

## Antidiabetic activity of *Carallia brachiata* Lour. leaves hydro-alcoholic extract (HAE) with antioxidant potential in diabetic rats

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Ethnomedicinal surveys have documented the traditional use of *Carallia brachiata* (Lour.) Merrill (Rhizophoraceae) leaves in the management of diabetes mellitus in the Northeastern region of India. This study screens the hydro-alcoholic extract (HAE) of *C. brachiata* leaves for antioxidant and antidiabetic activities. The HAE was prepared using ethanol:water (7:3) by cold maceration method. The antidiabetic activity of HAE was evaluated *in vivo* in streptozotocin-induced diabetic rats at the doses of 250 or 500 mg/kg body weight for 21 days. The extract was also evaluated for *in vitro* and *in vivo* antioxidant activity. Results revealed that HAE of *C. brachiata* leaves possesses good hypoglycemic activity in diabetic rats. The hypoglycemic activity of HAE was found significant as compared to normal rats. Results of antioxidant activity were found statistically significant compared to standard drugs, quercetin and gallic acid. Results indicated a possible role of the HAE of *C. brachiata* leaves as herbal antioxidants in the prevention and/ or treatment of oxidative stress-induced diabetes. Results suggested that antioxidant plant phenolics/ flavonoids might be responsible for the antidiabetic efficacy of HAE. Further research can be undertaken on the HAE of *C. brachiata* leaves for exploration of biochemical mechanisms of antidiabetic action with the isolation of bioactive flavonoids having antidiabetic potential.

**Keywords:** Antidiabetic activity, Antioxidant activity, *Carallia brachiata*, Hydro-alcoholic extract, Plant flavonoids.

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### Introduction

Diabetes mellitus (or diabetes) is a metabolic disease characterized by hyperglycemia with abnormal carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. Hyperglycemia is usually accompanied by polyuria, polydipsia, weight loss, sometimes polyphagia, blurred vision and also susceptibility to infectious illness. The chronic hyperglycemic condition of diabetes may lead to several health complications including cardiovascular (cardiomyopathy), neurological (neuropathy), renal (nephropathy) and ocular (retinopathy)<sup>1</sup>. The prevalence of diabetes is increasing with the global rise of obesity and related lifestyle disorders. It has been estimated that there were 422 million adults living with diabetes mellitus in 2016, according to a global report by the World Health Organization (WHO). Type 2 diabetes has accounted for the majority (>85%) of diabetes worldwide<sup>2</sup>.

Oxidative stress (OS) is believed to be the underlying cause of cellular injury, tissue damage or

organ dysfunctions commonly associated with diabetic complications. OS refers to elevated intracellular levels of reactive oxygen species (ROS) that cause damage to biomolecules such as lipids, proteins and DNA. Cellular OS may be reduced to a considerable extent by the action of various antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH) and glutathione peroxidase (GPx)<sup>3</sup>. Besides antidiabetic drugs, antioxidant medications are also used to reduce the long term complications of diabetes mellitus. Despite the availability of hypoglycemic agents from synthetic sources, diabetes is still life-threatening because of limited therapeutic utility of existing drugs. Traditional medicines derived from plants play a significant role in the management of diabetes mellitus. WHO recommended the evaluation of traditional plant remedies used in the treatment of diabetes because they are effective with less or no toxicities as compared to synthetic oral hypoglycemic agents<sup>4,5</sup>. Many indigenous Indian medicinal plants have been found to be useful in the treatment of diabetes mellitus.

*Carallia brachiata* (Lour.) Merrill (Rhizophoraceae) is an evergreen tree distributed widely in India, Sri

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Lanka, Southern China, Thailand, and other parts of Southeast Asia. Fruits and seeds of the plant are used for edible purposes by different ethnic communities in various parts of India. The seed oil is used as a substitute for ghee<sup>6</sup>. Different parts of *C. brachiata* are used traditionally in the treatment of a variety of human disorders. Leaf decoction mixed with benzoin, turmeric and rice powder is used in the treatment of sapraemia<sup>7</sup>. The traditional uses of bark in the treatment of itching, cuts and wounds, oral ulcers, throat inflammation and stomatitis are all well documented<sup>8</sup>. According to Ayurveda, the Indian System of Medicine, leaves of *C. brachiata* are used as a medicine for diabetes mellitus<sup>9</sup>. Modern literature reported that ethyl acetate and methanol bark extracts of *C. brachiata* possess anti-inflammatory, wound healing and antimicrobial activities<sup>10</sup>. A group of proanthocyanidins (carallidin, mahuanin and *para*-hydroxy benzoic acid) having antioxidant activities were reported from leaves and bark of this plant. Other phytoconstituents which include megastigmane diglycoside (3-hydroxy-5,6-epoxy- $\beta$ -ionol-3-O- $\beta$ apiofuranosyl (1 $\rightarrow$ 6)- $\beta$ -glucopyranoside), hygroline alkaloid, flavonoids and glyceroglycolipids have also been isolated from the leaves of *C. Brachiata*<sup>11</sup>.

Ethnomedicinal survey documents the use of leaves infusion for the management of diabetes by the tribal people in Dibrugarh forest region of Dibrugarh district, Assam (India). There are no scientific reports in modern literature on the antidiabetic efficacy of *C. brachiata* leaves. The objective of the present study was to ascertain the scientific basis of using this particular plant species traditionally in the management of diabetes, using streptozotocin-induced diabetic rats. According to WHO guidelines, the extract of *C. brachiata* leaves was prepared using hydro-alcoholic solvent and evaluated for the antidiabetic activity. It has been reported that traditional medicinal plants having antidiabetic activity possess antioxidant potential in experimental animals. Moreover, since a biochemical relationship exists between diabetic hyperglycemia and cellular oxidative stress, the antidiabetic activity evaluation of the HAE of *C. brachiata* leaves was carried out along with the antioxidant activity study.

## Materials and Methods

### Chemicals

All chemicals and reagents used in the study were of analytical grade and were procured from Rankem,

Mumbai and Himedia Laboratories Ltd., Mumbai. Streptozotocin (STZ) was procured from Sigma-Aldrich, Germany. Commercial reagent kits used for determination of biochemical parameters and enzymatic assays were purchased from SPAN Diagnostics Ltd., Surat (India).

### Plant material

Fresh leaves of *Carallia brachiata* (Lour.) Merrill (Rhizophoraceae) were collected from forest areas of Dibrugarh district, Assam (India) during the month of December 2014. The plant sample was identified and authenticated (BSI/ERC/2014/Plant identification/360, dt. 26.08.2014) by Dr A. A. Mao, Scientist E, BSI, Eastern Regional Centre, Botanical Survey of India, Eastern Regional Centre, Shillong (India). A voucher specimen (DU/PSC/HRB/B-11/2014) of the identified plant species was deposited in the Herbarium of the Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh.

### Preparation of HAE

The air-dried leaves were coarsely powdered (Sieve no. 40) using a cutter mill, powdered leaves (50 g) were extracted using sufficient quantity (400 mL) of ethanol:water (7:3) mixture by cold maceration for 24 hours. The extraction was carried out successively thrice and the combined extract was then concentrated under reduced pressure to dryness in a rotary vacuum evaporator to obtain a thick semisolid-like paste. The crude extract was dried at -40 °C in a lyophilizer and the dried extract (dark brown colour) so obtained was stored in a desiccator until further use. The % yield of the dried HAE was calculated per dry weight of powdered leaves.

### Test animals

Healthy Wistar male albino rats (240-260 g) were maintained under standard environmental conditions (temperature 25 $\pm$ 2 °C, relative humidity 50 $\pm$ 5%) with a 12 h light/dark cycle. They were fed on with normal laboratory chow pellet diet and drinking water was given *ad libitum*. Animals were allowed to acclimatize for 7 days before commencement of the experiment. The animals were used with the approval (no. IAEC/DU/50 dt. 24.9.13) of the Institutional Animal Ethics Committee under guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi (India).

### Estimation of phenolic and flavonoid contents

The total phenolic content of the HAE was evaluated following the Folin-Ciocalteu colourimetric

method<sup>12</sup> and results were expressed as mg of gallic acid equivalent (GE) per g of dry weight of the extract. The total flavonoid content was estimated using the aluminium chloride colourimetric method<sup>13</sup> and results were expressed as mg of quercetin equivalent (QE) per g of dry weight of the extract.

#### *Estimation of total phenolic content (TPC)*

Briefly, 1 mL of HAE (1 mg/mL, in 90% ethanol) were made up to 3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin-Ciocalteu reagent for 5 minutes, followed by the addition of 2 mL of 20% aqueous (w/v) sodium carbonate. The mixture was allowed to stand for further 60 minutes in the dark, and absorbance was measured at 650 nm. The TPC was calculated from the calibration curve of gallic acid (20, 40, 60, 80, 100 µg/mL, 90% ethanol). Results were obtained as mean±SEM of three replicate studies.

#### *Estimation of total flavonoids contents (TFC)*

In brief, 0.5 mL of HAE (1 mg/mL, in 90% ethanol) were made up to 1 mL with ethanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% NaNO<sub>2</sub> solution, 0.5 mL of 10% AlCl<sub>3</sub> solution were added after 5 minutes of incubation, and the mixture was allowed to stand for 5 minutes. To the above solution, 2 mL of 1 mol/L NaOH solution was added, and the final volume of the reaction mixture was made up to 10 mL with distilled water. The mixture was allowed to stand for 15 minutes, and absorbance was measured at 510 nm. The TFC was calculated from a calibration curve of quercetin (20, 40, 60, 80, 100 µg/mL, 90% ethanol). Results were obtained as mean±SEM of three replicate studies.

#### *In vitro antioxidant activity*

The *in vitro* antioxidant activity of HAE was carried out by the following three assay methods in accordance with previously reported procedures with minor modifications.

#### *Superoxide radical scavenging activity*

Superoxide radical scavenging activity of HAE was determined by the nitro blue tetrazolium (NBT) reduction method. In this assay, the non-enzymatic phenazine methosulfate/nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce NBT to a purple colour formazan. The reaction mixture contained phosphate buffer (0.5 mL, 100 mM, pH 7.4), 1.0 mL of NADH (0.4 mM), 1.0 mL of NBT (0.156 mM), 0.1 mL of

PMS (0.06 mM) and 3 mL of the HAE/standard drugs (quercetin and gallic acid) of various concentrations (50-250 µg/mL, in 90% ethanol). After incubation at 25 °C for 1 hour, the absorbance of the reaction mixture was measured at 560 nm against an appropriate blank to determine the quantity of formazan formed.

#### *Hydroxyl radical scavenging activity*

Hydroxyl radicals were generated by the Fenton reaction using Fe<sup>3+</sup>/ascorbate/EDTA/H<sub>2</sub>O<sub>2</sub> system. The hydroxyl radical generated in the system attacks deoxyribose which eventually results in the formation of thiobarbituric acid (TBA) reacting substance (TBARS) which was estimated. The reaction mixture contained 0.1 mL of 2-deoxy-2-ribose (10 mM), 0.33 mL of phosphate buffer (50 mM, pH 7.4), 0.1 mL of FeCl<sub>3</sub> (0.1 mM), 0.1 mL ethylenediamine tetra-acetic acid (EDTA) (0.1 mM), 0.1 mL of H<sub>2</sub>O<sub>2</sub> (2 mM), 0.1 mL of ascorbic acid (1 mM) and 1.0 mL of various concentrations (50-250 µg/mL) of the HAE or standards (quercetin and gallic acid). After incubation for 45 minutes at 37 °C, 1.0 mL of 2.8% (v/v) TCA, and 1.0 mL of [thiobarbituric acid, TBA, 0.5% (v/v) in 0.025 mol/L NaOH solution containing 0.2% (w/v) of butylated hydroxyl anisole, BHA] were added in the reaction mixture, and the mixture was incubated at 95 °C for 15 minutes to develop the pink chromogen. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution.

#### *Lipid peroxidation scavenging activity*

The Fe<sup>3+</sup>/ascorbic acid-dependent non-enzymatic lipid peroxidation in the liver extract was performed as follows. Reaction mixture (0.5 mL) containing rat liver homogenate (0.1 mL, 25% w/v) in Tris-HCl buffer (40 mM, pH 7.0), KCl (30 mM), FeCl<sub>3</sub> (0.16 mM) and ascorbic acid (0.06 mM) was incubated for 1 hour at 37 °C in the presence and absence of the HAE/ standard drugs (quercetin and gallic acid) at various concentrations (50-250 µg/mL). The lipid peroxide formed was measured by TBARS formation. For this incubation mixture, 0.4 mL was treated with sodium dodecyl sulphate (8.1%, 0.2 mL), TBA (0.8%, 1.5 mL) and acetic acid (20%, 1.5 mL, pH 3.5). The total volume was then made up to 4.0 mL by adding distilled water and kept in a water bath at 100 °C for 1 hour. After cooling, 1 mL of distilled water and 5.0 mL of a mixture of *n*-butanol and pyridine (10:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at

4000 rpm for 10 min. The butanol-pyridine layer was removed and its absorbance at 532 nm was measured to quantify TBARS.

In all the three above methods, the percent inhibition of scavenging activity was calculated using the following equation (Eq. 1).

$$\text{Percent inhibition (\%)} = [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100 \quad \dots (1)$$

where,  $A_{\text{control}}$  is the absorbance of the control and  $A_{\text{test}}$  represents the absorbance of a test (HAE/ standard drug).

Tests were performed in triplicate and values were obtained as mean  $\pm$  SEM of three independent studies. Results were evaluated by comparing the percent inhibition of the activity of HAE with that of standard drugs. Quercetin and gallic acid were used as standard drugs.

#### Acute oral toxicity study

Over-night fasted rats were randomly divided into six groups of six animals each. Rats of different groups were administered with increasing doses (250, 500, 1000, 2000, and 5000 mg/kg b.w.) of HAE. One group was maintained as normal control and was given vehicle alone. The animals were observed individually for the first hour for any gross behavioural changes including drowsiness, restlessness, writhing, convulsions and symptoms of toxicity and mortality if any, and then periodically for the next 24 hours, and then at every 24 hours for any signs of acute toxicity over a period of 14 days. The acute toxicity study was carried out as per OECD guideline 425<sup>14</sup>.

#### Oral glucose tolerance (OGT) test

This test was performed in overnight fasted normal rats according to the method reported by Junejo and co-workers<sup>15</sup>. Animals were divided into four groups of six each. Group I rats (normal control) were treated with vehicle alone. Group II and Group III rats were treated with HAE at 250 and 500 mg/kg b.w. doses, respectively. Group IV rats were treated with metformin hydrochloride (5 mg/kg b.w.). Carboxymethyl cellulose (CMC) solution (0.5% w/v in normal saline) was used as vehicle. Glucose 2 g/kg was fed orally 30 min after the administration of HAE and metformin hydrochloride. Blood was withdrawn from the tail vein at 0, 30, 60, 90 and 120 minutes, and glucose levels in blood were estimated by the GOD-POD method (Accu-Chek, Roche Diabetes Care India Pvt. Ltd., Mumbai, India).

#### Hypoglycemic activity in streptozotocin-induced diabetic rats

Diabetes was induced in overnight fasted animals by a single intraperitoneal (*i.p.*) injection of streptozotocin (STZ, 55 mg/kg b.w. in normal saline). The animals confirmed as diabetic (after 72 h of STZ injection) by the elevated plasma glucose levels (200-300 mg/dL) was used for the experiment. The animals were divided randomly into five groups of six rats in each group. Group I rats served as normal control and were given vehicle (0.5% CMC w/v in normal saline) alone. Group II rats served as diabetic control and were administered with vehicle alone. Group III and IV were treated with HAE at 250 and 500 mg/kg b.w., respectively. Group V rats received the standard drug, metformin hydrochloride (5 mg/kg b.w.). Treatments were given orally using a canula once daily for a period of 21 days. Blood was collected from the tail vein each time for the determination of glucose levels on 0, 7, 14 and 21 days. Blood glucose levels were measured by the GOD-POD method<sup>16</sup>.

#### Liver and kidney function tests

The initial and final body weights were measured. Liver tissues were excised, blotted, weighed and stored at -70 °C for assay of glycogen content. Liver glycogen was estimated by the method of Carroll *et al.*<sup>17</sup>. Blood was collected by cardiac puncture in dry test tubes containing a mixture of potassium oxalate and sodium fluoride (1:3) and allowed to coagulate in ambient temperature for 30 minutes. The serum was separated by centrifugation (2000 rpm, 10 minutes) for estimation of various biochemical parameters. Serum insulin levels were measured by the microplate ELISA method using a commercial kit (SPAN Diagnostics Ltd.). Serum lipid profile was estimated using commercially available kits (SPAN Diagnostics kit). Triglycerides (TG) and total cholesterol (TC) were estimated by enzymatic methods, HDL (High density lipoprotein) cholesterol by phosphotungstate method and LDL (low density lipoprotein) cholesterol were calculated by Friedewald's formula.

Serum was used to estimate glutamate oxaloacetate transaminase (GOT) glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP), total protein (TPR) and creatinine (CRTN). SGOT and SGPT were measured by UV kinetic method and ALKP was estimated by PNPP method. TPR was measured by Bradford Macro method, while CRTN was by picrate method.

#### *In vivo* antioxidant activity

On the 21<sup>st</sup> day, all the groups of animals were anaesthetized using diethyl ether, the liver was

dissected out, washed with normal saline and one part was preserved in 10% formalin for histopathological studies. The other part of the liver was homogenized by ice-chilled Tris-HCl buffer and used for activities/levels of superoxide dismutase (SOD), catalase, reduced glutathione (GSH), glutathione peroxidase (GPx), and malondialdehyde MDA. The malondialdehyde (MDA) production is a direct indicator of lipid peroxidation (LPO) process that was measured by TBA reaction using an ELISA reader (at 532 nm).

#### Histopathological studies

At the end of 21<sup>st</sup> day of treatment, the animals were fasted for 12 hours, anaesthetized using diethyl ether and sacrificed by cervical dislocation. Pancreas and liver were instantly dissected out, excised and rinsed in ice-cold saline solution. Tissues were processed as follows. A portion of pancreas/liver tissue was fixed in 10% formalin fixative solution for 4 days. After fixation, tissues were dehydrated in ethanol (70-95%), cleared in xylene, and embedded in paraffin was, solid transverse sections of 4-5  $\mu\text{m}$  thickness were obtained by using a rotary microtome. The sections were stained with hematoxylin-eosin and histopathological observations were carried out under a light microscope (40 $\times$ ).

#### Statistical analysis

Values are represented as mean $\pm$ SEM of three replicate studies. Statistical analysis was performed using the IBM SPSS 19.0 statistical software package, for Windows. Statistical differences at 5% level of probability ( $P < 0.05$ ) between the groups were analyzed by one-way ANOVA followed by Student's *t*-test<sup>5</sup>.

## Results

#### TPC and TFC of HAE

The TPC of HAE, calculated from the calibration curve of gallic acid ( $R^2 = 0.984$ ), was  $56.24 \pm 2.32$  gallic acid/g, and the total flavonoid content ( $R^2 = 0.983$ ), calculated from the calibration curve of quercetin was  $45.52 \pm 2.65$  quercetin/g.

#### In vitro antioxidant activity

##### Superoxide radical scavenging activity

The HAE showed superoxide radical scavenging activity in a concentration-dependent manner. HAE, quercetin and gallic acid exhibited  $78.34 \pm 0.56$ ,  $82.38 \pm 0.54$  and  $86.37 \pm 83\%$  of scavenging effect, respectively at the highest tested concentration of

250  $\mu\text{g/mL}$ . The inhibitory activity of HAE was found significant ( $P < 0.05$ ) compared to standard drugs, quercetin and gallic acid (Fig. 1).

##### Hydroxyl radical scavenging activity

The percent inhibition of hydroxyl radicals were  $84.72 \pm 0.27$ ,  $88.40 \pm 0.22$  and  $87.26 \pm 0.12\%$  for HAE, quercetin and gallic acid, respectively at the concentration of 250  $\mu\text{g/mL}$ . Results were statistically significant ( $P < 0.05$ ) as compared to standard drugs (Fig. 2).

##### Lipid peroxidation scavenging activity

LP induced by  $\text{Fe}^{2+}$ /ascorbate in rat liver homogenate was found to be inhibited by the HAE in

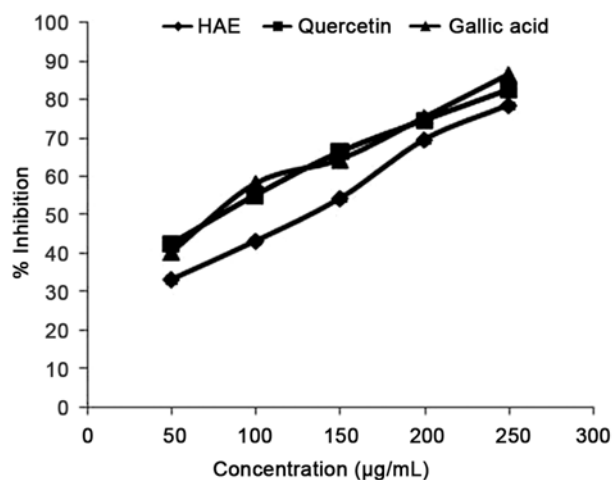


Fig. 1 — Superoxide radical scavenging activity. Values are mean $\pm$ SEM of three replicate experiments. % Scavenging activity of HAE is statistically significant at  $P < 0.05$ , compared to quercetin and gallic acid (standards)

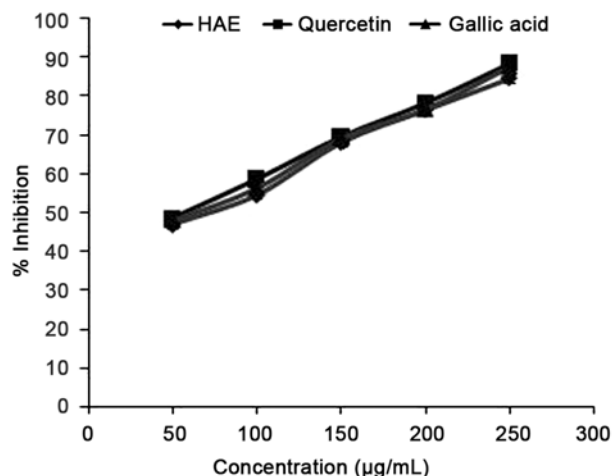


Fig. 2 — Hydroxyl radical scavenging activity. Values are mean $\pm$ SEM of three replicate experiments. % Scavenging activity of HAE is statistically significant at  $P < 0.05$ , compared to quercetin and gallic acid (standards)

a concentration-dependent manner and a considerable amount of lipid peroxidation inhibitory effect was observed by  $62.13 \pm 0.18\%$ , while quercetin and gallic acid inhibited by  $72.12 \pm 0.12$ , and  $70.23 \pm 0.11\%$ , respectively, at  $250 \mu\text{g/mL}$  (Fig. 3). Test results were considered statistically significant when compared to standard drugs ( $P < 0.05$ ).

**Acute toxicity study**

No sign and symptoms of acute toxicity and mortality up to  $2000 \text{ mg/kg}$  body weight dose were observed during the whole experimental period. The body weight and food consumption were normal compared to vehicle-treated rats. For further studies, the doses were fixed as  $250$  and  $500 \text{ mg/kg}$  body weight. The dosages were fixed judiciously considering the efficacy and toxicity levels of extracts as per OECD guidelines.

**Effect of HAE on OGT test in normal rats**

In OGT, HAE ( $250$  and  $500 \text{ mg/kg}$ ) showed significant ( $P < 0.05$ ) reduction of glucose load (plasma glucose level) as compared to normal control group. The metformin ( $5 \text{ mg/kg}$ ) treated group also showed significant ( $P < 0.05$ ) activity compared to the normal control group (Fig. 4).

**Effect of HAE on blood glucose levels in diabetic rats**

STZ-treated diabetic rats exhibited a significant increase in the levels of blood glucose in comparison to normal rats. After treatment with HAE, the blood glucose levels were significantly ( $P < 0.05$ ) reduced compared to the diabetic control rats at both the doses, viz.  $250$ ,  $500 \text{ mg/kg}$ . The metformin ( $5 \text{ mg/kg}$ ) treated rats also showed significant ( $P < 0.05$ ) reduction in plasma glucose level when compared to normal rats. Results of the effect of HAE on blood glucose levels in normal and diabetic rats are depicted in Table 1.

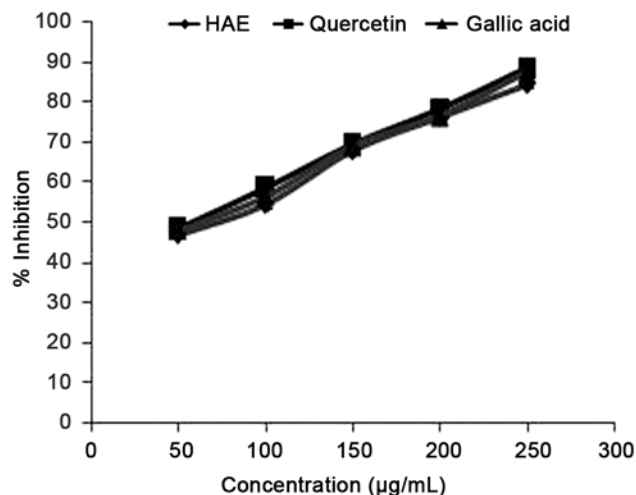


Fig. 3 — Lipid peroxidation scavenging activity. Values are mean $\pm$ SEM of three replicate experiments. % Scavenging activity of HAE is statistically significant at  $P < 0.05$ , compared to quercetin and gallic acid (standards)

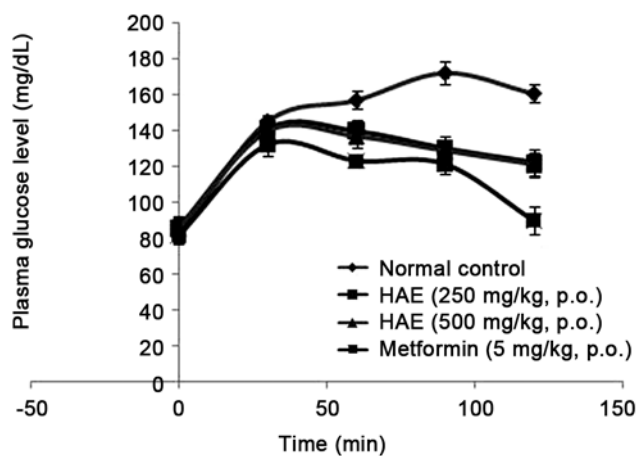


Fig. 4 — OGT test. Values are mean $\pm$ SEM of three replicate experiments. Activities of HAE and metformin (metformin) are statistically significant at  $P < 0.05$ , compared to normal control.

Table 1 — Effect of HAE on blood glucose levels in normal and diabetic rats

Group	Days			
	0 day	7 <sup>th</sup> day	14 <sup>th</sup> day	21 <sup>st</sup> day
Normal control	94.39 $\pm$ 2.71	102.86 $\pm$ 3.51	103.49 $\pm$ 3.99	99.88 $\pm$ 2.58
Diabetic control STZ (55 mg/kg, i.p.)	266.72 $\pm$ 8.29	289.21 $\pm$ 9.57	298.12 $\pm$ 6.58	322.64 $\pm$ 11.86
Diabetic + HAE (250 mg/kg, p.o.)	259.82 $\pm$ 9.48	226.91 $\pm$ 8.23*	159.84 $\pm$ 6.56*	110.18 $\pm$ 9.31*
Diabetic + HAE (500/kg, p.o.)	258.62 $\pm$ 8.38	225.72 $\pm$ 7.43*	138.62 $\pm$ 5.38*	103.29 $\pm$ 8.09*
Diabetic + Metformin (5 mg/kg, p.o.)	264.56 $\pm$ 7.26	170.66 $\pm$ 8.95	116.89 $\pm$ 9.04	76.99 $\pm$ 8.63

Values indicate mean  $\pm$  SEM ( $n = 6$ ); \* $P < 0.05$ , compared with normal control values

#### Effect of HAE on body weight, plasma insulin and liver glycogen in diabetic rats

Table 2 depicts the effect of HAE on body weight, levels of plasma insulin and liver glycogen in STZ-induced diabetic rats. In diabetic rats, the body weight, insulin level and glycogen content were significantly decreased. After 21 days of treatment with HAE at 250 or 500 mg/kg, the body weight was significantly ( $P < 0.05$ ) increased, insulin level and glycogen content were also significantly ( $P < 0.05$ ) increased as compared to diabetic rats. The activity of HAE was found less than that of metformin (5 mg/kg) treated group.

#### Effect of HAE on lipid profile in diabetic rats

The effect of HAE on lipid profile of diabetic rats is displayed in Table 3. In diabetic rats, the levels of TG, TC, and LDL were significantly increased and HDL level was significantly decreased. In HAE (250 & 500 mg/kg) treated groups, the TG, TC and LDL levels activities were significantly ( $P < 0.05$ ) reduced and the HDL level was significantly ( $P < 0.05$ ) increased as compared to diabetic control rats, which is, in turn, comparable to metformin (5 mg/kg) treated group.

#### Effect of HAE on SGOT, SGPT, ALKP, TPR and CRTN in diabetic rats

There was a significant increase in activities of SGOT, SGPT and ALKP in diabetic rats. After treatment with HAE (250 and 500 mg/kg) the activities of SGOT, SGPT and ALKP activities were significantly ( $P < 0.05$ ) reduced as compared to diabetic control rats. A significant decrease in serum TPR level and a significant increase in CRTN level were observed in diabetic rats. After treatment with HAE at 250 or 500 mg/kg doses for 21 days, the TPR level was significantly increased ( $P < 0.05$ ) and CRTN level was significantly ( $P < 0.05$ ) decreased compared to diabetic control rats. Metformin (5 mg/kg) treated rats also showed significant effects on blood levels of SGOT, SGPT, ALKP, TPR and CRTN in diabetic rats (Table 4).

#### Effect of HAE on liver enzymes and MDA

Table 5 displays the activities of SOD, CAT, GSH and GPx in normal and diabetic rats. In STZ-treated diabetic rats, the activities of SOD, CAT, GSH and GPx were significantly increased. There was a significant ( $P < 0.05$ ) reduction in the activities of these antioxidant enzymes in diabetic rats as compared to normal rats. Metformin (5 mg/kg) also

Table 2 — Effect of HAE on body weight, plasma insulin and liver glycogen in normal and diabetic rats

Group	Body weight in g		Plasma insulin ( $\mu\text{U/mL}$ )	Liver glycogen (mg/g tissue)
	0 day	21 <sup>st</sup> day		
Normal control	200.50 $\pm$ 2.84	205.38 $\pm$ 11.49	15.31 $\pm$ 3.62	72.14 $\pm$ 2.23
Diabetic control	205.67 $\pm$ 4.88	130.38 $\pm$ 7.27	6.23 $\pm$ 6.45	32.18 $\pm$ 2.25
STZ (55 mg/kg, i.p.)				
Diabetic + HAE (250 mg/kg, p.o.)	205.31 $\pm$ 4.06	171.21 $\pm$ 7.96	8.23 $\pm$ 3.65*	54.20 $\pm$ 4.14
Diabetic + HAE (500 mg/kg, p.o.)	208.84 $\pm$ 4.49	180.64 $\pm$ 6.29	12.59 $\pm$ 4.0	62.34 $\pm$ 5.62
Diabetic+ Metformin (5 mg/kg, p.o.)	206.66 $\pm$ 2.32	192.59 $\pm$ 8.30	15.65 $\pm$ 6.72	72.80 $\pm$ 6.67

Values indicate mean  $\pm$  SEM ( $n = 6$ ); \* $P < 0.05$ , compared with normal control values

Table 3 — Effect of HAE on lipid profile in normal and diabetic rats

Groups	TG (mg/dL)	TC (mg/dL)	HDL (mg/dL)	LDL(mg/dL)
Normal control	86.89 $\pm$ 7.26	152.20 $\pm$ 6.56	38.29 $\pm$ 2.14	95.32 $\pm$ 4.92
Diabetic control	210.43 $\pm$ 6.84	270.83 $\pm$ 14.96	30.61 $\pm$ 2.60	199.33 $\pm$ 15.67
STZ (55 mg/kg, i.p.)				
Diabetic + HAE (250 mg/kg, p.o.)	164.38 $\pm$ 7.86	190.71 $\pm$ 9.51	31.79 $\pm$ 4.41	156.82 $\pm$ 7.49
Diabetic + HAE (500 mg/kg, p.o.)	144.84 $\pm$ 6.23	181.29 $\pm$ 8.37	36.95 $\pm$ 2.34*	136.32 $\pm$ 5.86
Diabetic + Metformin (5 mg/kg, p.o.)	115.29 $\pm$ 3.79	146.59 $\pm$ 11.15	54.69 $\pm$ 3.28	78.89 $\pm$ 6.74

Values indicate mean  $\pm$  SEM ( $n = 6$ ); \* $P < 0.05$ , compared with normal control values

Table 4 — Effect of HAE on SGOT, SGPT, ALKP, TPR and CRTN in normal and diabetic rats

Group	SGOT (U/L)	SGPT (U/L)	ALKP (U/L)	TPR (mg/dL)	CRTN (mg/dL)
Normal control	49.78±6.69	46.89±6.23	116.61±4.98	8.89±0.46	0.436±0.026
Diabetic control STZ (55 mg/kg, i.p.)	102.80±8.43	88.39±4.59	320±8.95	4.62±0.92	0.826±0.037
Diabetic + HAE (250 mg/kg, p.o.)	79.28±6.71*	69.83±7.42*	160.63±5.33	6.82±0.34*	0.628±0.042
Diabetic + HAE (500 mg/kg, p.o.)	71.32±7.85	64.82±6.28	144.31±9.13	8.20±0.54	0.573±0.056
Diabetic + Metformin (5 mg/kg, p.o.)	58.79±7.61	53.91±8.56	132±6.52	8.98±2.02	0.434±0.062

Values indicate mean ± SEM (n = 6); \*P <0.05, compared with normal control values

Table 5 — Effect of HAE on SOD, CAT, GSH, GPx and MDA in normal and diabetic rats

Treatment	SOD (U/mg protein)	CAT (U/mg protein)	GSH (U/mg protein)	GPx (U/mg protein)	MDA (LPO) (U/mg protein)
Normal control	8.28±0.06	72.16±4.32	16.20±0.48	23.12±1.89	0.52±0.12
Diabetic control STZ (55 mg/kg, i.p.)	4.23±0.08	42.60±3.69	6.20±0.28	9.12±0.48	0.82±0.03
Diabetic + HAE (250 mg/kg, p.o.)	6.22±0.09	58.02±4.20	9.23±0.28*	12.28±0.44	0.72±0.06*
Diabetic + HAE (500 mg/kg, p.o.)	7.67±0.06	64.74±7.26	12.67±0.64	18.43±0.65	0.64±0.04
Diabetic + Metformin (5 mg/kg, p.o.)	8.51±0.05	69.86±3.64	15.20±1.20	20.34±0.82	0.50±0.10

Values indicate mean±SEM (n = 6); \*P <0.05, compared with normal control values

showed significant ( $P < 0.05$ ) reduction of these enzymes. Increased levels of MDA, an indicator of LPO, in diabetic rats were significantly ( $P < 0.05$ ) reduced after treatment with HAE (250 or 500 mg/kg) as compared to the normal rats.

#### Histopathological observations

##### *Effect of HAE on the pancreatic section in normal and diabetic rats*

Histopathological studies of STZ-treated diabetic rat's pancreas (Plate 1) exhibited a reduction in the dimensions of islets, damaged  $\beta$ -cell population and extensive necrotic changes followed by fibrosis and atrophy (B). HAE (500 mg/kg) and metformin-treated rats restored the necrotic and fibrotic changes and also increased the number and increased the size of the islets (C). In the normal control group, normal acini and normal cellular population in the islets of Langerhans in the pancreas were observed (A). The changes in pancreas morphology in metformin treated group (D) are similar to HAE treated rats.

##### *Effect of HAE on the liver section in normal and diabetic rats*

Photomicrographs of STZ-treated diabetic rat's liver (Plate 2) showed normal hepatic cells with well-preserved cytoplasm, nucleus, nucleolus and central

vein (a). In the case of diabetic rats, the normal lobular structure was preserved. The central vein was prominent and prominently congested. Focal areas of haemorrhage were also seen. Vacuolization and fatty change were evident. The portal tracts appeared normal (b). In diabetic treated group (HAE, 500 mg/kg), the hepatocytes portal tracts and central veins appeared normal (c). HAE treated group is comparable with the metformin-treated group (d).

#### Discussion

*C. brachiata* leaves hydro-alcoholic extract did not exhibit toxicities up to a dose of 2000 mg/kg b.w. in experimental animals which indicated a high margin of safety of bioactive principles present in the extract. HAE treated rats lowered glucose level when compared to normal rats which indicated that the increased glucose tolerance in HAE treated rats was due to insulin secretion from  $\beta$ -cells and increased glucose utilization by the tissues<sup>18</sup>. The HAE treated group exhibited a significant reduction of fasting plasma glucose levels as compared to the diabetic control group. Administration of HAE to diabetic rats showed a significant reduction in the levels of blood glucose and an increase in the levels of serum insulin.



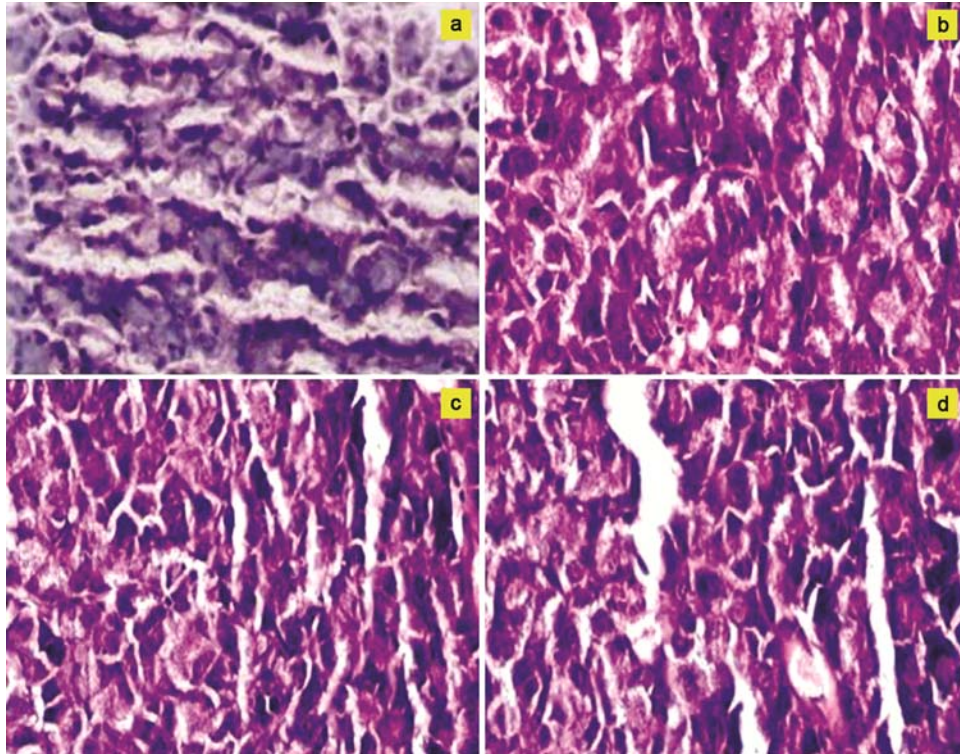


Plate 1 — Histology of pancreas of experimental rats after treatment with HAE, 500 mg/kg. a) Normal control, b) Diabetic control, c) Diabetic treated with HAE (500 mg/kg), and d) Diabetic treated with metformin

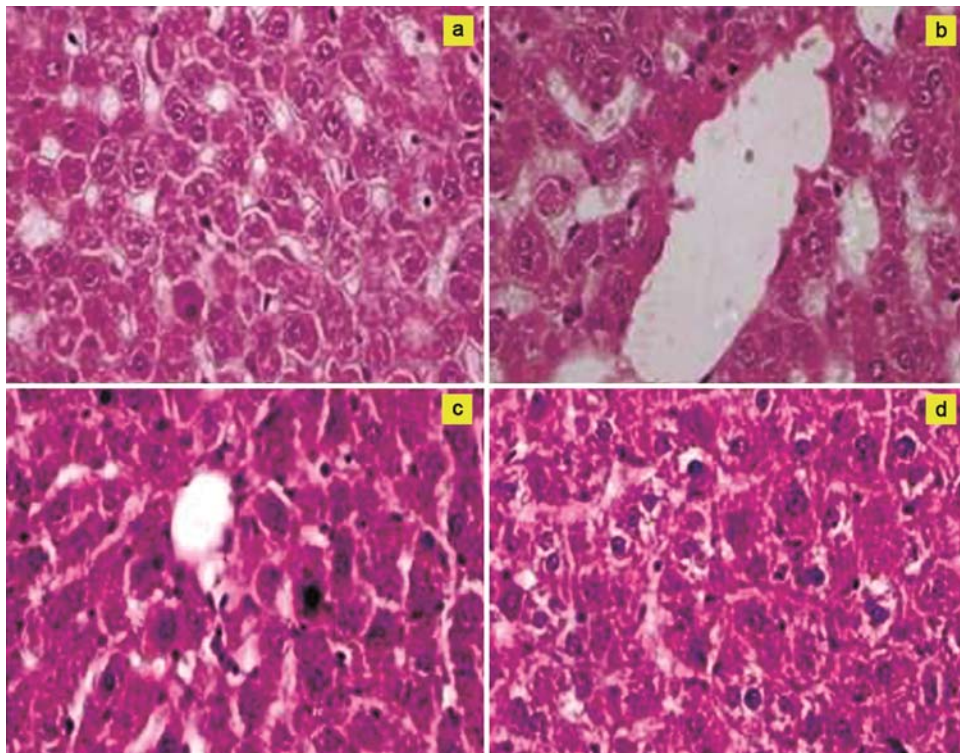


Plate 2 — Histology of liver of experimental rats after treatment with HAE, 500 mg/kg. a) Normal control, b) Diabetic control, c) Diabetic treated with HAE (500 mg/kg), d) Diabetic treated with metformin

The possible mechanism by which HAE brought about its hypoglycemic action might be by improving glycaemic control mechanism and by increasing insulin secretion from regenerated  $\beta$ -cells of pancreas<sup>19</sup>. It was further supported by histopathological observations which clearly revealed the presence of shrinkage, necrosis and damaged  $\beta$ -cell population in the endocrine region of the pancreas in STZ-induced diabetic rats. Our finding is consistent with an earlier report by Irudayaraj and co-authors<sup>2</sup>.

Diabetic rats treated with HAE showed an improvement in body weight in comparison to the diabetic control rats and standard metformin-treated rats, signifying the protective effect of HAE in controlling muscle wasting. Moreover, the ability of HAE to protect body weight loss might be the result of its ability to reduce hyperglycemia. The liver glycogen content was markedly reduced in diabetic animals, which was in proportion to insulin deficiency. Diabetic rats treated with HAE increased significantly the liver glycogen content as compared to diabetic control, which could be due to increased insulin secretion. The significant increase in the glycogen levels of the HAE treated diabetic animals might be because of the reactivation of the glycogen synthase system.

Elevation of plasma lipid concentration in diabetes is well documented. The most common lipid abnormalities in diabetes are hypertriglyceridemia and hypercholesterolemia. The increase in serum TG and TC observed in untreated diabetic rats is in agreement with the findings of Shirwaikar *et al.*<sup>20</sup>. Under normal circumstances, insulin activates enzyme lipoprotein lipase and hydrolyses triglycerides. Insulin deficiency results in failure to activate the enzymes leading to hypertriglyceridemia. Diabetic rats treated with HAE significantly improved serum TG and TC. The significant control of the levels of serum lipids in the HAE treated diabetic rats might be attributed to improvements in insulin levels. Significant lowering of LDL cholesterol and raise in HDL cholesterol were observed in treated diabetic rats. The HAE extract-treated animals showed a weight loss, which probably is due to the lipid-lowering activity of the extract or indirectly due to the influence on various lipid regulation systems. The lipid-lowering activity of the HAE may help in the prevention of diabetic complications like atherosclerosis and ischaemic conditions.

An increase in the activities of SGOT, SGPT and ALP in plasma of diabetic rats might be mainly due to

the leakage of these enzymes from the liver cytosol into the bloodstream which was an indicator of the hepatotoxic effect of STZ<sup>21</sup>. Treating diabetic rats with HAE reduced the activity of these enzymes compared to the diabetic control group. Reduction in plasma TPR was observed in diabetic rats. This might be due to the distinct metabolic renal alterations in the diabetic state, leading to a negative nitrogen balance, enhanced proteolysis and decreased protein synthesis. The plasma protein level was improved in diabetic rats after treatment with HAE. Diabetic rats showed a significantly increased level of CRTN, a reliable marker of renal dysfunction, in the serum. The significant reduction in the level of CRTN in HAE treated diabetic rats indicated that the HAE prevented the progression of renal damage in diabetic rats.

In diabetes mellitus, high glucose level can inactivate antioxidant enzymes SOD, CAT, GSH and GPx by glycosylating these proteins thus producing induced oxidative stress, which in turn, causes lipid peroxidation. Furthermore, malonaldehyde (MDA) is one of the end products in the LPO process. LPO in the tissue homogenate was determined by measuring the amounts of MDA produced primarily<sup>22</sup>. SOD, CAT, GSH and GPx activities were increased to normal indicating the efficacy of HAE in attenuating the oxidative stress (OS) and eventual inhibition of LPO in diabetic liver. A decrease in MDA level indicated a reduced rate of LPO in HAE treated diabetes.

Increasing evidence in both experimental and clinical studies suggest that OS plays a crucial role in the pathogenesis of both types (type 1 and type 2) of diabetes mellitus. Plant antioxidants play a vital role in the alleviation of diabetes. STZ produces oxygen radicals in the body, which cause pancreatic injury and could be responsible for increased blood sugar level in animals. Moreover, abnormally high levels of free radicals and the simultaneous decline of antioxidant defence mechanisms can lead to the development of insulin resistance<sup>23,24</sup>. In our study, a marked increase in the concentration of TBARS and MDA were observed in STZ induced diabetic rats indicating the LPO of tissues under OS. HAE significantly decreased TBARS levels as well as MDA in the liver of diabetic rats, indicating strong lipid peroxidation scavenging activity of HAE as an antioxidant agent. Superoxide directly initiates LPO and plays an important role in the formation of other ROS like hydroxyl radicals, which induce oxidative damage in lipids, proteins and DNA. Hydroxyl

radicals are more potent than superoxides, and HAE could effectively scavenge these radicals together with the inhibition of LPO, wherein it scavenged active oxygen species by preventing the propagation of free radical chain reaction as an antioxidant. The reducing property of HAE indicates it acts as electron donors, which reduce the oxidized intermediates of LPO process and subsequent reaction mechanism<sup>25-27</sup>. Studies<sup>28,29</sup> suggest that high molecular weight phenolic compounds including plant flavonoids comprising hydroxyl group and aromatic ring serve as potent free radical scavengers. The phenolic compounds may suppress LPO through different chemical mechanisms, including free radical quenching, electron transfer, radical addition, or radical recombinations. It is now assumed that the antioxidant activity is responsible for the antidiabetic action of the HAE, and phenolic compounds and flavonoids present in the HAE may be involved in reducing underlying cellular OS and eventual hypoglycemic reactions. Kusirisin *et al.*<sup>30</sup> have shown that phenolic compounds possess free radical scavenging properties and hence reduce the oxidative stress associated with diabetes mellitus. Aromatic plants are especially rich in phenolic and flavonoid contents and have been widely used in traditional medicine as a treatment for many diseases including diabetes. In fact, it was found that traditional medicines used in human diabetes also have significant antioxidant activity. Plants rich in phenolics, flavonoids and related substances, have antioxidant activity due to their redox properties, and as their free radicals scavenging ability is facilitated by hydroxyl groups. It has been reported that antioxidant properties of plant-derived phenolic compounds are brought about mainly via scavenging of superoxide anion radicals<sup>31,32</sup>. Literature reports<sup>33,34</sup> also tell us plant flavonoids have antioxidant activity *in vitro* and also act as antioxidants *in vivo*. Abdelmoaty *et al.*, (2010)<sup>35</sup> studied that quercetin, a plant flavonoid with antioxidant properties, has antidiabetic efficacy in diabetic rats. Flavonoids suppress reactive oxygen formation, chelated trace elements involved in free-radical production, scavenge reactive species and also protect antioxidant defences and thus help in the prevention of diseases.

### Conclusion

The findings of the present investigation justify the traditional use of *C. brachiata* (Lour.) Merrill (Rhizophoraceae) leaves in ethnomedicine of northeast India for the treatment of diabetes. The antioxidant activity of *C. brachiata* leaves reported herein

signifies the potential of the plant as a herbal antioxidant with a possible role in the prevention of oxidative stress-induced diabetes and associated disease complications. Studies are in progress in our laboratory to isolate bioactive principles from HAE extract of *C. brachiata* leaves and further exploration of biochemical mechanisms involved in antidiabetic action of isolated compounds. As this is the first report on the antioxidant activity of *C. brachiata*, thorough phytochemical analyses need to be executed in order to identify the possible antioxidant phenolic and flavonoid components responsible for antidiabetic activity.

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### Conflict of interest

The authors declare that there are no conflicts of interest.

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