoids, tannins, saponins, terpinoids, glycocides, volatile oils and phenol or phenolic compounds) following our previous standard qualitative methods [17,18].

**Separation and purification of compound U$_1$ and U$_2$**

Further separation and purification of the isolated compounds were carried out on CombiFlash Companion (Teledyne-Iscio, USA) chromatographic system using Redisep normal phase column (size- 20 g) as stationary phase. For this purpose 5 g of combined column fraction (No-2) was adsorbed on Silica gel (230-400 ASTM Mesh) to obtain a free flowing powder. The free flowing adsorbed powder was then filled in sample cartridge (size-5 gm) and then subjected to chromatographic separation using CHCl$_3$ and MeOH as mobile phase with linear gradient elution (CHCl$_3$, 100-0%; MeOH 0-100%) technique. Flow rate of the mobile phase was maintained at 40 mL/min and detection of the compounds was done at 254 nm. The column eluents were collected in different collection tubes. The identification of pure fraction was further confirmed by TLC (Normal phase and Reverse phase) using various solvent systems as mobile phase. Pure fractions containing U$_1$ were combined together and dried under reduced pressure at 40°C to obtain pure compound (8.9 mg w/w) as pale yellow powder. Similarly pure fractions containing U$_2$ were combined together to obtain pure U$_2$ (3.1 gm w/w) as white crystalline powder. Both the compounds; U$_1$ and U$_2$ were finally subjected to bioactivity study at various dose levels (2 mg, 5 mg, 10 mg and 20 mg/Kg of body weight) to establish their antihyperglycaemic potency.

**UV- Visible Spectroscopy: Determination of λ max**

UV-Visible spectra (for λ max) were recorded using Perkin-Elmer Lambda 35 UV/VIS dual beam spectrophotometer fitted with quartz cells. Both U$_1$ and U$_2$ were separately dissolved in and diluted with HPLC grade methanol to prepare the solutions of 25µg/mL and the compounds were scanned over entire UV range (400 –199 nm) for recording of UV spectra to determine the λ max of the compounds.

**High performance thin layer chromatography (HPTLC) Finger printing**

HPTLC finger printing was also recorded on Merck HPTLC silica gel 60F$_{254}$ plates by Camag LINOMAT5 automatic HPTLC sampler fitted with 100µl Hamilton syringe[19]. HPTLC was performed on 10 cm x 10 cm precoated silica gel G 60F$_{254}$ plates (E. Merck). For this purpose ethyl acetate fraction, U$_1$ and U$_2$ were separately dissolved in and diluted with HPLC grade methanol to prepare the solutions of 0.5 mg/mL. 5 µL of each sample solution was applied to the HPTLC plates by spray-on technique. TLC plates were developed in a Camag twin-through TLC chamber (10 cm x 10 cm) previously saturated with mobile phase, CHCl$_3$:MeOH:H$_2$O (100:13.5:10 v/v) for 30 min. The plates were dried under stream of hot air and then examined in a Camag UV cabinet at λ = 254nm.

**High performance liquid chromatography (HPLC) analysis**

HPLC analysis was performed on Waters alliance HPLC system fitted with 2695 Separation Module. RP-HPLC analysis was carried on Thermo Hypersil BDS C18 (4.6 x 250 mm, 5 µm) column using a premixed solvents Water: ACN: MeOH at 40:30:30 v/v as mobile phase with a flow rate of 1.0 mL/min and isocratic elution technique. The column temperature was maintained at 30°C and detection was performed at 220 nm. For HPLC analysis U$_1$ and U$_2$ were dissolved in and diluted with the mobile phase to prepare the final solution containing 50 µg/mL of each of the compound. The sample solution was then...
sonicated for 30 min and then filtered through Millipore Millex syringe filter unit (0.45 μm). The sample solution (10 μL) was then injected through auto injector. The compounds were scanned over entire UV range on 3D spectral mode and HPLC chromatograms of both the compounds were recorded at 220nm.

RESULTS

Carbohydrate metabolomics

Mean fasting blood glucose level was significantly (p<0.05) elevated with a value of greater than 250 mg/dL in untreated diabetic animals when compared with non-diabetic control rats. Treatment with ethyl acetate fraction or glibenclamide to diabetic animals resulted in significant (p<0.05) recoveries with the control group. The administration of the ethyl acetate fraction or glibenclamide to diabetic animals resulted in significant (p<0.05) recoveries with the control group. After 28 days treatment with non-diabetic control rats. Treatment with ethyl acetate fraction or glibenclamide to diabetic animals for 28 days resulted in a significant (p<0.05) lowering in fasting blood glucose level (Fig.1).

Table 1. Protective efficacy of ethyl acetate fraction of E. jambolana or glibenclamide on serum insulin, glycated hemoglobin and glycogen contents in liver and skeletal muscle in streptozotocin-induced diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum insulin level (mg/ml)</th>
<th>Glycated hemoglobin level (GH%)</th>
<th>Glycogen content (μg/mg of tissue)</th>
<th>Liver</th>
<th>Skeletal muscle</th>
<th>Cardiac muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Control group)</td>
<td>4.11 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.48 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.0 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II (Diabetic group)</td>
<td>1.81 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.85 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.79 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III (Ethyl acetate fraction treated group)</td>
<td>3.13 ± 0.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.14 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.03 ± 0.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group IV (Glibenclamide treated group)</td>
<td>3.26 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.22 ± 0.15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.89 ± 0.39&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM, n=6. ANOVA followed by multiple comparison two tail ‘t’ test. Values with different superscripts (a, b, c, d) differ from each other significantly (p<0.05).
Oxidative stress markers profile assessment
Catalase, peroxidase, superoxide dismutase and glutathione-s-transferase enzyme activities in hepatic, skeletal and cardiac muscle were significantly decreased in diabetic group in respect to control group. After the treatment with this ethyl acetate fraction or glibenclamide to the STZ-treated diabetic rats, the levels of these parameters were restored significantly (p<0.05) towards their control levels (Fig. 3).

Figure 3. Remedial effect of ethyl acetate fraction or glibenclamide on antioxidative enzyme activities and levels of lipid peroxidative end products in hepatic, skeletal and cardiac muscle in streptozotocin-induced diabetic rats. Each bar represents Mean ± SEM, n=6 for each group. ANOVA followed by multiple comparison two tail ‘t’ test. Values of bar diagram with different superscripts (a,b,c,d) differ from each other significantly at the level of p<0.05.
Levels of end products of the lipid peroxidation i.e. conjugated diene and thiobarbituric acid reactive substance in said tissues were increased significantly (p<0.05) in diabetic group when compared to the control group. There was a significant (p<0.05) recovery in the levels of the above parameters in target tissues after treatment with ethyl acetate fraction or glibenclamide to the diabetic group animals (Fig. 3).

Histology and histometric study

Diameter of pancreatic islets as well as count of islets were significantly decreased (p<0.05) in streptozotocin induced diabetic group in respect to the control group. The values of these parameters were significantly restored towards the control group after ethyl acetate fraction or glibenclamide treatment in diabetic rat (Table 3, Fig. 4).

Table 3. Effect of ethyl acetate fraction of hydromethanolic extract of E. jambolana or glibenclamide on islet number and islet diameter in streptozotocin-induced diabetic rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Islet number (count per field at 100X magnification)</th>
<th>Islet diameter (micron)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Control group)</td>
<td>19.9 ± 3.1 a</td>
<td>149.3 ± 4.06 b</td>
</tr>
<tr>
<td>Group II (Diabetic group)</td>
<td>7.2 ± 2.1 b</td>
<td>56 ± 0.93 c</td>
</tr>
<tr>
<td>Group III (Ethyl acetate fraction treated group)</td>
<td>11.3 ± 2.5 b</td>
<td>128.6 ± 3.31 c</td>
</tr>
<tr>
<td>Group IV (Glibenclamide treated group)</td>
<td>13.4 ± 2.9 a</td>
<td>118 ± 2.38 b</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM, n=6. ANOVA followed by multiple comparison two tail ‘t’ test. Values with different superscripts (a, b, c, d) differ from each other significantly (p<0.05).

Analysis of phytoconstituents

Qualitative screening of phytochemicals

Following the addition of ferric chloride (2 mg) to each of the solutions of isolated compounds U₁ and U₂ (1mg/10 ml water) turned into bluish black colour confirmed that the isolated compounds were chemically gallic acid in nature.

UV-Visible Spectroscopy: Determination of λ max

UV-Visible spectra (for λ max) were recorded for both U₁ and U₂ separately. Compounds were scanned over entire UV range (400 –199 nm) for recording of UV spectra to determine the λ max of the compounds (Fig. 5).

Figure 4. Histology of pancreas, 400 X (Haematoxylin-Eosin Stain)

Toxicity assessment

Body weight of the diabetic animals was decreased significantly in comparison with the animals of control group. Ethyl acetate fraction or glibenclamide treatment to the diabetic rat for 28 days resulted in a significant (p<0.05) recovery of this parameter (Table 2).

Activities of serum GOT and GPT were significantly (p<0.05) increased in diabetic group compared to the control group. A significant (p<0.05) attenuation of the enzyme activities towards the control level were found after treatment with ethyl acetate fraction or glibenclamide (Table 2).

Table 2. Rectification in body weight and neutralization of elevated toxicity biomarkers serum GOT and GPT activities towards the control levels after treatment with ethyl acetate fraction of hydromethanolic extract of E. jambolana or glibenclamide in streptozotocin-induced diabetic rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body Weight (gm)</th>
<th>SGPT (IU/L)</th>
<th>SGOT (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Control group)</td>
<td>124 ± 1.5 a</td>
<td>130 ± 2.4 b</td>
<td>35 ± 1.46 c</td>
</tr>
<tr>
<td>Group II (Diabetic group)</td>
<td>120 ± 1.1 a</td>
<td>115 ± 1.4 b</td>
<td>55 ± 2.50 c</td>
</tr>
<tr>
<td>Group III (Ethyl acetate fraction treated group)</td>
<td>121 ± 2.1 b</td>
<td>128 ± 1.9 b</td>
<td>53 ± 2.33 c</td>
</tr>
<tr>
<td>Group IV (Glibenclamide treated group)</td>
<td>122 ± 2.3 c</td>
<td>129 ± 2.2 b</td>
<td>58 ± 2.11 c</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM, n=6. ANOVA followed by multiple comparison two tail ‘t’ test. Values with different superscripts (a, b, c, d) differ from each other significantly (p<0.05).

Ethyl acetate fraction of hydromethanolic extract of E. jambolana at its maximum dose level of 300 mg/100 gm of body weight did not produce any significant changes in the autonomic, behavioral or neurological alteration. Acute toxicity study revealed the non-toxic nature of the ethyl acetate fraction of hydromethanolic extract of E. jambolana at the applied dose.
HPLC analysis
The reverse phase HPLC chromatogram of the sample at λmax 220 nm has been shown in figure with two well resolved peaks at 2.041 min and 3.104 min. Therefore pure two compounds were separated (Fig. 7).

DISCUSSION
Streptozotocin causes selective destruction of insulin secreting pancreatic β-cells by reactive oxygen species dependent oxidative damage [10] resulting insulin dependent diabetes mellitus, supported here by histological and histometric studies of pancreatic histoarchitecture.

Present findings, the elevation in levels of fasting blood glucose and glycated hemoglobin along with diminution in liver and skeletal muscle glycogen levels in streptozotocin induced diabetic rat due to low levels of serum insulin are parallel with our previous reports [19, 27].

The streptozotocin induced hyperglycaemic state supported here by the diminution in the activities of principal carbohydrate metabolic enzymes hexokinase and glucose-6-phosphodehydrogenase in liver, skeletal and cardiac tissues as these enzymes are under the positive regulation of insulin [19]. The activities of glucose-6-phosphatase, lactate dehydrogenase enzymes, which were increased in above tissues in diabetes are also in same line as these are under negative control of insulin [24].

The interdependent association between diabetes mellitus and oxidative stress has been established here from the diminished activities of free radical scavenging enzymes viz. catalase, peroxidase, superoxide dismutase and glutathione-s-transferase along with the elevated levels of free radicals peroxidative end products TBARS and CD in hepatic, skeletal and cardiac tissues may be due to low serum insulin [20] that resembles the findings of our previous studies [14, 24]. In diabetic state the oxidative stress in tissues may also develop due to high level of uncoupler protein synthesis in hypnotic state as diabetes results glycated hemoglobin formation that interfere oxygen delivery at target tissues [22].

The antidiabetic activity of ethyl acetate fraction of hydromethanolic extract of E. jambolana was established through this in-vivo study. The altered homeostasis in fasting blood glucose, glycated hemoglobin and hepato-skeletal glycogen levels were restored after this fraction treatment by elevated synthesis and secretion of serum insulin due to insulinotropic activity recognized from the pancreatic histoarchitectural study. This finding was parallel to the remedial activity of glibenclamide treatment and supported by our previous study [22].

The ethyl acetate fraction established its antioxidative activity by putrefaction of lipid peroxidative end products and promoting the free radical scavenging enzyme activities evaluated from diminished TBARS and CD levels and elevated activities of CAT, Px, SOD, GST enzymes after 28 days treatment with this fraction. One more possible remedial activity of this fraction was the insulin dependent inhibition in glycation of hemoglobin that resulted in augmented oxygen delivery at target tissues with reduced oxidative stress development [21].

In the entire experiment, ethyl acetate fraction established its potency against diabetes and oxidative stress may be due to its phytoingredient viz. gallic acid compounds present in it as they have major antioxidative activity with redox properties, adsorption and neutralization capacity to free radicals, potency to extinguish singlet and triplet oxygen and scavenging of peroxides [15, 27, 28]. From previous study higher positive antioxidative efficacy of this phytochemical has been established [16, 27, 29].

This fraction has no toxicity which has been indicated here from the improved body weight as well as correction in serum GOT and GPT activities seems to be its ability to enhance glucose utilization and reduce hepato-renal dysfunction as these are the indicators of general and metabolic toxicity [3, 23]. In respect to maximum non-fatal doses studied revealed the non-toxic nature of this fraction of this plant. There was no lethality or any toxic reactions found at any of these doses selected until the end of the study period. According to toxicity classification [11], this ethyl acetate fraction of hydro methanol extract of E. jambolana is non toxic.

The structural deriveritization and final identification of the isolated two compounds U1 and U2 purified from the bioactive ethyl acetate fraction by HPTLC fingerprinting and reversed phase HPLC analysis is necessary for antihyperglycaemic and antioxidative drug development.

ACKNOWLEDGEMENT
The Financial support from Department of Science & Technology, Govt. of India (Project No.-SR/SO/HS-88/2006) to conduct this project work is gratefully acknowledged.

REFERENCES


