

## Original Article

# Alcoholic Stem Extract of *Coscinium fenestratum* Regulates Carbohydrate Metabolism and Improves Antioxidant Status in Streptozotocin–Nicotinamide Induced Diabetic Rats

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Alcoholic extract of the stems of *Coscinium fenestratum*, a medicinal plant indigenous to India and Sri Lanka used in ayurveda and siddha medicine for treating diabetes, was studied for its carbohydrate metabolism effect and antioxidant status in streptozotocin–nicotinamide induced type 2 diabetic rats. Oral administration of *C. fenestratum* stem extract in graded doses caused a significant increase in enzymatic antioxidants such as catalase, superoxide dismutase, glutathione synthetase, peroxidase, and glutathione peroxidase and in the nonenzymatic antioxidants ascorbic acid, ceruloplasmin and tocopherol. Effects of alcoholic extract on glycolytic enzymes such as glucose-6-phosphate dehydrogenase, lactate dehydrogenase and hexokinase showed a significant increase in their levels, whereas a significant decrease was observed in the levels of gluconeogenic enzyme, glucose-6-phosphatase and alanine aminotransferase in treated diabetic rats. Serum creatinine and urea levels also declined significantly. This investigation demonstrates significant antidiabetic activity of *C. fenestratum*.

**Keywords:** carbohydrate metabolism – *Coscinium fenestratum* – enzymatic antioxidants – nonenzymatic antioxidants – streptozotocin–nicotinamide induced diabetes

## Introduction

Free radicals may play an important role in the causation and complication of diabetes mellitus. The increased oxidative stress and accompanying decrease in antioxidants may be related to the causation of diabetes (1). In diabetes mellitus, alterations in the endogenous free radical scavenging defense mechanisms may lead to ineffective scavenging of reactive oxygen species, resulting in oxidative damage and tissue injury (2). It has been proposed that streptozotocin acts as a diabetogenic owing to its ability to destroy pancreatic  $\beta$ -islet cells, possibly by a free radical mechanism. The level of lipid peroxidation in cells is controlled by various cellular defense mechanisms consisting of enzymatic and nonenzymatic scavenger systems (3,4), the levels of which are altered in diabetes (5).

*Coscinium fenestratum* Colebr. (Menispermaceae), commonly known as tree turmeric, grows widely in the Western Ghats (India) and Sri Lanka. The plant has been mainly used for treating diabetes mellitus in the traditional Ayurvedic and Siddha systems of medicine (6). The stem contains berberine, ceryl alcohol, hentriacontane, sitosterol, palmitic acid, oleic acid and saponin, together with some resinous material (7). Isolation of tertiary alkaloids, berlambine, dihydroberlambine and noroxyhydrastinine from the roots has been reported (8). The stem is used for dyspepsia, and as a febrifuge. Its hypotensive (9) and hepatoprotective (10) actions have also been reported. An earlier study carried out by the authors established the significant antidiabetic activity of *C. fenestratum* (11). In the present investigation we attempted to investigate further the alcoholic stem extract of *C. fenestratum* for its antioxidant status and its effect on key enzymes of carbohydrate metabolism in streptozotocin and nicotinamide induced type 2 diabetic rats.

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## Methods

### Chemicals and Instruments Used

The following chemicals were used for the study: glucose-6-phosphate dehydrogenase, glucose-6-phosphate, lactate dehydrogenase, streptozotocin (Sigma Aldrich Co., Germany); ascorbic acid, meta phosphoric acid, *O*-phosphoric acid, magnesium chloride, EDTA, sodium citrate (NICE Chemicals Pvt. Ltd, Cochin, India); phenazine methosulfate, nitroblue tetrazolium chloride, NADH, NADPH, ATP, glutathione, 5, 5' dithio nitro *bis* benzoic acid, tocopherol (Himedia Laboratories Ltd, Mumbai, India); disodium hydrogen phosphate, potassium hydrogen phosphate (E. Merck India Ltd, Mumbai, India); 2,4 dinitro phenylhydrazine (Sarabhai M. Chemicals, Baroda, India); sodium pyruvate (S.D. fine-chem Ltd, Mumbai, India); Tris buffer (SISCO Research Laboratories Ltd, Mumbai, India); sodium pyro phosphate (Thomas Baker Chemicals Ltd, Mumbai, India); nicotinamide (Qualigens Fine Chemicals, a division of Glaxo, Mumbai, India).

A UV spectrophotometer (Shimadzu 160 IPC), homogenizer, centrifuge and pH meter were the instruments used for the study.

### Albino Rats

Healthy adult male Wistar albino rats weighing ~250–300 g were used. Rats were housed in polypropylene cages, maintained under standard conditions (12 h light/12 h dark cycle;  $25 \pm 3^\circ\text{C}$ ; 35–60% humidity), and were fed with a standard rat pellet diet (Hindustan Lever Ltd, Mumbai, India) and water *ad libitum*. The study was approved by the Institutional Animal Ethical Committee of Kasturba Medical College, Manipal, India (IAEC/KMC/03/2003-04).

### Plant Material

The *C. fenestratum* plant material, purchased from Jogappa Shanbag Ayurvedic Store, Udupi, Karnataka, India during August 2003, was authenticated by Dr Gopalakrishna Bhat, Professor, Department of Botany, Poorna Prajna College, Udupi, Karnataka, India. A voucher specimen (PP 526) has been deposited at the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, India.

### Preparation of Alcoholic Stem Extract

Approximately 160 g of the stem powder was placed in a soxhlet extractor and extracted with ethanol for 72 h. The solvent was recovered by distillation *in vacuo*, and the residue (yield 18 g), stored in the desiccator, was used for subsequent experiments (12,13).

### Induction of Experimental Diabetes

An rat model of type 2 diabetes mellitus (non-insulin dependent diabetes mellitus, NIDDM) was induced (14) in overnight-fasted rats by a single intraperitoneal injection of 60 mg kg<sup>-1</sup> streptozotocin 15 min after the intraperitoneal

administration of 120 mg kg<sup>-1</sup> nicotinamide. Hyperglycemia was confirmed by elevated blood glucose levels determined at 72 h and then on day 7 after injection. Only rats confirmed to have permanent NIDDM were used for the antidiabetic study (15).

### Experimental Design

Rats were divided into four groups ( $n = 6$ ): normal rats administered with 2% gum acacia solution, diabetic rats administered with 2% gum acacia solution, diabetic rats administered with *C. fenestratum* alcoholic extract 250 mg kg<sup>-1</sup> and diabetic rats administered with *C. fenestratum* alcoholic extract 500 mg kg<sup>-1</sup>, respectively, for 12 days orally (16).

### Sample Collection

#### Blood Sample

At the end of day 12, blood samples were collected under light ether anesthesia retro-orbitally from the inner canthus of the eye using capillary tubes (Micro Hematocrit Capillaries, Mucaps). Blood was collected in fresh vials containing anticoagulant, and serum was separated in a centrifuge at 2000 r.p.m. for 2 min.

#### Collection of Organs

Rats were euthanized using an overdose of intraperitoneal anesthesia, and tissue samples collected for assessing the following parameters.

### Estimation of Enzymes in Carbohydrate Metabolism

The following parameters were evaluated.

#### Hexokinase (EC 2.7.1.1)

The hexokinase assay is based on the reduction of NAD<sup>+</sup> through a coupled reaction with glucose-6-phosphate dehydrogenase. The excised liver tissue homogenate was prepared in saline. To 0.1 ml of homogenate were added 2.28 ml of Tris (200 mmol l<sup>-1</sup>)-MgCl<sub>2</sub> buffer (20 mol l<sup>-1</sup>), pH 8, 0.5 ml of 0.67 M glucose, 0.1 ml of 16 mM ATP, 0.1 ml of 6.8 mM NAD and 0.01 ml of 300 U ml<sup>-1</sup> glucose-6-phosphate dehydrogenase. The solution was mixed thoroughly, and the absorbance was measured at 340 nm (17).

#### Glucose-6-Phosphate Dehydrogenase (EC 1.1.1.49)

The measure of glucose-6-phosphate dehydrogenase activity is the rate of increase in absorbance. Addition of maleimide inhibits oxidation of reaction products by 6-phospho gluconolactone. Liver tissue was excised and rinsed with saline solution and the homogenate prepared in saline solution. To 0.02 ml of homogenate were added 0.6 ml of distilled water, 0.1 ml of 3.8 mmol l<sup>-1</sup> NADP, 0.1 ml of 0.5 mol l<sup>-1</sup> Tris buffer (pH 7.5), 0.1 ml of 0.63 mol l<sup>-1</sup> MgCl<sub>2</sub> and 0.1 ml of 33 mol l<sup>-1</sup> glucose-6-phosphate. Then ~0.5 mg of maleimide was added, with gentle mixing to dissolve the maleimide. The absorbance was measured at 339 nm. One unit of enzyme

activity is defined as that quantity which catalyses the reduction of 1  $\mu\text{M}$  of NADP per minute (18).

#### Lactate Dehydrogenase (EC 1.1.1.27)

Lactate dehydrogenase catalyzes the conversion of L-lactate to pyruvate with simultaneous reduction and oxidation of NAD to NADH. Change in absorbance with time as a result of converting NAD to NADH is directly proportional to LDH activity. The liver and kidney homogenate was prepared in saline. To 0.05 ml of tissue homogenate was added 2.5 ml of Tris (81.3 mmol, pH 7.2)/NaCl (203.3 mmol)/NADH (0.244 mmol  $\text{l}^{-1}$ ), and the solution was mixed thoroughly. To this solution was added 0.5 ml of Tris (81.3 mmol, pH 7.2)/NaCl (203.3 mmol)/pyruvate (9.76 mmol  $\text{l}^{-1}$ ). The solution was mixed well and the absorbance measured at 339 nm (19).

#### Glucose-6-Phosphatase (EC 3.1.3.9)

Glucose-6-phosphatase catalyzes the conversion of glucose-6-phosphate to glucose. The liver was homogenized in ice-cold sucrose (250 mM) solution. To 0.1 ml of sucrose/EDTA buffer were added 0.1 ml of glucose-6-phosphate (100 mM), 0.1 ml of imidazole buffer (100 mM, pH 6.5) and 0.1 ml of homogenate, with thorough mixing. The tubes were incubated at 37 °C for 15 min. The enzymatic activity was terminated by the addition of 2 ml of TCA/ascorbate (10%/2%, w/v), and the solution was centrifuged at 3000 r.p.m. for 10 min. To 1 ml of clear supernatant were added 0.5 ml of ammonium molybdate (1%, w/v) and 1 ml of sodium citrate (2%, w/v). The absorbance was measured at 700 nm. The enzyme activity was expressed as unit per gram per minute in tissue (20).

#### Alanine Amino Transferase (EC 2.6.1.2)

Alanine amino transferase (ALT) catalyzes the transamination of L-alanine to 2-oxo glutarate, forming a glutamate and a pyruvate. The pyruvate formed is reduced to lactate by lactate dehydrogenase with simultaneous oxidation of reduced NADH to NAD. The change in absorbance with time as a result of the conversion of NADH to NAD is directly proportional to the ALT activity. The kidney homogenate was prepared in liver homogenate. To 0.2 ml homogenate were added 2.3 ml L-alanine (610 mmol) in Tris buffer (0.10 mol  $\text{l}^{-1}$ , pH 7.15), 0.1 ml of NADH (4.2 mmol  $\text{l}^{-1}$ ), 0.1 ml of pyridoxal 6 phosphate (3.4 mmol  $\text{l}^{-1}$ ) and 0.1 ml of lactate dehydrogenase 72 000 U  $\text{l}^{-1}$ . The reaction was initiated by the addition of 0.2 ml of 2-oxo glutarate solution. The absorbance was measured at 339 nm (21).

#### Urea and Creatinine

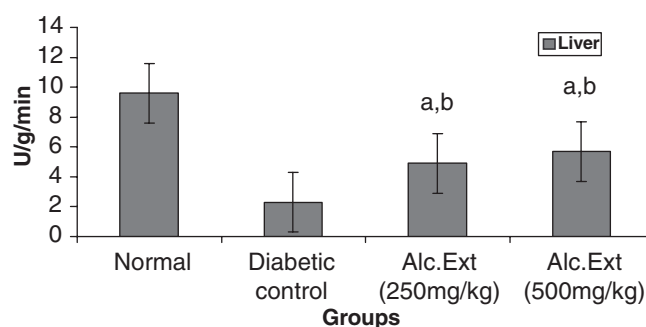
Serum urea level was determined using a Urease enzyme kit modified Berthlot method (Agappe Diagnostics, Thane, India). The creatinine level in serum was estimated using the alkaline picrate-modified Jaffe's method with a creatinine kit (Agappe Diagnostics).

### Estimation of Antioxidant Parameters

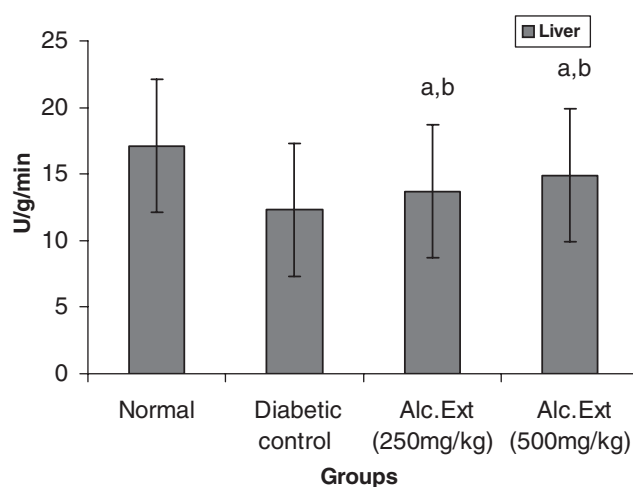
Enzymatic antioxidants [glutathione synthetase and glutathione peroxidase (22), catalase (EC 1.11.1.6) (23,24), peroxidase (EC 1.11.1.7) (25), superoxide dismutase (EC 1.15.1.1) (26)] and nonenzymatic antioxidants [ceruloplasmin (27), tocopherol (28) and ascorbic acid (29)] were determined. The protein content in the tissue homogenate was estimated following the standard methodology (30).

### Statistical Analysis

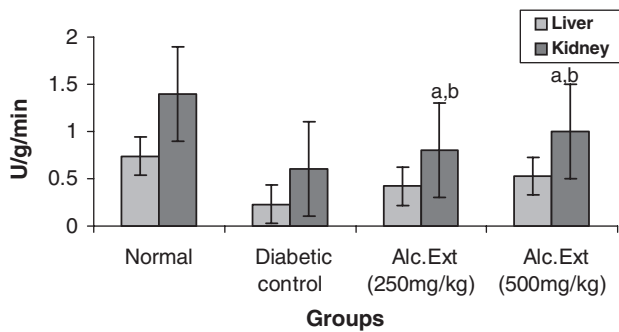
Data were statistically evaluated using one-way ANOVA, followed by a post hoc Scheffe's test using the SPSS computer software, version 7.5. The values were considered significant when  $P < 0.05$ .



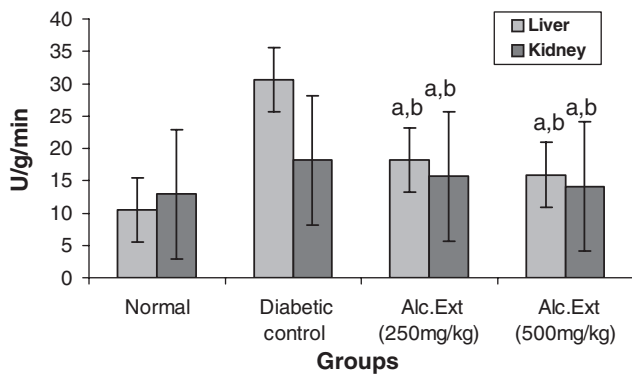
**Figure 1.** Alcoholic extract of *C. fenestratum* significantly increases hexokinase levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ); U,  $\mu\text{mol}$  reduction of NAD<sup>+</sup> per minute.



**Figure 2.** Alcoholic extract of *C. fenestratum* significantly increases glucose-6-phosphatase levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ); U,  $\mu\text{mol}$  conversion of NAD to NADH per minute.



**Figure 3.** Alcoholic extract of *C. fenestratum* significantly increases lactate dehydrogenase levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ); U,  $\mu\text{mol}$  conversion of NAD to NADH per minute.



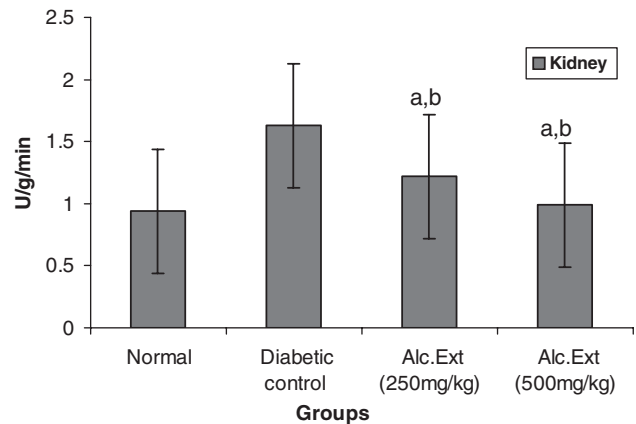
**Figure 4.** Alcoholic extract of *C. fenestratum* significantly decreases glucose-6-phosphatase levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ); U,  $\mu\text{mol}$  conversion of glucose-6-phosphate to glucose.

## Results and Discussion

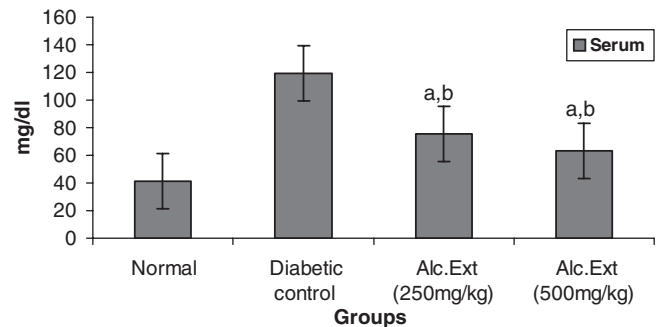
### Carbohydrate Metabolism

The activities of different key enzymes (hexokinase, glucose-6-phosphate dehydrogenase and lactate dehydrogenase) are represented in Figs 1, 2 and 3. Diabetic animals treated with 500 mg kg<sup>-1</sup> of the alcoholic extract of *C. fenestratum* showed better enzyme activity than those treated with the lower (250 mg kg<sup>-1</sup>) dose of the alcoholic extract. The significant increase in the levels of hexokinase, a key glycolytic enzyme known to decrease in the diabetic state (31), may be due to the direct stimulation of glycolysis in tissues with increased glucose removal from the blood. The significant reversal of diabetes induced decreased levels of glucose-6-phosphate dehydrogenase and lactate dehydrogenase may be attributed to an increase in glucose utilization through the pentose phosphate pathway (32), interfering with the mitochondrial respiratory chain and promoting the peripheral glucose utilization by enhancing anaerobic glycolysis (33).

Treated groups exhibited a significant decrease in the levels of glucose-6-phosphatase (Fig. 4) and alanine amino transferase



**Figure 5.** Alcoholic extract of *C. fenestratum* significantly decreases alanine amino transferase levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ); U,  $\mu\text{mol}$  conversion of NADH to NAD per minute.



**Figure 6.** Alcoholic extract of *C. fenestratum* significantly decreases urea levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ).

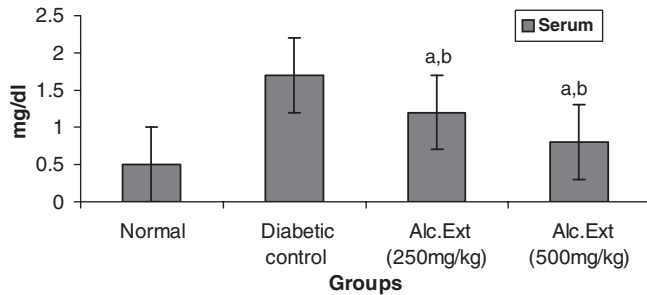
(Fig. 5). Glucose-6-phosphatase, a key enzyme in gluconeogenesis, plays an important role in glucose homeostasis in the liver and kidney (34). The decreased levels observed in treated diabetic animals may be because of the suppression of hepatic gluconeogenesis and glucose output from liver. The elevation in alanine amino transferase in liver and kidney observed in streptozotocin induced diabetic rats corroborates earlier findings (35,36) which attribute the increased gluconeogenesis and ketogenesis observed in diabetes to the high activity of transaminases. In our study, supplementation with the alcoholic extract decreased the enhanced transaminase activity significantly. Serum urea (Fig. 6) and creatinine levels (Fig. 7) were also decreased significantly compared with the diabetic control.

### Antioxidant Status

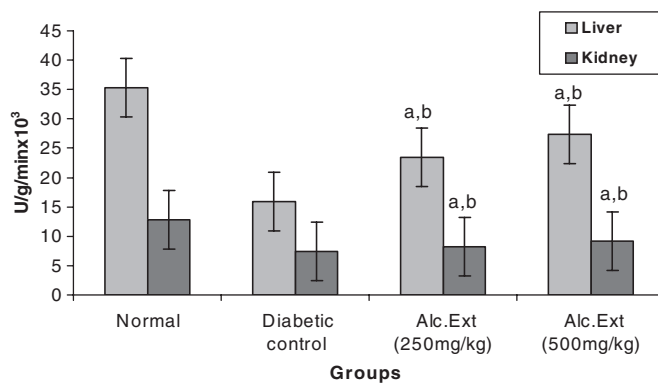
#### Enzymatic Antioxidants

Antioxidants are of two types: enzymatic and nonenzymatic antioxidants. Catalase, superoxide dismutase, peroxidase, glutathione synthetase and glutathione peroxidase are examples

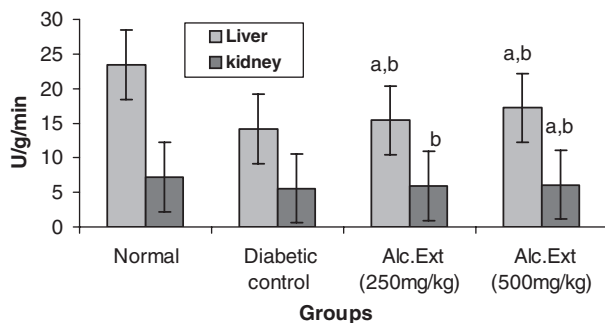
of enzymatic antioxidants. Superoxide dismutase and catalase are considered primary enzymes since they are involved in the direct elimination of reactive oxygen species (37). Superoxide dismutase is an important defense enzyme which catalyzes the dismutation of superoxide radicals (38), and catalase is a hemoprotein which catalyzes the reduction of hydrogen peroxides and protects tissues from highly reactive hydroxyl radicals (39). The reduced activity of superoxide dismutase



**Figure 7.** Alcoholic extract of *C. fenestratum* significantly decreases creatinine levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ).



**Figure 8.** Alcoholic extract of *C. fenestratum* significantly increases catalase levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ); U,  $\mu$ mol of hydrogen peroxide consumed per minute.

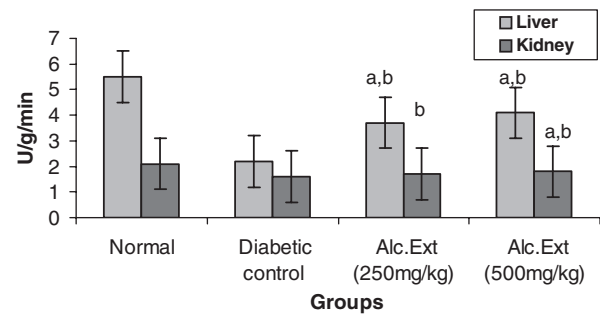


**Figure 9.** Alcoholic extract of *C. fenestratum* significantly increases glutathione peroxidase levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ); U,  $\mu$ mol of glutathione consumed per minute.

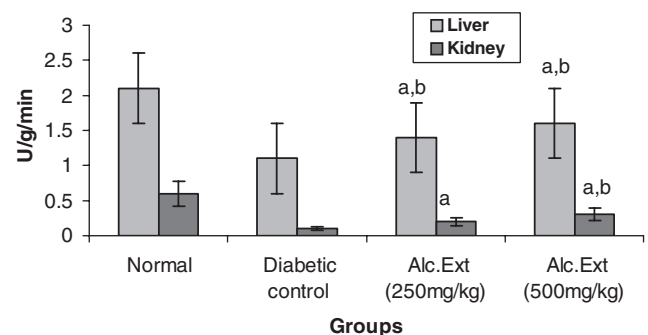
and catalase in the liver and kidney observed during diabetes may result in deleterious effects as a result of the accumulation of superoxide anion radicals and hydrogen peroxide (40). Glutathione synthetase, the most important biomolecule protecting against chemical induced toxicity, participates in the elimination of reactive intermediates by reduction of hydroperoxide in the presence of glutathione peroxidase (41,42). The decreased level of glutathione synthetase observed in the diabetic animals represents increased utilization resulting from oxidative stress (43). Glutathione peroxidase, a selenium-containing enzyme present in significant concentrations, detoxifies  $H_2O_2$  to  $H_2O$  through the oxidation of reduced glutathione (44). Depression of glutathione peroxidase activity, observed in diabetic liver and kidney, has been shown to be an important adaptive response to increased peroxidative stress (45). The activity of enzymatic antioxidants [catalase, glutathione peroxidase, glutathione synthetase, peroxidase and superoxide dismutase (Figs 8–12)] increased significantly in extract-treated animals ( $P < 0.05$ ).

#### Nonenzymatic Antioxidants

Tocopherol, ceruloplasmin and ascorbic acid are nonenzymatic antioxidants. Whereas  $\alpha$ -tocopherol reduces lipid

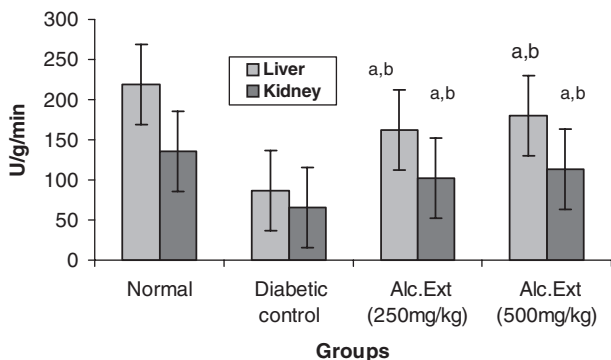


**Figure 10.** Alcoholic extract of *C. fenestratum* significantly increases glutathione synthetase levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ); U,  $\mu$ mol of 1-Chloro-2,4-dinitro benzene (CDNB)–glutathione synthetase conjugate formed per minute.

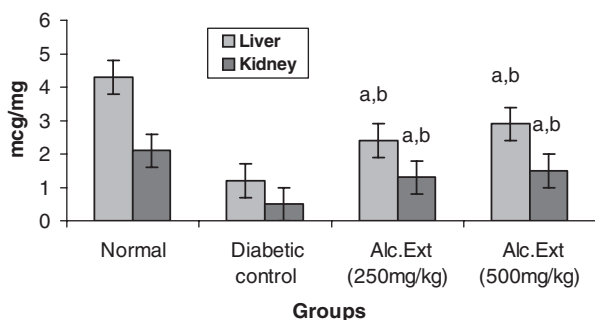


**Figure 11.** Alcoholic extract of *C. fenestratum* significantly increases peroxidase levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ); U,  $\mu$ mol of hydrogen peroxide consumed per minute.





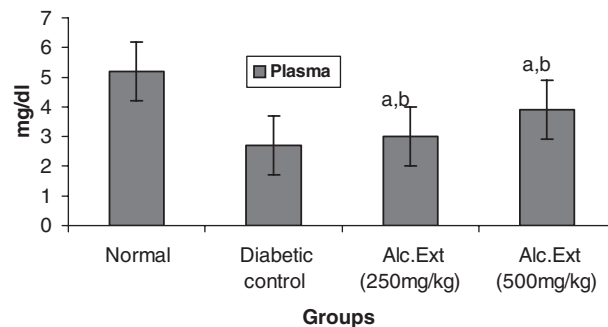
**Figure 12.** Alcoholic extract of *C. fenestratum* significantly increases superoxide dismutase levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ); U,  $\mu$ mol inhibition of Nitro blue tetrazolium chloride reduction per minute.



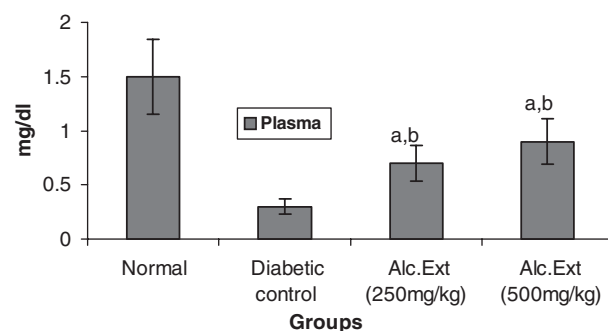
**Figure 13.** Alcoholic extract of *C. fenestratum* significantly increases tocopherol levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ).

hydroperoxides generated during the process of peroxidation and protects cell structures against damage (46), the powerful nonenzymatic antioxidant ceruloplasmin inhibits lipid peroxidation by binding to copper (47). Vitamin C or ascorbic acid is an excellent hydrophilic antioxidant in plasma and disappears faster than other antioxidants on exposure to reactive oxygen species (48). The decreased level of ascorbic acid in diabetic rats may be due either to increased utilization as an antioxidant defense against increased reactive oxygen species or to a decrease in glutathione level, since glutathione is required for the recycling of ascorbic acid (49,50). The effect of treatment with *C. fenestratum* on the nonenzymatic antioxidants tocopherol, ceruloplasmin and ascorbic acid is presented in Figs 13, 14 and 15.

In our investigations, the levels of both enzymatic and nonenzymatic antioxidants, which declined in the diabetic animals, were significantly restored on treatment with the alcoholic extract. The overexpression of these antioxidant parameters in diabetic rats treated with *C. fenestratum* implies that this potential oxidant defense is reactivated by the active principles of *C. fenestratum*, with an increase in the capacity for detoxification through enhanced scavenging of oxy radicals.



**Figure 14.** Alcoholic extract of *C. fenestratum* significantly increases ceruloplasmin levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ).



**Figure 15.** Alcoholic extract of *C. fenestratum* significantly increases ascorbic acid levels in diabetic rats. Each value represents mean  $\pm$  SE,  $n = 6$ ; a, statistical significance versus control ( $P < 0.05$ ); b, statistical significance versus normal ( $P < 0.05$ ).

The tetrahydro protoberberine analogue of berberine has been reported to inhibit lipid peroxidation and to scavenge hydroxyl free radicals (51). Preliminary phytochemical screening of *C. fenestratum* showed the presence of phenolic compounds (11), which have been reported to have antioxidant activity (52). Hence in our study the antioxidant activity of *C. fenestratum* may be due to the presence of berberine and phenolic compounds.

The present investigation draws out a sequential metabolic correlation between increased glycolysis and decreased gluconeogenesis stimulated by *C. fenestratum*, which may have been the biochemical mechanism through which glucose homeostasis was regulated. The enhanced effect and the protective action on cellular antioxidant defense of *C. fenestratum* suggest protection against oxidative damage in streptozotocin–nicotinamide induced diabetes.

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