Antidiabetic and Antioxidative activity of Ethyl acetate Fraction of Hydromethanolic Extract of Seed of *Eugenia jambolana* Linn Through *In-Vivo* and *In-Vitro* Study and its Chromatographic Purification

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ABSTRACT

Introduction: *Eugenia jambolana* of Myrtaceae family is widely distributed and used as diabetic therapeutic traditional medicine in rural India. Antidiabetic potentiality of ethyl acetate fraction of hydromethanolic (40:60) extract of seed of *E. jambolana* was investigated following *in-vivo* models in experimental diabetic rat and antioxidative efficacy following *in-vitro* models. **Materials and Methods:** Alteration in carbohydrate metabolism during hyperglycaemia was assessed by increased fasting blood glucose, glycated hemoglobin levels along with diminished body weight, level of serum insulin and glycogen contents in liver, skeletal and cardiac tissues of diabetic rat. *In-vitro* antioxidative potentiality was measured by inhibitory activity of lipid peroxidation, scavenging activity against hydrogen peroxide, nitric oxide, hydroxyl, ABTS and DPPH radicals along with preliminary phytochemical analysis. Bioactive phytoingredients were isolated from ethyl acetate fraction through column chromatography, HPTLC fingerprinting and RP-HPLC analysis. **Results:** Oral administration of 20 mg ethyl acetate fraction or 0.6 mg glibenclamide 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 carbohydrate metabolomics towards the control levels. This fraction established its primary antioxidant attribute by scavenging hydroxyl, nitric oxide, hydrogen peroxide, ABTS and DPPH radicals along with the inhibition of lipid peroxidation with IC_{50} values 26.49,28.14,18.45,13.65,10.46, 28.88 µg/mL respectively. Phytochemical screening confirmed isolated compounds were chemically gallic acid in nature. Two separate spots of ethyl acetate fraction were recorded after scanning of HPTLC fingerprinting. RP-HPLC study also shows two completely resolved peaks. Its biosafety profile was established following guidelines. **Conclusion:** On the basis of experimental studies ethyl acetate fraction of *E. jambolana* proved its antihyperglycemic and antioxidant nature.

Keywords: antihyperglycemic agent; *Eugenia jambolana*; hplc; hptlc; *in-vitro* study

INTRODUCTION

Diabetes mellitus caused due to endocrine disorders in insulin secretion and/or insulin action both, characterized with chronic hyperglycemic state that results chronic metabolic impairment in carbohydrate, lipid and protein homeostasis.[1] Oxidation process, a principal way for producing free radicals in living systems is enhanced by high glucose concentration induced oxidative stress mediated progression of cellular destruction has been proved from previous studies.[2] The oxidative stress induction in diabetic state may be due to high level of uncoupler protein synthesis in tissues.[3]

Principal free radicals or reactive oxygen species (ROS) produced due to biochemical reactions or by leakage from respiratory chain those cross membranes and diffuse away from the site of generation and induce lipid peroxidation mediated cellular destruction by loss of membrane fluidity,

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DNA breakage or destruction of enzymes or receptor activity.[4] Antioxidant with its low concentration significantly delays or reduces oxidation process by reacting with free radicals and protects cellular destruction.[5] Diseases linked with hyperglycaemia induced oxidative stress medicated free radicals generation can be prevented by antidiabetic and antioxidative therapy.[6]

Despite progress in the management of diabetes by synthetic drug and oxidative stress using synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), Rutin, tertiary butylated hydroquinon and gallic acid esters are less effective and suspected to cause health hazard.[7] So, current researches are directed towards improved, safe and natural antidiabetic and antioxidative plant products as widespread traditional medical treatment,[8] applied either in extract or fraction form for treatment.[9] It is also recommended by World Health Organization.[10] In rural sectors of India, various parts of *E. jambolana* (Family-Myrtaceae) are being used for treatment and management of diabetes related disorders. Its medicinal values have been published earlier by others[11,12,13] and by us.[14,15,16] The present study was designed to investigate the antihyperglycaemic and antioxidative potentiality of ethyl acetate fraction of hydromethanolic (40:60) extract of *Eugenia jambolana* through *in-vivo* and *in-vitro* models along with isolation of active plant ingredients through chromatographic purification.

MATERIALS AND METHODS

Plant material preparation

Fresh seeds of *E. jambolana* were collected from rural areas of Paschim Medinipore District, West Bengal, India in the month of May-July. Preliminary identification of the plant was made by a taxonomist and a voucher specimen (HPCH 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 ^{0}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 (H_2O: 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 filtrate was evaporated under reduced pressure (10 to 200 mbar) using rotavapour instrument (HAHN-SHIN HS-2000NS, Korea) at 38 °C for complete removal of methanol. Finally, plain aqueous filtrate was lyophilized on benchtop K Lyophilizer 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* was dissolved with 2 L hydromethanolic (H_2O: MeOH :: 40:60) 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 studies 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 *in-vivo* experiments.

Chemicals

Streptozotocin (STZ) was obtained from Sigma, USA. Organic solvents of analytical grade like n-hexane, chloroform, ethyl acetate and methanol were purchased from Himedia, Mumbai, India. 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, Bodford, USA) was used in analysis. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and other chemicals like Butylated hydroxy anisole (BHA), N-(1-naphthyl) ethylene diamine dihydrochloride (NED), sodium nitroprusside (SNP), trichloroacetic acid (TCA), thiobarbituric acid (TBA) , 2-deoxy-2-ribose, Aluminium chloride ($AlCl_3$), Sodium nitrite ($NaNO_2$), Sodium hydroxide (NaOH), Potassium persulphate ($K_2S_2O_8$), quercetin were purchased from Sigma Chemical Company Inc., St. Louis, USA and Sisco Research Laboratory (SRL), India.

In-Vivo study for antihyperglycaemic activity assessment

Selection of animal and animal care

Normoglycaemic (Fasting Blood Glucose level 70-80 mg/dL) wistar male albino rats having body weight about 120 ± 10 g

were used in this experiment. 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[17] and instruction given by our "Institutional Ethical Committee" (VU/IAEC/BioMed/08/2008-2009) were followed throughout the experiment.

Animal grouping and treatment modules

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.[15] 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 350 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/0.l mL citrate buffer/100 g body weight/rat.

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

Group IV (Glibenclamide treated group) diabetic rats were fed with standard drug glibenclamide by gavage at a dose of 0.6 mg/0.5 mL distilled water /100 g body weight/rat for twice a day at fasting state on and from 7th day for 28 days.

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

Every day, the first oral dose of the above mentioned fraction was given 1 hour before supply of animal feeds in the morning (at 8:00 am) and a second oral dose was administered 2 hours after cleaning the feed box in the afternoon (at 5:00 pm). Feeds were supplied again to the animals 1 h after the second oral administration of the fraction.

From the day of fraction or glibenclamide treatment to rats of *Group III or Group IV*, fasting blood glucose level in all the groups was monitored using single touch glucometer (Ascensia ENTRUST glucometer, Bayer, Germany) in every two days interval. On the 29th day of the experiment (considering the day of treatment of ethyl acetate fraction or glibenclamide as the 1st day), all the animals were sacrificed by decapitation after recording the final body weight and organs wet weight. Blood was collected from dorsal aorta by syringe and the serum was separated by centrifugation at 3000 g for 5 min for insulin and metabolic toxicity parameters assessment. Packed cell pellet was used for glycated hemoglobin (HbA_1c) measurement.

Hyperglycaemic profile measurement

Fasting blood glucose (FBG) level was measured using the single touch glucometer by collecting blood from tip of the tail of all experimental and control animals in all groups at the initial time of experiment and every two days interval throughout the experiment.[15] Serum insulin level was measured using solid phase-conjugated sandwich ELISA kit for rat (EZRMI-13K, Millipore, USA).[18] Glycated hemoglobin (HbA_1c) level along with hepatic and skeletal muscle glycogen contents were measured following standard protocol.[15] The activities of principal carbohydrate metabolic enzymes viz. hexokinase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and lactate dehydrogenase were assayed following the established methods of our laboratory.[19]

Metabolic and acute toxicity studies

Serum Glutamate Oxaloacetate Transaminase and Glutamate Pyruvate Transaminase activities were measured as metabolic toxicity bio-markers following the instructions of specific supplied kits (Span Diagnostics Ltd., Surat, India). The activities of these enzymes were expressed as relative units.[15]

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.[20]

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 $p < 0.05$.[15]

In-Vitro study for antioxidative activity evaluation

Scavenging activity against hydrogen peroxide radical, nitric oxide radical, hydroxyl radical, lipid peroxidation inhibitory activity along with $ABTS^{\cdot+}$ radical and $DPPH^{\cdot}$ radical scavenging activities in comparison to BHA and Rutin standards were measured following our published standard methods.[21,22] All tests were performed in triplicate manner. % of Inhibition activity was computed by comparing absorbance of the control with the sample using the equation,

$(A_0\text{-}A_1)/A_0 \times 100$. Where, A_0 = absorbance of control and A_1 = absorbance of sample. IC_{50} values were calculated using STATISTICA 6.0 software.

Chromatographic isolation

Glass column chromatography[23] with silica 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 gm of ethyl acetate fraction was subjected to column chromatography and Chloroform, Ethyl acetate as well as Methanol ($CHCl_3$, EtOAc, MeOH) was 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 Laborota-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 were found to be anti hyperglycaemic in nature.

Separation and purification of compound U_1 and U_2

Further separation and purification of the isolated compounds was carried out on CombiFlash Companion (Teledyne-Isco, USA) chromatographic system using Redisep normal phase column (size- 20 gm) as stationary phase. For this purpose 4 gm 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 U_1 (810 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 antihyperglycemic potency.

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, glycosides, volatile oils and phenol or phenolic compounds) following our previous standard qualitative methods.[21-24]

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) fingerprinting

HPTLC finger printing was also recorded on Merck HPTLC silica gel G $60F_{254}$ plates by Camag LINOMAT5 automatic HPLTC sampler fitted with 100 µl Hamilton syringe.[14] HPTLC was performed on 10 cm × 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.[22] TLC plates were developed in a Camag twin-through TLC chamber (10 cm × 10 cm) previously saturated with mobile phase (EtOAc:MeOH:H_2O :: 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 × 250 mm, 5 µm) column using a premixed solvents (Water: ACN: MeOH :: 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 220 nm.

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 for 28 days resulted in a significant ($p<0.05$) lowering in fasting blood glucose level (Figure 1).

A significant ($p<0.05$) decrease in the levels of serum insulin, hepatic and skeletal muscle glycogen content along with higher value of glycated hemoglobin level were found in the untreated diabetic group when compared with the control group. The administration of the ethyl acetate fraction or glibenclamide to diabetic animals resulted in significant ($p<0.05$) recoveries in the above said parameters towards their respective control levels (Table 1).

Hepatic, skeletal and cardiac muscle hexokinase and glucose-6-phosphate dehydrogenase enzymes activities were found deviated significantly ($p<0.05$) in a downward manner accompanied by significant ($p<0.05$) increase in glucose-6-phosphatase and lactate dehydrogenase activities in STZ induced diabetic group when compared to the control

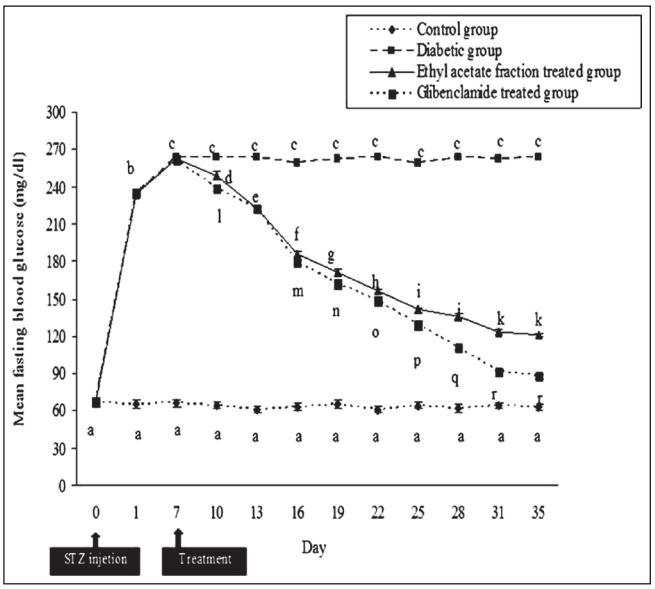


Figure 1: Correction in the mean fasting blood glucose level after treatment with ethyl acetate fraction of *E. jambolana* or glibenclamide in streptozotocin-induced diabetic rats. Each line represents Mean ± SEM, n=6 for each group. ANOVA followed by multiple comparison two tail 't' test. Values of line diagram with different superscripts (a,b,c,d, e,f,g,h,i,j,k,l,m,n,o,p,q,r) differ from each other significantly at the level of $p<0.05$.

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

Group	Glycated hemoglobin level (GHb%)	Glycogen content (µg of glucose/mg of tissue)		Serum insulin level (ng/mL)
		Liver	Muscle	
Group I (Control group)	2.48 ± 0.12[a]	13.0 ± 0.57[a]	7.14 ± 0.33[a]	4.11 ± 0.14[a]
Group II (Diabetic group)	4.85 ± 0.33[b]	4.79 ± 0.21[b]	2.81 ± 0.13[b]	1.81 ± 0.04[b]
Group III (Ethyl acetate fraction treated group)	3.14 ± 0.14[c]	6.03 ± 0.32[c]	4.27 ± 0.19[c]	3.13 ± 0.10[c]
Group IV (Glibenclamide treated group)	3.22 ± 0.15[c]	6.89 ± 0.39[d]	4.71 ± 0.24[c]	3.26 ± 0.10[c]

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 ($p<0.05$).

group. After 28 days treatment with ethyl acetate fraction or glibenclamide to diabetic rats significant ($p<0.05$) revitalization was observed in the activities of these above enzymes (Figure 2).

Toxicity assessment

Body weight of the diabetic animals was decreased significantly ($p<0.05$) in comparison with the control group.

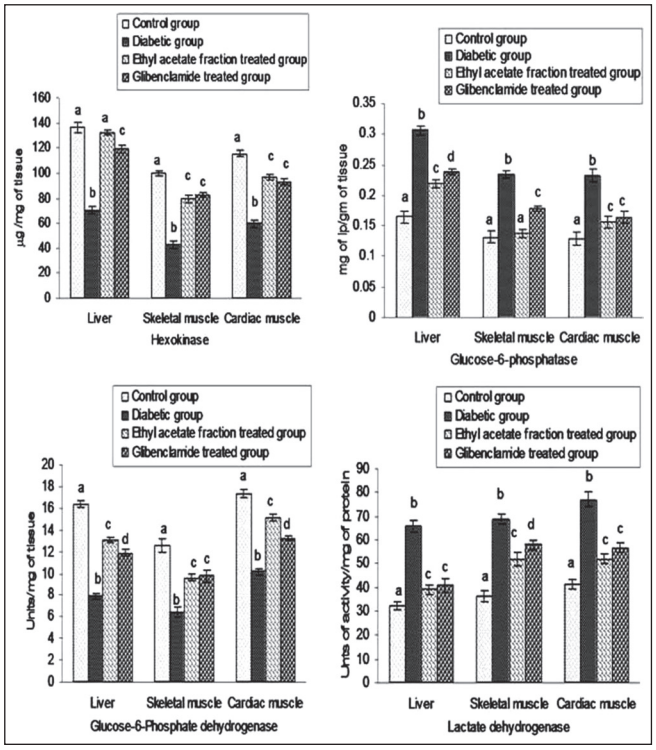


Figure 2: Hyperglycaemia induced alteration in the activities of carbohydrate metabolic enzymes (hexokinase, glucose-6-phosphatase glucose-6-phosphate dehydrogenase and lactate dehydrogenase) in liver, skeletal and cardiac tissues and its correction after treatment with ethyl acetate fraction or glibenclamide treatment 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 $p<0.05$.

Ethyl acetate fraction or glibenclamide treatment to the diabetic rat for 28 days resulted in a significant recovery of this parameter (Table 2).

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

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, behavioural or neurological alteration. Acute toxicity studies revealed the non-toxic nature of the ethyl acetate fraction of hydromethanolic extract of *E. jambolana*.

In-Vitro anti oxidative potentiality evaluation

For scavenging of hydroxyl radicals the ethyl acetate fraction showed the inhibitory activity with an IC_{50} value of 26.49 µg/mL. It has potent neutralization capacity against the nitric oxide and hydrogen peroxide with IC_{50} value of 28.14 µg/mL and 18.45 µg/mL. The inhibitory capacity of the ethyl acetate fraction against lipid peroxidation was effective with IC_{50} value of 28.88 µg/mL. All the above assessments were compared with their respective standard (BHA) having the IC_{50} values of 22.29 µg/mL, 25.53 µg/mL, 26.41 µg/mL and 29.19 µg/mL respectively (Figure 3).

Ethyl acetate fraction was effective scavenger of the $ABTS^{\cdot+}$ radical and this activity was compared with rutin as standard. For *E. jambolana* and rutin IC_{50} values were 13.65 µg/mL and 1.27 µg/mL respectively. From the dose-response curve for $DPPH^{\cdot}$ radical scavenging activity of ethyl acetate fraction or rutin the IC_{50} values obtained were 10.46 µg/mL and 5.35 µg/mL respectively (Figure 3).

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

Groups	Body Weight (gm)		SGPT (IU/L)	SGOT (IU/L)
	Initial	Final		
Group I (Control group)	124 ± 1.5^{a}	130 ± 2.4^{b}	35 ± 1.46^{a}	16 ± 1.57^{a}
Group II (Diabetic group)	120 ± 1.1^{a}	113 ± 1.4^{c}	117 ± 4.77^{b}	55 ± 2.50^{b}
Group III (Ethyl acetate fraction treated group)	121 ± 2.1^{a}	128 ± 1.9^{b}	53.2 ± 2.33^{c}	22 ± 1.52^{c}
Group IV (Glibenclamide treated group)	122 ± 2.3^{a}	129 ± 2.2^{b}	58.1 ± 2.11^{d}	27 ± 1.29^{c}

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 ($p<0.05$).

Qualitative screening of phytochemicals

Following the addition of ferric chloride (2 mg) to each of the solutions of isolated compounds U_1 and U_2 (1 mg/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 U_1 and U_2 were separately compounds were scanned over entire UV range (400–199 nm) for recording of UV spectra to determine the λ max of the compounds (Figure 4).

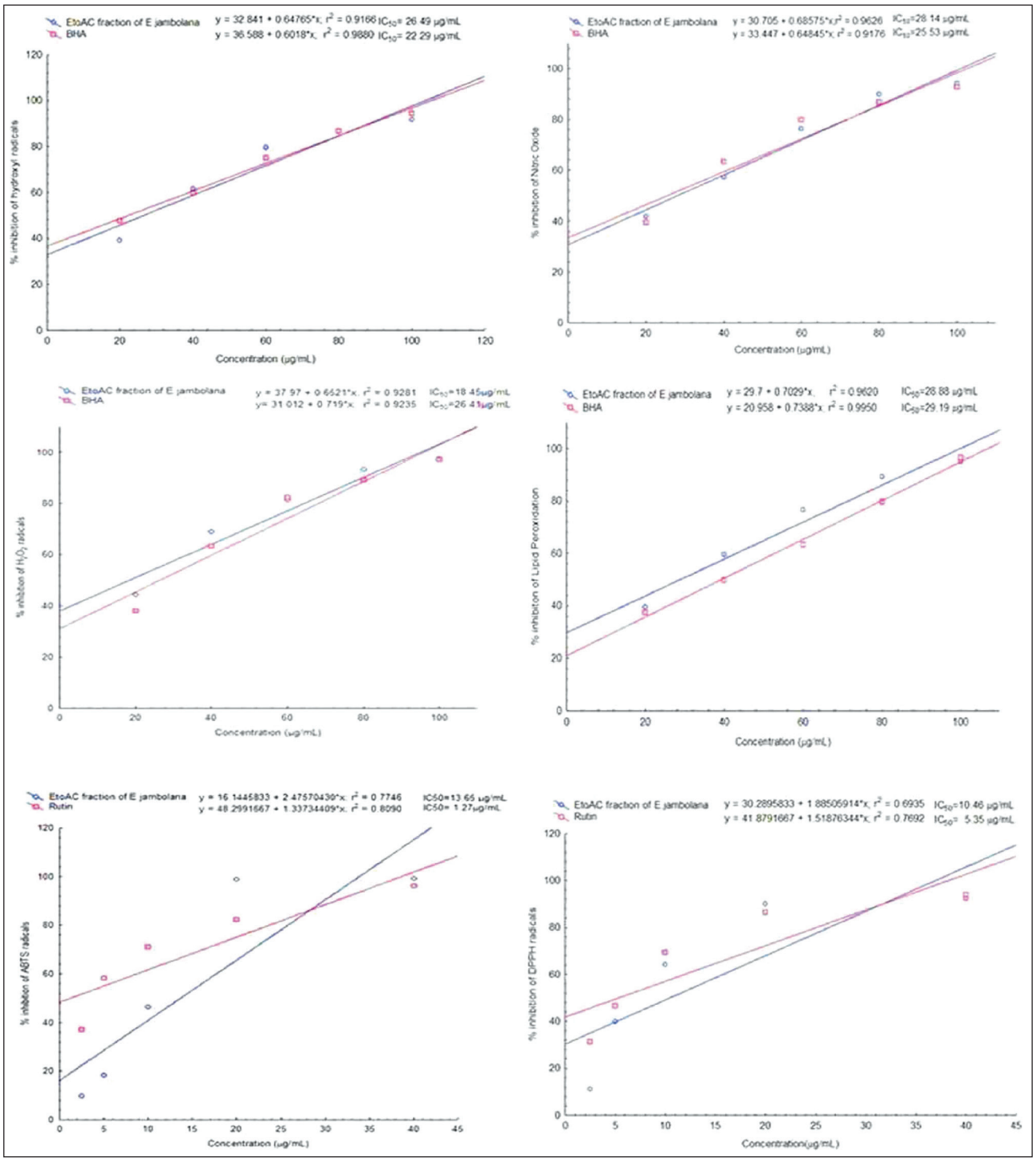


Figure 3: Evaluation of scavenging activities of ethyl acetate fraction of *E. jambolana* or standard antioxidant against hydroxyl radical, hydrogen peroxide radical, nitric oxide radicals production and lipid peroxidation inhibitory potentiality along with ABTS and DPPH radicals scavenging activities through *in vitro* study model.

HPTLC fingerprinting

After development of TLC plates it was found that the bioactive ethyl acetate fraction contains three different compounds among which two bands resemble with bands of U_1 (R_f value 0.83) and U_2 (R_f value 0.54) (Figure 5).

HPLC analysis

The reversed phase HPLC chromatogram of the sample at λ_{max} 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 (Figure 6).

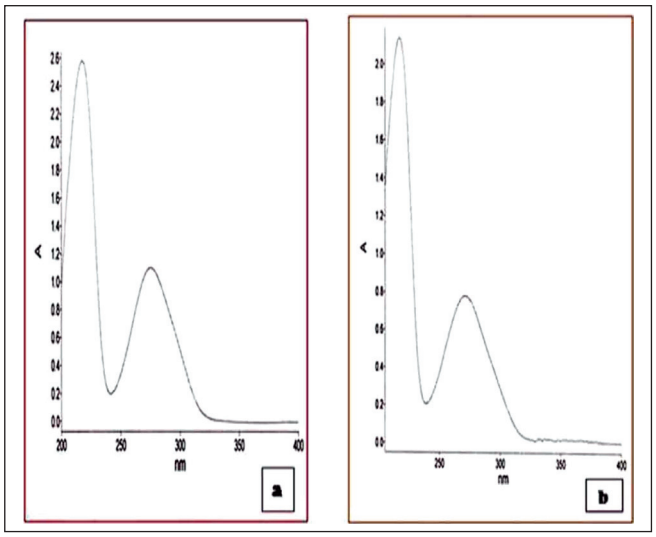


Figure 4: UV-Visible spectroscopy (400–199 nm) for determination of λ_{max} of the compounds U_1 and U_2
a. UV Spectrum of compound U_1
b. UV Spectrum of compound U_2

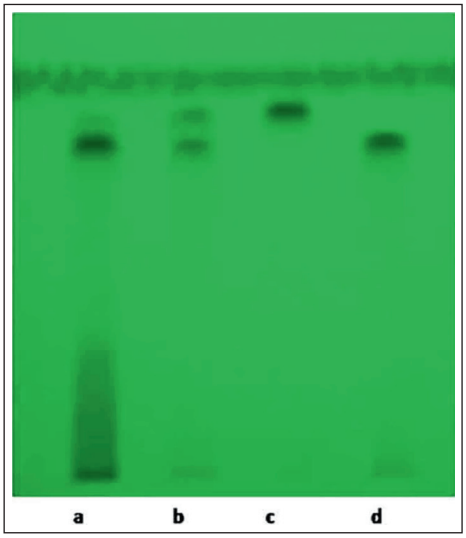
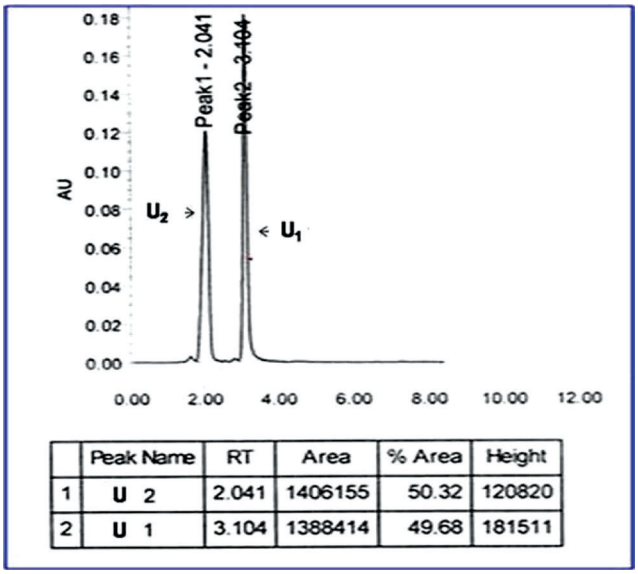


Figure 5: HPTLC finger printing of ethyl acetate fraction of *E. jambolana* L. with isolated compounds U_1 and U_2
a. Ethyl acetate fraction with separate bands of three different compounds
b. Isolated compound U_1 and U_2.
c. Isolated compound U_1.
d. Isolated compound U_2.



	Peak Name	RT	Area	% Area	Height
1	U 2	2.041	1406155	50.32	120820
2	U 1	3.104	1388414	49.68	181511

Figure 6: RP-HPLC Chromatogram (at 220 nm) of bioactive ethyl acetate sub fraction of hydromethanolic extract of *E. jambolana* shows two well resolved peaks of the isolated compounds U_1 and U_2

DISCUSSION

Insulin dependent diabetes mellitus study model has been developed here to explore the anti hyperglycaemic activity of ethyl acetate fraction of hydromethanolic extract of *E. jambolana* as streptozotocin causes selective destruction of insulin secreting pancreatic β-cells through reactive oxygen species dependent oxidative damage[25] supported here by diminished serum insulin level.

The insulin dependent alteration in the carbohydrate metabolism results 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. Present findings bear a resemblance with our previous reports.[15,19]

The altered carbohydrate metabolism in streptozotocin induced hyperglycaemic state is associated with the divergence in key carbohydrate metabolic enzyme activities. Diminished hexokinase and glucose-6-phosphodehydrogenase enzyme activities in liver, skeletal and cardiac muscles are positively regulated by insulin.[26] 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.[15]

The *in-vitro* findings indicated that this ethyl acetate fraction impaired formation of hydroxyl radical, the major reactive oxygen species that causes damage to DNA, lipids and proteins in a cascading process of molecular oxygen reduction.[27] This fraction also inhibits stable nitrite compound production from unstable nitric oxide which is associated with diseases like diabetes by direct competition with oxygen.[21] Hydrogen peroxide, an oxidizing agent, inactivates enzymes by means of essential thiol groups oxidation that leads to oxidative stress development[28] is also inhibited by this fraction. Increased lipid peroxidation is one of the characteristic features of chronic diabetes, which impairs cellular function by decreasing the activity of enzymes and receptors.[29] Ethyl acetate fraction established its attribute of antioxidant with scavenging activity for the protonated radical $ABTS^{\cdot+}$. The proton-donating ability of this fraction was evaluated through $DPPH^{\cdot}$ Assay, one of the antioxidant activities as it measures hydrogen atom donating capability of plant fraction and by means free radical scavenging.[6] So, their inhibition is an important approach for the management of oxidative stress induced ailment.

In the entire experiment, ethyl acetate fraction established its potency against diabetes and oxidative stress may be due to its phytoingredients 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.[25,30,31] From previous study higher positive antioxidative efficacy of this phytochemical has been established.[32]

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.[15,26] 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[20] this ethyl acetate fraction of hydro methanol extract of *E. jambolana* is non toxic.

The structural derivetization and final identification of isolated two compounds U_1 and U_2 from the bioactive ethyl acetate fraction by HPTLC fingerprinting and reversed phase HPLC analysis is necessary for antihyperglycaemic and antioxidative drug development.

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ABBREVIATIONS

$ABTS^{+}$ / ABTS	:	2,2`-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
$DPPH^{\cdot}$ / DPPH	:	1,1-diphenyl-2-picrylhydrazyl
$K_2S_2O_8$	:	Potassium persulphate
NaOH	:	Sodium hydroxide
$NaNO_2$	:	Sodium nitrite
$AlCl_3$	:	Aluminium chloride
TBA	:	Thiobarbituric acid
TCA	:	Trichloroacetic acid
SNP	:	Sodium nitroprusside
NED	:	N-(1-naphthyl) ethylene diamine dihydrochloride
BHA	:	Butylated hydroxy anisole
FBG	:	Fasting blood glucose
HbA_1c	:	Glycated hemoglobin
ELISA	:	Enzyme-linked immunosorbent assay
HPTLC	:	High Performance Thin Layer Chromatography
RP-HPLC	:	Reverse Phase High Performance Liquid Chromatography
SGPT	:	Serum glutamic pyruvate transaminase
SGOT	:	Serum glutamic oxaloacetic transaminase
E. jambolana	:	*Eugenia jambolana*