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ORIGINAL ARTICLE

Experimental Validation of Antidiabetic Potential of *Cayratia trifolia* (L.) Domin: An Indigenous Medicinal Plant

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Abstract The present study was undertaken to evaluate antidiabetic and antioxidant activities of *Cayratia trifolia* root extract against streptozotocin induced diabetes in experimental rats to scientifically validate its use against diabetes in some parts of India. Ethanolic extract, showing the highest activity in in vitro experiments, was prepared in saline and administered orally to streptozotocin induced albino Wistar diabetic rats for 21 days. Biochemical parameters liver and muscles glycogen and in vivo antioxidant activity in normal, diabetic control, standard (metformin) and treated animals were determined and compared. Attempt was made to isolate, purify and characterize one of the major secondary metabolites in extract by range of chromatographic and spectroscopic techniques. Treatment of streptozotocin induced diabetic rats with ethanolic root extract (500 mg/kg) caused significant ($P < 0.01$) reduction in blood glucose (312–178 mg/dL), increase in body weight (181–219 g) and serum insulin (1.28–2.26 IU/dL). It also maintained lipid profile and tests of liver and kidney functions within normal range as compared to diabetic control rats and almost *at par* with standard drug metformin. The oxidative stress induced decline in glutathione and catalase in liver and kidney tissues showed recovery nearly to normal level as a function of treatment. The GC–MS profile of the extract showed relatively high concentration of β-sitosterol which was characterized by different spectroscopic and chromatographic techniques. The result scientifically and comprehensively validate the reported use of roots of this indigenous plant against diabetes. A strong antioxidant activity of the ethanolic root extract suitably compliments the antidiabetic effect.

Keywords *Cayratia trifolia* · Hypoglycemic · Metformin · Reactive oxygen species · Catalase · Diabetes · β Sitosterol

Introduction

Diabetes mellitus is a group of syndromes characterized by disturbance in the metabolism of carbohydrate, fats and proteins. Globally, there is increase in number of people suffering from diabetes mellitus in all age groups, from estimated 2.8 % (170 million) in 2000 to 4.4 % (366 million) up to year 2030 [1, 2]. It is also estimated that diabetes causes about 5 % of all deaths globally every year [3, 4]. Diabetes is classified as insulin dependent (type 1), due to reduced insulin secretion by pancreatic β-cells and, insulin independent (type 2), due to low biological activity of the secreted insulin [5]. The main defect in type 2 diabetes is that cells becomes insulin resistant and less responsive to it, which leads to impairment in insulin signaling pathway and failure in glucose uptake in target tissues like muscles and fats [6]. Currently available therapies to treat diabetes include insulin and various oral antidiabetic agents such as sulfonylureas, biguanides and glinides. Despite considerable progress in treatment of diabetes by oral hypoglycemic agents, there is a need of new drugs because existing synthetic drugs have several limitations and harmful effects [7, 8]. It has been reported that treatment with plant extract resulted in activation of β-cells, regranulation and insulinogenic effects [9, 10]. In view of adverse effects of synthetic drugs and considering the natural medicine as low cost, safer and effective, there is an

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enhanced focus on exploring or scientifically validating indigenous medicinal plants with antidiabetic potential [11, 12]. Hypoglycemic effects of several plants used to treat diabetes is already known, and the mechanisms of hypoglycemic activity of these plants are also being studied [13, 14].

Cayratia trifolia (*Vitaceae*), known as fox grape in English, a perennial climber having trifoliate leaves (2–3 cm), is native to India, Asia and Australia. Various parts of the plant have a range of medicinal properties and roots are used against diabetes in some parts of India. The paper describes a comprehensive study on in vivo antidiabetic and antioxidative potential of root extract of this plant. Attempt has also been made to characterize a major secondary metabolite β-sitosterol which in association of others, might be responsible for the observed effect.

Materials and Methods

Collection of Plant Material

The roots of *C. trifolia* were collected from Eklagna village near Jalgaon [21°00′53.5 N, 075°28′41.3E (elevation: 198 m)] Maharashtra during the month of January 2014. The plant species was identified and authenticated by Dr. J Jayanthi from Botanical Survey of India, Pune and a specimen voucher number *MSMI-II* was deposited in the School of Life Sciences, North Maharashtra University, Jalgaon.

Preparation of Extract

Extract for the study were prepared from washed, shade dried and powdered root of *C. trifolia*. It was put into a thimble made of cellulose filter paper and extracted with hexane (69 °C), ethyl acetate (48 °C) and ethanol (78 °C; 30 g per 300 mL of each separately) in Soxhlet apparatus for 8 h. The extracts were then concentrated to dryness in a rotary vacuum evaporator (R-215, Buchi, Switzerland) under reduced pressure. The dried residues were collected and stored desiccated at 4 °C until further use.

Chemicals

Streptozotocin (STZ) was purchased from Hi-media, India. Total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoproteins (LDL), very low lipoprotein (VLDL), bilirubin, aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), serum urea, uric acid, serum creatinine were analyzed using standard kits from Erba Diagnostic,

(Mannheim GmbH, Germany) by an auto analyzer (Erba Mannheim, Chem-5 plus V2, Germany).

In Vitro Antidiabetic Activity

This was evaluated by (1) α Amylase inhibition assay as per modified method of Bernfeld [15], (2) estimating degree of non-enzymatic glycosylation of hemoglobin as per Daksha et al. [16], with alpha tocopherol as standard and (3) estimating glucose uptake by yeast cells as described by Harish et al. [17] with metronidazole as standard.

Experimental Animals

Healthy albino Wistar rats, 8 weeks old of either sex weighing between 180 and 200 g, were purchased from National Bio Sciences, Pune, Maharashtra (1091/PO/07/CPCSEA), India. Rats in polypropylene cages (6 rats per cage) lined with husk were housed in a registered animal house of Moolji Jaitha College, Jalgaon (M.S), India under standard environmental conditions of temperature 25 ± 2 °C and dark/light cycle of 12/12 h with 40–60 % relative humidity. The animals were fed with standard commercial diet (Amrut Agro Ltd, Sangli) and provided with water ad libitum during the experiment.

Induction of Diabetes and Experimental Setup

Induction of diabetes and experimental setup for the study were same as described previously [18]. Diabetes was induced in overnight fasted rats by single intra peritoneal (ip) injection of freshly prepared streptozotocin (STZ) in 0.1 M cold citrate buffer of pH-4.5 (Hi-Media, Mumbai, India) at a dose of 50 mg/kg body weight. The animals were fed with 20 % sterile glucose solution for 24 h to prevent STZ induced hypoglycemic mortality. Diabetic animals were identified after 96 h of STZ administration by measuring the vein blood glucose levels by a digital glucometer (one touch select, Johnson and Johnson, USA) based on glucose oxidase peroxidase method. Albino Wistar rats with blood glucose level above 250 mg/dL were considered as diabetic and used for study [7, 19].

The experimental setup for the study comprised six groups with 6 animals in each group. Plant extract was prepared in saline and fed daily to the experimental rats by using oral gavage feeding needle as per following detail.

Group A and B rats were fed with saline alone. Group C animal were fed with standard, metformin (oral hypoglycemic agent) prepared in saline at a dose of 10 mg/kg body weight.

Group D, E and F rats were fed with 50, 250 and 500 mg/kg body weight of *C. trifolia* ethanolic root extract

(CTERE) prepared in saline. Blood was collected for the measurement of glucose from the tail vein after 0, 7, 14, and 21 days and measured using digital glucometer (One touch select, Johnson and Johnson, USA).

At the end of 21 days, animals were fasted overnight. Blood samples were collected from retro orbital plexus in fresh vials for measurement of various biochemical parameters from serum on the next day under mild anesthesia and rats were sacrificed.

Biochemical Parameters

Various biochemical parameters such as serum insulin level, total cholesterol, triglycerides, HDL, LDL, VLDL, total protein, bilirubin, AST, ALT, ALP, serum urea, serum creatinine and uric acid were determined using auto analyzer (Erba Mannheim, Chem-5 plus V2, Germany) using commercially available kits according to instructions of manufacturer (Erba Diagnostic Mannheim GmbH, Germany). Serum insulin was determined using rat insulin enzyme linked immunosorbent assay (ELISA) kit (DRG International, NJ, USA).

In Vivo Antioxidant Activity

In vivo antioxidant activity in liver and kidney tissues were evaluated as a function of activities of superoxide dismutase [20], catalase and level of reduced glutathione [21] in normal, diabetic and treated rats.

Acute Oral Toxicity Study

Acute oral toxicity study was carried out according to OECD guidelines 425 of 2001 [22]. *C. trifolia* ethanolic root extract (CTERE) (100–1000 mg/kg) was given orally by gavage to different groups, each group having six animals. After administration of dose, animals were observed for body weight, acclimatization, behavior and mortality for 7 days.

Characterization of Bioactive Molecule

GC–MS Analysis of Ethanolic Extract

Gas chromatography mass spectroscopy (GC–MS) analysis of CTERE was performed on an instrument (TSQ 8000, Thermo Scientific, USA) equipped with RACE 1300 GC along with auto-sampler for automated sample handling and HP-5 capillary column (30 m × 0.25 mm ID × 0.25 μm coating thickness). The injector temperature was set at 280 °C, the oven temperature was initially set at 40 °C and programmed to increase up to 300 °C at the rate of 10 °C/min and finally held at 200 °C for 5 min. Helium gas was maintained at a flow rate 1.0 mL/min as a carrier gas. One microliter of the sample diluted with ethanol in 1:10 ratio was injected in the split mode. The percentage of constituents in CTERE was calculated and identified based on the comparison of their retention time (RT) and mass spectra of WILEY, NIST library data of the GC–MS.

Isolation of β-Sitosterol

Five gm CTERE dissolved in ethanol was passed through a column of silica gel (60–120 mesh) and eluted with hexane chloroform and methanol in a gradient manner with increasing polarity. Twenty-two fractions each of 5 mL, were collected and tested for presence of steroid by Salkowski test [23]. Fractions showing positive results were pooled, concentrated using rotary vacuum evaporator (R-215, Buchi, Switzerland) to dryness and stored desiccated.

Spectroscopic Analyses

The purified β-sitosterol fraction of CTERE and standard were scanned between 200 and 800 nm in a double beam scanning spectrophotometer (Model- UV-1601, Shimadzu, Japan) using appropriate control. The FT-IR spectra of the purified compound and standard were recorded between 4000 and 650 cm^{-1} on a FT-IR spectrophotometer (Perkin Elmer, Spectrum 2, USA) using ATR method. Mass spectroscopic analysis of both purified and standard compounds were performed at Sophisticated Analytical Instrumentation Facility (SAIF), Punjab University Chandigarh, on Q-TOF Micro, LC–MS system (Waters, USA).

Chromatographic Analyses

TLC was performed with standard silica gel 60 F254 plates (10 cm × 10 cm) and (5 cm × 10 cm) (Merck Germany). The purified fraction and standard, dissolved in ethanol, were applied thrice with the help of a thin capillary. The plates were air dried and developed in ascending manner in a chromatography twin trough chamber (CAMAG, Switzerland) pre-saturated for 10 min with the respective mobile phase. The mobile phase consisted of hexane and ethyl acetate in the ratio of 60:40. The plates were derivatized with 5 % concentrated sulfuric acid in methanol. After the development, the plates were observed in visible light and Rf value of the bands were calculated in the standard way.

HPLC analysis of isolated fraction was performed on a Shimadzu LC-20AT prominence binary HPLC system (Shimadzu, Japan). Pre-filtered (20 μL, 1 mg/mL concentration) purified compound and standard β-sitosterol were individually injected with the help of a micro syringe in a
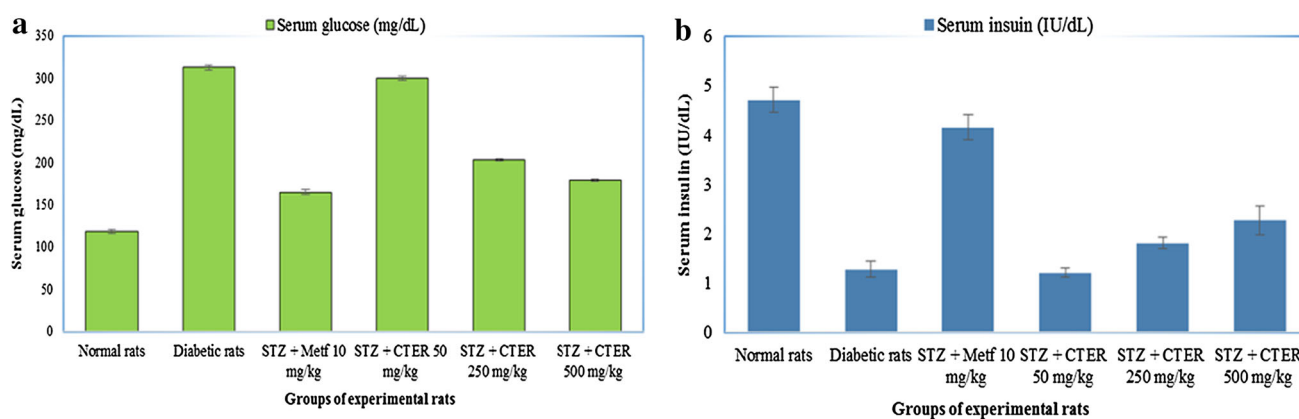
Fig. 1 Effect of CTERE on **a** Serum glucose. **b** Serum insulin in streptozotocin induced diabetic rats after 21 days of treatment

C-18 reverse phase column (Phenomenix Proteo Jupiter, 250 × 10 mm id). Elution was done with methanol: water (95:05) at a flow rate of 1 mL/min and peaks were detected using UV detector at 210 nm.

Statistical Analysis

All the results were expressed as mean ± standard error of mean (S.E.M) using GraphPad Prism® 6, IC_{50} value were calculated using statistical software *Stats Direct 2.8.0.* and bar diagram were plotted using Microsoft office Excel 2013.

Results

Yield of Leaf Extracts

The % yield of extract residues in ethanol, ethyl acetate and hexane were 4.313, 2.990 and 2.506 g, respectively. The residues of three solvents were semi solid, sticky and reddish brawn in appearance.

In Vitro Antidiabetic Activity

The *C. trifolia* root extracts in three solvents were rapidly screened for in vitro antidiabetic activity by their ability to inhibit enzyme α-amylase, non-enzymatic glycosylation of hemoglobin and glucose uptake by yeast cells. The IC_{50} values of three extracts and standards (acarbose, α tocopherol and metronidazole, respectively) were evaluated. The IC_{50} values in the ethanolic extract by the three assay procedures mentioned above were 86.65, 92.33 and 121 μg/mL, respectively and were the lowest among the three extracts indicating that antidiabetic principle is primarily getting partitioned in ethanol and therefore, ethanolic root extract (CTERE) was used for all further in vivo experiments using albino Wistar rats.

Acute Oral Toxicity Study

The acute oral toxicity profile of CTERE, in rat model as per OECD guidelines, did not produce any signs of toxicity or deaths in experimental animals (no observed adverse effect level, NOAEL) up to a dose of 1000 mg/kg body weight indicating minimal or no chance of toxicity of the extract at the likely therapeutic doses in human which are lower by several orders of magnitude than NOAEL.

Effect of CTERE on Blood Glucose Level and Serum Insulin

Diabetes in experimental animal was induced by a single intra-peritoneal injection of STZ at a dose of 50 mg/kg body weight. It caused an increase in blood glucose from 118.60 to 312.70 mg/dL. Treatment with CTERE, at 3 different doses 50, 250 and 500 mg/kg body weight, resulted in a dose dependent decline in the level of blood glucose (Fig. 1a). It declined to 178.96 mg/dL from 312.70 mg/dL at 500 mg/kg body weight dose of CTERE. Similarly, Intra peritoneal injection of STZ caused significant decrease in serum insulin in comparison to control. After oral administration of CTERE in increasing dose of 50, 250 and 500 mg/kg body weight for 21 days, a dose dependent recovery in insulin level was visible and, at 500 mg/kg body weight dose, it was almost at 58 % of the standard drug, metformin (Fig. 1b).

Effect of CTERE on Lipid Profile

Lipid profile gets severely affected in diabetes. All its parameters TC, TG, LDL, VLDL, except HDL showed a significant increase in experimentally induced diabetic rats, compared to normal control rats (Compare group B with group A, Table 1). However, the trends were found to reverse in all the parameters as a function of oral administration of CTERE in diabetic rats (Table 1). Total cholesterol

Table 1 Effect of CTERE on lipid profile of streptozotocin induced diabetic rats after 21 days of treatment

Group	Description	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
A	Normal rats	82.05 ± 0.81	104.20 ± 1.03	36.65 ± 0.69	24.73 ± 0.50	19.87 ± 0.80
B	Diabetic rats	197.81 ± 1.61	177.10 ± 1.29	16.03 ± 0.27	144.10 ± 0.11	35.14 ± 0.46
C	Diabetic rat + metformin 10 mg/kg	93.19 ± 0.87**	88.42 ± 1.00**	39.00 ± 0.63**	34.66 ± 0.62***	17.18 ± 0.22***
D	Diabetic rat + CTERE 50 mg/kg	192.68 ± 1.23*	176.41 ± 1.56	19.16 ± 0.72*	137.37 ± 1.21*	36.22 ± 0.31
E	Diabetic rat + CTERE 250 mg/kg	177.81 ± 1.01**	159.46 ± 1.32*	21.36 ± 0.18**	118.41 ± 1.27**	37.66 ± 0.72
F	Diabetic rat + CTERE 500 mg/kg	101.86 ± 1.23**	125.21 ± 0.86**	25.39 ± 0.84**	51.36 ± 1.32**	25.86 ± 0.45*

The data is expressed as mean ± S.E.M. (n = 6)

CTERE C. trifolia ethanolic root extract

* Represent statistically significant versus diabetic control ($P < 0.05$)

** Represent statistically significant versus diabetic control ($P < 0.01$)

*** Represent statistically significant versus diabetic control ($P < 0.001$)

declined from 197.81 mg/dL in diabetic rats to 101.86 mg/dL in rats treated with CTERE at 500 mg/kg body weight dose. Similarly, HDL was found to increase from 16.03 mg/dL in diabetic rats to 25.39 mg/dL in rats treated with the highest dose of CTERE in this study (Table 1).

Effect of CTERE on Liver and Kidney Functions Tests

Results of liver and kidney functions tests of control and experimental rats are shown in Table 2. STZ induced diabetes severely affects parameters of liver function-bilirubin, AST, ALT and ALP. For example, bilirubin went up from 0.38 mg/dL in normal rats to 0.96 mg/dL in diabetic rats whereas, the level of remaining three enzymes was found to be doubled in diabetic rats compared to control (Compare group A and B, Table 2 A). CTERE, at the doses studied, reversed the trend and the level of all and significant recovery was recorded in all the four parameters (Compare group B with D, E and F). The recovery was found to be dose dependent, with the highest being at 500 mg/kg body weight dose of CTERE. Similarly, kidney functions, represented by serum urea, creatinine, uric acid and protein, showed a steep increase in STZ induced diabetic rats compared to control (Table 2B). A statistically significant dose dependent recovery was recorded in all the parameters as a function of treatment with CTERE. At the highest dose, 500 mg/kg body weight, the extent of recovery was almost at par with the standard, metformin (Compare group F, E and Table 2B).

Effect of CTERE on Glycogen Content of Liver and Skeleton Muscles and Body Weight of Diabetic Rats

The effect of CTERE administration in diabetic rats on liver and muscle glycogen content and body weight is summarized in Table 3. The decrease in glycogen content in both tissues and overall body weight showed a dose dependent recovery by CTERE and at the highest dose, the results were comparable with the standard (Table 3).

In Vivo Antioxidant Activity

The antioxidant effects of CTERE in liver and kidney of treated animals was evaluated as a function of activities of superoxide dismutase (SOD), catalase and level of reduced glutathione (Table 4). Induction of diabetes severely affected the redox balance in experimental animals reflected by a sharp decline in the level of SOD, catalase and reduced glutathione in both the tissues (Table 4). Treatment with CTERE significantly restored the redox balance with increase in the levels of all the three parameters. In fact, the increase in levels of reduced glutathione in kidneys and catalase in both liver and kidney tissue was more than the standard at the highest dose of CTERE (Table 4).

Characterization of a Major Secondary Metabolite

The GC–MS profile of CTERE showed a total of 41 constituents accounting for 99.96 %, metabolites. Nine major constituents of CTERE, identified on the basis of WILEY, NIST library data base, were found to possess antidiabetic, antioxidant and hypoglycemic activity (Table 5). Of these 9 constituents, β-sitosterol and stigmasterol are known as potent antidiabetic, antioxidant and antihypercholesterolemia agents present in plants [24, 25]. In CTERE, β-sitosterol and stigmasterol together constituted 5.21 % and therefore, attempts were made to purify β-sitosterol by column chromatography. Fractions from column chromatography showing sitosterol activity were pooled. The pooled fractions were evaporated to dryness under vacuum

Table 2 Effect of CTERE on liver and kidney functions tests of streptozotocin induced diabetic rats after 21 days of treatment

| Tests | Normal rats | Diabetic rats | Diabetic rat + metformin 10 mg/kg | Diabetic rat + CTERE | | |
| | | | | 50 mg/kg | 250 mg/kg | 500 mg/kg |
	Group A	Group B	Group C	Group D	Group E	Group F
Liver function tests						
Total protein (g/dL)	8.54 ± 0.23	4.70 ± 0.25	7.29 ± 0.26*	6.73 ± 0.31	7.13 ± 0.41*	9.52 ± 0.71**
Bilirubin (mg/dL)	0.38 ± 0.05	0.96 ± 0.01	0.41 ± 0.02***	0.95 ± 0.03*	0.86 ± 0.12**	0.64 ± 0.15**
AST (U/L)	48.74 ± 0.48	105.2 ± 0.96	60.73 ± 0.53***	92.15 ± 0.15**	87.16 ± 0.25***	65.13 ± 0.71***
ALT (U/L)	60.15 ± 0.94	115.1 ± 1.92	58.60 ± 0.50***	110.21 ± 1.36	101.47 ± 0.98*	93.12 ± 0.77*
ALP(U/L)	120.60 ± 2.33	203.50 ± 0.54	150.30 ± 0.94***	198.86 ± 1.31	177.68 ± 2.34**	162.67 ± 2.36***
Kidney function tests						
Serum urea (mg/dL)	36.69 ± 0.51	70.08 ± 0.90	37.06 ± 0.19**	68.06 ± 0.86	61.31 ± 0.92*	43.78 ± 0.78**
Serum creatinine (mg/dL)	0.84 ± 0.05	1.21 ± 0.16	0.86 ± 0.01	1.12 ± 0.16	0.98 ± 0.08	0.89 ± 0.06
Uric acid (mg/dL)	8.68 ± 0.19	21.08 ± 0.23	8.48 ± 0.43***	18.13 ± 1.12	14.21 ± 0.98**	11.36 ± 0.51***

The data is expressed as mean ± S.E.M. (n = 6)

CTERE C. trifolia ethanolic root extract

* Represent statistically significant versus diabetic control ($P < 0.05$)

** Represent statistically significant versus diabetic control ($P < 0.01$)

*** Represent statistically significant versus diabetic control ($P < 0.001$)

Table 3 Effect of CTERE on liver and muscles glycogen content of streptozotocin induced diabetic rats after 21 days of treatment

| Group | Description | Glycogen content (mg/100 mg weight) | | Body weight (g) |
		Liver glycogen	Muscle glycogen	
A	Normal rats	18.92 ± 0.24	8.57 ± 0.41	220.33 ± 1.37
B	Diabetic rats	6.38 ± 0.17	1.92 ± 0.05	181.30 ± 1.01
C	Diabetic rat + metformin 10 mg/kg	18.55 ± 0.18***	7.77 ± 0.41***	216.90 ± 1.43***
D	Diabetic rat + CTERE 50 mg/kg	10.16 ± 0.56	3.16 ± 0.21	191.46 ± 1.34
E	Diabetic rat + CTERE 250 mg/kg	16.18 ± 0.92**	5.38 ± 0.38*	204.28 ± 1.61**
F	Diabetic rat + CTERE 500 mg/kg	18.18 ± 0.72***	8.57 ± 0.41**	219.42 ± 1.95***

The data is expressed as mean ± S.E.M. (n = 6)

CTERE C. trifolia ethanolic root extract

* Represent statistically significant versus diabetic control ($P < 0.05$)

** Represent statistically significant versus diabetic control ($P < 0.01$)

*** Represent statistically significant versus diabetic control ($P < 0.001$)

to yield a white powdery substance with brownish tinge. It was reconstituted in ethanol and subjected to different spectroscopic and chromatographic techniques along with standard β-sitosterol (Fig. 2). The purified compound showed a single band at an Rf 0.48 along with standard in TLC after derivatization with 5 % sulfuric acid in methanol (Fig. 2a). It also yielded a single, sharp, symmetrical peak at a retention time 3.64 min along with standard in HPLC study (Fig. 2b). The FTIR spectrum of purified compound was found to be almost overlapping with that of standard (Fig. 2c) and, in mass spectroscopy (Fig. 2d, e), its molecular weight was calculated to be 398.61, similar to that of standard and in agreement with couple of earlier

reports [26]. Collectively these results prove presence of β-sitosterol in CTERE. It is found to be present in relatively higher concentration in CTERE as revealed by GC–MS analysis (Table 5). This corroborates well with the known and reported antidiabetic activity of β-sitosterol [25] along with, antihypercholesterolemic, antioxidant and anticancer activities [27]. However, it should be pointed out here that both, purified compound and standard β-sitosterol did not show appreciable antidiabetic activity in in vitro assays when tested individually. The observed antidiabetic and antioxidant activities of CTERE, therefore, appears to be a synergistic action of more than one compounds present in the extract and that needs to be further investigated.

Table 4 In vivo antioxidant activity of CTERE extract in liver and kidney tissues

Group	Description	Superoxide dismutase (U/gm fresh weight)		Reduced glutathione (U/gm fresh weight)		Catalase (U/gm fresh weight)	
		Liver	Kidney	Liver	Kidney	Liver	Kidney
A	Normal rats	13.21 ± 0.33	26.4 ± 0.36	73.69 ± 0.90	132.70 ± 1.30	20.76 ± 0.60	70.77 ± 0.53
B	Diabetic rats	5.68 ± 0.29	9.3 ± 0.28	21.36 ± 0.61	45.20 ± 0.55	7.25 ± 0.06	9.73 ± 0.15
C	Diabetic rat + metformin 10 mg/kg	12.33 ± 0.63**	21.3 ± 0.74**	68.36 ± 0.78**	78.20 ± 2.77**	20.87 ± 0.51**	61.01 ± 0.89***
D	Diabetic rat + CTERE 50 mg/kg	6.15 ± 0.56	12.36 ± 0.36	32.18 ± 1.36	89.86 ± 2.17**	14.36 ± 0.71**	13.78 ± 0.89
E	Diabetic rat + CTERE 250 mg/kg	7.39 ± 0.81	14.19 ± 0.58*	44.15 ± 2.13*	119.87 ± 1.91**	18.19 ± 0.36***	46.78 ± 0.16**
F	Diabetic rat + CTERE 500 mg/kg	9.56 ± 0.78**	18.78 ± 0.64**	54.71 ± 0.98**	126.13 ± 1.56**	21.96 ± 0.49***	81.16 ± 0.78**

The data is expressed as mean ± S.E.M. (n = 6)

CTERE *C. trifolia* ethanolic root extract

* Represent statistically significant versus diabetic control ($P < 0.05$)

** Represent statistically significant versus diabetic control ($P < 0.01$)

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Table 5 GC-MS profile of major peaks of CTERE

Sr. no	R.T	Name	Molecular formula	Area percent	Biological activity
1.	14.46	Hexadecane	$C_{16}H_{34}$	2.35	Antimicrobial, antioxidant, antidiabetic [40]
2.	17.99	Hexadecanoic acid	$C_{17}H_{34}O_2$	0.74	Antioxidant, hypocholesterolemic, antiinflammatory, antibacterial [41]
3.	18.65	Hexadecanoic acid	$C_{17}H_{34}O_2$	4.03	Antioxidant, hypocholesterolemic, antiinflammatory, antibacterial [41]
4.	20.25	9,12-Octadecadienoic acid		4.84	Antiinflammatory, hypocholesterolemic, antibacterial, hepatoprotective, antihistaminic, antiarthritic, anticoronary [41]
5.	20.50	Heptadecanoic acid (Margaric acid)	$C_{20}H_{36}O_2$	1.52	Antioxidant [41]
6.	26.48	11,20-Didecyl triacontane	$C_{50}H_{102}$	0.56	Antimicrobial, antioxidant, antispasmodic [40]
7.	32.12	Vitamin E	$C_{29}H_{50}O_2$	1.27	Analgesic, antidiabetic, antiinflammatory, antioxidant, antidermatitic, antileukemic, antitumor, anticancer, hepatoprotective, antispasmodic [42]
8.	32.12	β-Sitosterol	$C_{28}H_{48}O$	2.92	Antidiabetic [25], Antihypercholesterolemia, reduces blood cholesterol, antioxidant activity, anticancer [27]
9.	32.80	Stigmasterol	$C_{29}H_{48}O$	2.29	Antihepatotoxic [43], antioxidant, hypoglycemic and thyroid inhibiting properties, precursor of progesterone, antimicrobial, anticancer [24]
Total				20.52 %	

CTERE *C. trifolia* ethanolic root extract

Discussion

A multipronged antidiabetic activity of CTERE along with an excellent antioxidant activity support and validate folk lore use of this plant in diabetes management in some parts of India. A strong antioxidant action has been proposed to be useful in limiting the conflagration of diabetes [28, 29]. This synergistic combination of the two could prove to be very effective and valuable for pharmacological approach in the management of diabetes. Streptozotocin mediated necrosis of pancreatic β-cells has opposing effects on serum glucose and insulin levels. Maintenance of glucose
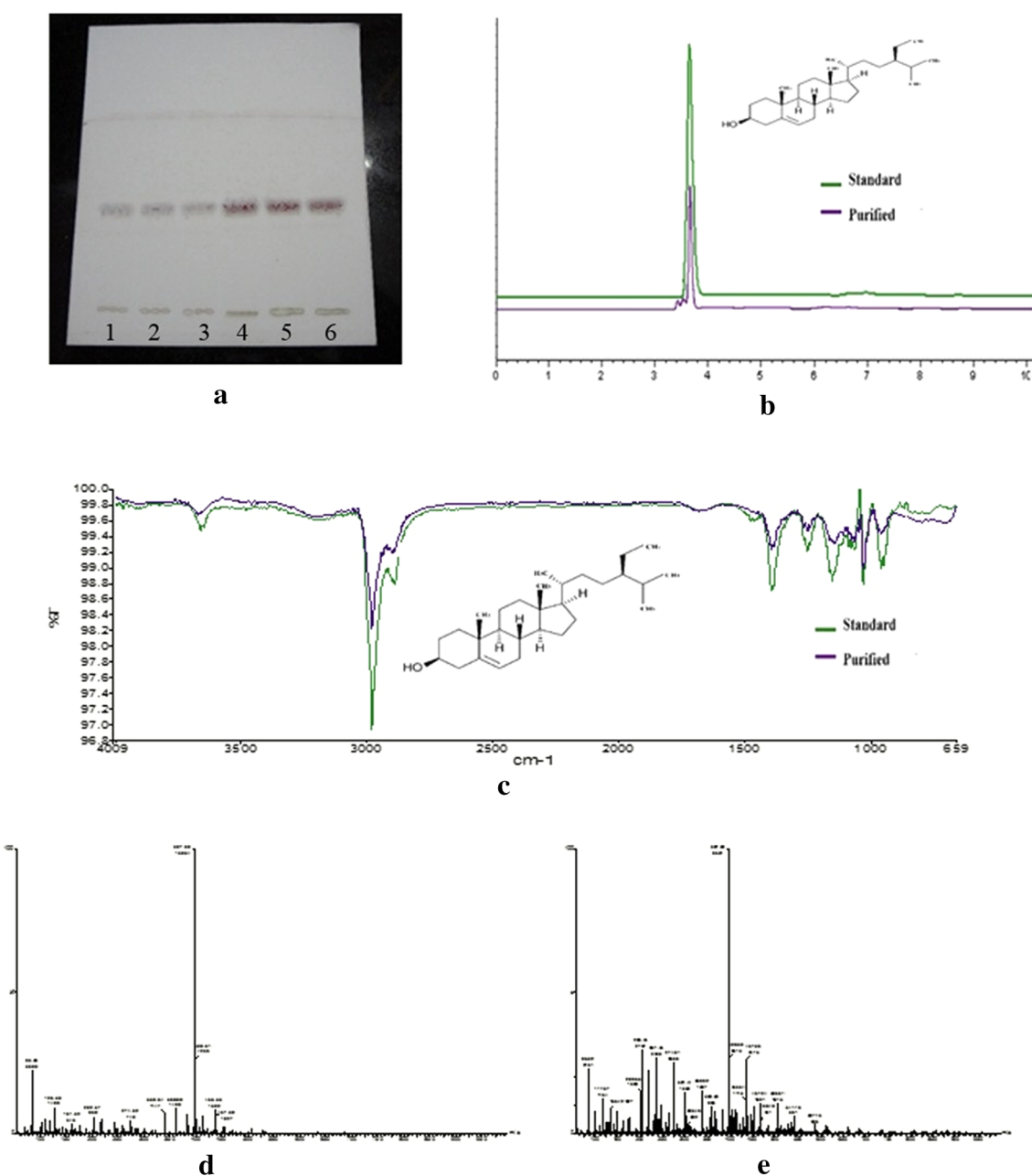
Fig. 2 Characterization of major secondary metabolite fraction of CTERE. **a** TLC of purified compound (*lane 1–3*) and standard β-sitosterol (*lane 4–6*) showing *purple pink color* bands. **b** Comparative chromatograms of purified compound and standard showing peaks at the same RT, 3.641 min. **c** Comparative IR spectra of purified compound and standard β-sitosterol and **d**, **e** LC–MS spectra of standard β-sitosterol and purified compound, respectively

homeostasis in the body within normal limit is a responsibility of pancreatic β-cells which are highly specialized in producing insulin. Defect in their generation or maintenance leads to impaired insulin metabolism leading to diabetes [30]. Streptozotocin is not only a β-cells specific toxin but also facilitates generation of reactive oxygen species (ROS) resulting in redox imbalance and diabetic state. A strong recovery in both serum glucose and insulin level as a function of treatment of CTERE could be attributed to strong antioxidant activity [25] and potentiating insulin effect [31]. Antioxidant enzymes and non-enzymatic antioxidants are first line of defense against ROS induced oxidative damage in the body [32]. Oxidative stress, resulting from redox imbalance, is suggested as mechanism underlying diabetes and ensuring complications. A strong recovery, almost at par with standard drug metformin, in the level of superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH), both in

liver and kidney tissues as a function of treatment with CTERE indicate improved redox balance. Decrease in level of oxidative enzymes SOD and CAT may be due to their inactivation caused by ROS [29]. This improvement apparently has a cascading effect on the liver and kidney function tests in diabetic rats treated with CTERE and our results suggest that it has good renal and hepatoprotective potential and is non-toxic in nature. The level of ALT and AST was found to be more in diabetic rats because of increased oxidative stress cause leakage of theses enzymes into blood indicating hepatic tissue damage [8]. Elevated level of markers of liver and kidney functions are indicative of toxicity to these tissues due to STZ mediated oxidative stress and hyperglycemia.

The activating effect of insulin on lipoprotein lipase get abolished in its deficiency leading to elevated lipid profile [33]. Restoration in lipid profile in diabetic rats as a function of treatment of CTERE suggests that it may have insulin like activity which would be helpful in alleviating incidences of lipid born complications associated with diabetes in agreement with previous reports [18, 34].

GC–MS analysis of CTERE revealed as many as 41 constituents of which 9 major were found to belong to broad group of steroids. We have purified and characterized one of the major constituents β-sitosterol whose level in the extract, in our opinion, is relatively high. The observed effects of extract could be due to presence of secondary metabolites as β-sitosterol in particular has been shown to inhibit formation of advanced glycation products (AGEs) whose level get elevated during severe diabetes [35, 36]. Type 2 diabetes is by far the most prevalent endocrine disorder of the world characterized by chronic hyperglycemia and associated complications [37]. Although insulin is one of the important therapeutic agents known to medicine and, technological breakthroughs have improved its access and availability, there is an increased focus on finding insulin substitutes, secretagogues or sensitizer from synthetic or plant source for the treatment of diabetes [38]. It should be pointed out here that plant derived natural compounds have established a proven platform for developing new drug synthesis with fewer side effects [39]. A strong antioxidative and antidiabetic activity of CTERE demands a wider role, application and research in insulin alternative strategies for diabetes management for it.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors. The study was approved by the Institutional Animal Ethics Committee (IAEC/16/CPCSEA/MJC/14-15) and was carried out in accordance with the current guidelines set by Organization for Economic Co-operation and Development (OECD), received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India for the care of laboratory animals.

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