Protective role of *Cassia auriculata* leaf extract on hyperglycemia-induced oxidative stress and its safety evaluation

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*Cassia auriculata* L. (Caesalpiniaceae) is widely used from the ancient period to treat diabetes mellitus. In the present study, the antioxidant activity of *C. auriculata* aqueous leaf extract (CLEt) was evaluated in streptozotocin-induced mild diabetic (MD) and severe diabetic (SD) rats. A short-term toxicity assessment was also conducted in healthy rats to examine toxic effects of the extract. Oral administration of CLEt to MD and SD rats (100, 200 and 400 mg/kg body weight per day for a period of 21 days) produced significant fall in fasting blood glucose (FBG) in a dose-dependent manner. Treatment with the extract (400 mg/kg) showed significant reduction in serum levels of thiobarbituric acid reactive substances (TBARS) and oxidized low-density lipoprotein (OxLDL) in both MD and SD rats. The antioxidant defense system was also found to be improved in CLEt-treated (400 mg/kg) MD and SD rats, as revealed by significant increase in activities of erythrocyte’s antioxidant enzymes i.e. superoxide dismutase (SOD) and catalase (CAT) with a concomitant elevation in erythrocyte’s reduced glutathione (GSH) content. Moreover, there were no toxic signs in rats treated with high doses of the extract (1000 and 2000 mg/kg body weight per day for 21 days). Blood glucose, hepatic and renal function parameters in these rats were found within normal limits. Phytochemical screening of CLEt revealed the presence of alkaloids, flavonoids, saponins, tannins and cardiac glycosides with antihyperglycemic and antioxidant properties. This study suggests that CLEt possesses potent antioxidant activity along with antihyperglycemic potential, hence protective against diabetic complications.

**Keywords:** *Cassia auriculata*, Diabetes mellitus, Streptozotocin, Phytochemicals, Antioxidant activity, Toxicity assessment

Oxidative stress, defined as a persistent imbalance between the production of free radicals and antioxidant defenses leads to many biochemical changes and is an important causative factor in several chronic diseases such as atherosclerosis, cancer, inflammatory disorders and aging process¹². It has been postulated that the etiology of the complications of diabetes involves oxidative stress as a result of hyperglycemia, which in turn may result in cellular damage³.

Though several pharmacological agents have been developed for management of diabetes, many traditional plant treatments are still used throughout the world. In India, several indigenous plant products have been used by the practioners of the Ayurvedic system to treat diabetes⁴. Recent years have witnessed a renewed interest in plant-based therapy, as plants synthesize a variety of secondary metabolites having antidiabetic and antioxidant potential⁵-⁷.

*Cassia auriculata* L. (Caesalpiniaceae) a shrub, characterized by presence of leafy stipules is found in the regions of Asia. In Ayurvedic medicine, it is widely used for the control of sugar levels and reduction of symptoms like polyuria and thirst in diabetics⁸. It has also been shown to have antiviral, antispasmodic and antipyretic activities⁹,¹⁰. Recently, we have demonstrated the antihyperglycemic and hypolipidemic effect of aqueous extract of *C. auriculata* leaves in streptozotocin (STZ)-induced diabetic rats¹¹. However, the reports are lacking on the antioxidant potential of aqueous extract of *C. auriculata* leaves in experimental diabetes. Thus, in the present study, the effect of *C. auriculata* aqueous leaf extract (CLEt) on hyperglycemia-induced oxidative stress has been investigated by measuring thiobarbituric acid reactive substances (TBARS), oxidized low-density lipoprotein (OxLDL), reduced glutathione (GSH) and antioxidant enzymes, such as superoxide dismutase (SOD) and catalase.
(CAT) in STZ-induced mild and severe diabetic rats. In addition, toxicity assessment of the extract has also been studied.

Materials and Methods

Animals
Male albino Wister rats (weighing 160-200 g) were procured from Central Animal House of University College of Medical Sciences (UCMS), Delhi, India. The animals were housed in standard conditions of temperature (22 ± 2°C) and 12 h light-dark cycle. The rats were fed with commercial diet (Hindustan Liver Ltd., Mumbai) and water ad libitum. The experimental protocol was approved by Institutional Animal Ethical Committee of UCMS, Delhi. All experimental procedures were conducted in accordance to the Ethical Guidelines of International Association for the Study of Pain.

Plant material and preparation of extract
The leaves of Cassia auriculata were collected from Nagercoil, Tamilnadu, India. The identification of the plant was done by Dr. Sudhanshu Shekher Dash, a taxonomist in the Botanical Garden of Indian Republic, Noida, India, where a voucher specimen was lodged for future reference (Voucher specimen no. 1605).

The leaves were dried under shade and machine grounded to soft powder. The leaf powder (100 g) was suspended in 500 ml of cold distilled water overnight and then filtered. The whole procedure was carried out at 4°C. The filtrate i.e. aqueous extract was lyophilized. The yield of aqueous extract was approximately 10 g/100 g of leaves.

Phytochemical screening
The methods as described previously were used to screen the aqueous extract of leaves for its chemical constituents.

Induction of experimental diabetes
To induce diabetes, a freshly prepared solution of STZ (45 mg/kg body weight in 0.1 M citrate buffer, pH 4.5) was injected intraperitoneally in a volume of 1 ml/kg to overnight fasted rats. After 48 h of STZ administration, fasting blood glucose (FBG) levels were measured and again repeated twice at intervals of three days. The rats with stabilized diabetes having FBG values between 150 and 250 mg/dl were considered as mild diabetic (MD) and those with FBG values of above 250 mg/dl as severe diabetic (SD).

Experimental procedure
The rats were divided into three groups (five animals in each group or subgroup). Group I served as normal control. Groups II and III served as MD and SD, respectively. Both groups II and III were divided into five subgroups as follows: Subgroup A, diabetic control rats; subgroups B, C and D, diabetic rats treated with C. auriculata aqueous leaf extract (100, 200 and 400 mg/kg body weight, respectively) and subgroup E, diabetic rats treated with glibenclamide (600 µg/kg body weight).

Control rats received vehicle i.e., distilled water and treated rats received CLEt or glibenclamide in 1 ml of distilled water. The vehicle and drugs were orally administered every morning for 21 days using a standard orogastric cannula. Blood samples were drawn from overnight fasted rats by retro-orbital venepuncture technique. FBG was measured before the treatment (day 0) and after the treatment on days 7 and 21 using the glucose oxidase-peroxidase method. Antioxidant parameters were analyzed at the end of the experimental period.

Antioxidant assay
Lipid peroxides were measured in serum as TBARS by using the standard method. TBARS were estimated by reaction with thiobarbituric acid in the presence of butanol and measuring the absorbance spectrophotometrically at 530 nm of pink colored chromogen formed. OxLDL in serum was estimated by the baseline levels of diene conjugates in lipid fraction of LDL. The activity of SOD in erythrocytes was determined according to the previously described method with some modifications. The method is based on the inhibition of pyrogallol autoxidation in the presence of SOD. Catalase in red blood cells and GSH content in erythrocytes were also assayed.

Toxicity assessment
To evaluate the toxicity profile of aqueous leaf extract, two separate groups of healthy rats (five animals per group) were treated with the extract at a dose of 1000 and 2000 mg/kg body weight per day for 21 days and one group is taken as control. The rats were observed during first 4 h and then after every 24 h up to 1 week for any physical signs of toxicity and/or mortality. FBG was determined on days 0 and 21. Hepatic function tests such as alanine aminotransferase (ALAT) and alkaline phosphatase (ALP) as well as renal function tests such as urea and
Creatinine were performed in serum at the end of the experiment (day 21) using standard kits. Body weight was also recorded, prior to treatment and then post-treatment.

Statistical analysis
All data were presented as mean ± SEM for five animals in each group. Statistical analysis was performed by using repeated measure analysis of variance (ANOVA), followed by Dunnett’s multiple comparison test and one-way ANOVA, followed by Tukey’s multiple comparison test. A value of \( p < 0.05 \) was considered significant.

Results
Phytochemical screening
The preliminary phytochemical screening of the aqueous extract of \( C. \) auriculata leaves revealed the presence of alkaloids, flavonoids, saponins, tannins, phenols and cardiac glycosides.

Antihyperglycemic activity
Table 1 gives the levels of FBG in normal, MD and SD rats at various day intervals. Oral administration of CLEt (100, 200 and 400 mg/kg) and glibenclamide produced significant reduction \((p<0.001)\) in FBG levels from day 7 to day 21 in both MD and SD rats, when compared with day 0. On the other hand, MD and SD controls maintained the marked hyperglycemia without any significant change throughout the experimental period. The antihyperglycemic effect of the extract was found to be dose-dependent. Therefore, further studies were carried out with the dose of 400 mg/kg. These results were consistent with our earlier study showing 400 mg/kg dose of the extract as an effective dose in a one-day treatment\(^\text{11}\).

Antioxidant activity
Fig. 1 demonstrates the effect of CLEt on TBARS, OxLDL, SOD, CAT and GSH in MD and SD rats. A significant elevation \((p<0.001)\) in the serum levels of TBARS (Fig. 1A) and OxLDL (Fig. 1B) was observed in both MD and SD control rats as compared to normal. The supplementation with the extract for 3 weeks significantly reverted back \((p<0.001)\) the TBARS (products of lipid peroxidation) and OxLDL levels towards normal in both MD and SD rats. Glibenclamide-treated MD and SD rats also showed reduction in the levels of TBARS and OxLDL, but it was found to be less significant.

As depicted in Fig. 1C, the activity of antioxidant enzyme SOD in erythrocytes of MD and SD control rats was found to be significantly lower \((p<0.001)\) than the normal group. Following treatment with the extract, significant increase \((p<0.001)\) in the activity of erythrocyte’s SOD was observed in both MD and SD rats. The activity of another important antioxidant enzyme CAT was also suppressed in MD \((p<0.01)\) and SD \((p<0.001)\) control rats compared to normal (Fig. 1D). However, erythrocyte’s CAT activity was significantly increased in extract-fed MD \((p<0.01)\) as well as SD \((p<0.001)\) rats. The effect of glibenclamide on antioxidant enzymes was lower than that of the extract in both models of diabetic rats.

| Table 1—Changes in the levels of fasting blood glucose in normal, mild and severe diabetic rats |
|------------------------|------------------------|------------------------|
| Group                  | Dose (mg/kg)           | Fasting blood glucose (mg/dl) |
|                        | Day 0                  | Day 7                  | Day 21                  |
| Normal control         | -                      | 78.0 ± 2.6             | 79.2 ± 1.6             | 82.0 ± 1.9               |
| Mild diabetic          |                         |                        |                        |
| Diabetic control       | -                      | 206.0 ± 13.2           | 211.4 ± 10.7           | 214.2 ± 11.1             |
| Diabetic + Glibenclamide | 600 mg             | 195.6 ± 11.4           | 152.8 ± 9.3*           | 24.6 ± 9.2*              |
| Diabetic + CLEt        | 100 mg                 | 198.4 ± 8.6            | 173.8 ± 9.1*           | 134.2 ± 8.5*             |
|                        | 200 mg                 | 200.2 ± 6.8            | 160.6 ± 7.2*           | 129.4 ± 6.2*             |
|                        | 400 mg                 | 197.8 ± 10.5           | 142.6 ± 11.0*          | 113.8 ± 9.2*             |
| Severe diabetic        |                         |                        |                        |
| Diabetic control       | -                      | 322.8 ± 15.5           | 315.2 ± 16.0           | 317.8 ± 17.1             |
| Diabetic + Glibenclamide | 600 mg             | 314.4 ± 13.6           | 253.4 ± 13.9*          | 196.8 ± 11.0*            |
| Diabetic + CLEt        | 100 mg                 | 315.2 ± 14.6           | 275.4 ± 8.8*           | 235.8 ± 12.9*            |
|                        | 200 mg                 | 310.4 ± 13.5           | 237.6 ± 14.7*          | 189.6 ± 10.1*            |
|                        | 400 mg                 | 312.6 ± 9.6            | 216.8 ± 10.2*          | 159.2 ± 7.7*             |

\*\( p < 0.001 \) compared to day 0
CLEt, Cassia auriculata leaf extract
As shown in Fig. 1E, both MD and SD control animals showed significantly low ($p<0.001$) levels of erythrocyte’s GSH, when compared with normal. The GSH content was found to be significantly elevated ($p<0.001$) in erythrocytes of MD as well as SD rats following supplementation with the extract. The increased erythrocyte’s GSH level was also observed in glibenclamide-treated MD and SD rats. However, it was significant only at 5% level in both diabetic models of rats.

Safety profile

Oral administration of CLEt up to a dosage of 2000 mg/kg per day for 21 days produced no adverse effect on the general behavior or appearance of the animals and all the rats survived during the test period. There were no signs of toxic symptoms such as restlessness, respiratory distress, diarrhea, convulsions and coma. After administration of high doses of the extract, none of the animals experienced the hypoglycemic episodes as shown in Table 2. Like control animals, body weight of treated animals was found to be significantly increased (Table 2). Table 3 shows the effect of the extract on hepatic and renal function parameters. The levels of ALP, urea and creatinine were significantly decreased in the animals treated with 2000 mg/kg dose of the extract, compared to control group. Although this dose also produced reduction in the level of ALAT, but it was not statistically significant.

Discussion

In recent years, natural products of plant origin are finding increasingly wide use for the treatment of various human diseases. They play a vital role in the treatment of diabetes and its associated complications. The plants synthesize a variety of secondary metabolites with antihyperglycemic and antioxidant potential which can play a major role in protection against hyperglycemia-induced oxidative stress$^{5-7}$. In the present study, the phytochemical screening of the aqueous extract of *C. auriculata* leaves revealed the presence of alkaloids, flavonoids, saponins, tannins, phenols and cardiac glycosides. Earlier, the presence of flavone glycoside (7',4'-dihydroxyflavone-5-O-β-D-galactopyranoside), anthocyanidin glycoside (pelargonidin-5-O-β-D-galactoside) and dimeric procyanidins such as fisetinidol-(4→8°)-catechin and fisetinidol-(4→8°)-gallocatechin has been reported in different parts of *C. auriculata* $^{24}$. These phytochemicals have been shown to be endowed with antidiabetic and antioxidant activities $^{7,25-30}$.
As alkaloids, flavonoids, saponins, tannins etc., act as antioxidants by their ability to scavenge reactive oxygen species (ROS) and/or inhibit lipid peroxidation\textsuperscript{31-35}, the presence of these constituents may account for the observed pharmacological effects of the extract. A number of other plants containing such active compounds have been reported to combat oxidative stress\textsuperscript{36-38}. STZ, a potent cytotoxic agent of pancreatic $\beta$-cells\textsuperscript{39} induces diabetes by rapid depletion of $\beta$-cells, resulting in a reduction of insulin secretion. In diabetes, persistent hyperglycemia causes oxidative damage by generation of ROS\textsuperscript{40}, which in turn leads to development of diabetic complications\textsuperscript{41,42}. Further, the STZ-induced diabetic animals may exhibit most of the diabetic complications including enhanced oxidative stress\textsuperscript{43}. Oxidative stress is a common pathway linking diverse mechanisms for the complications of diabetes and thus implicated in the pathophysiology of diabetes\textsuperscript{44,45}.

The deleterious effects of ROS can be prevented by antioxidant defense system of the body. This shows the importance of antioxidant defense system in maintaining the normal physiology. It suggests that the ideal treatment for diabetes should have favorable effect on antioxidant status, in addition to antihyperglycemic activity. The capacity of aqueous leaf extract of \textit{C. auriculata} to significantly bring down the elevated levels of blood glucose in both MD and SD rats shows its antihyperglycemic activity, which is an essential trigger for the development of normal homeostasis during experimental diabetes.

Lipid peroxidation, a common consequence of cell death is one of the features of chronic diabetes and lipid peroxide-mediated damage has been observed in both type I and type II diabetes mellitus. The studies have shown an elevation in lipid peroxidation in experimental diabetes\textsuperscript{46}. This is also evident in the present study, as STZ-induced diabetic rats showed increased serum levels of TBARS (products of lipid peroxidation). Administration of CLEt to MD and SD rats significantly brought down the TBARS levels, indicating the suppression of lipid peroxidation.

Overproduction of free radicals in diabetes leads to formation of OxLDL which has atherogenic role\textsuperscript{47}. The levels of this modified LDL were also decreased in extract-fed MD as well as SD rats.

Biological systems protect themselves against the damaging effect of ROS through two major antioxidant enzymes i.e., SOD and CAT\textsuperscript{48}. Reduced activity of these enzymes in erythrocytes has been observed in diabetes and this may result in a number of deleterious effects due to accumulation of superoxide radicals and $H_2O_2$\textsuperscript{49}. The present study also revealed low SOD and CAT activities in red blood cells of both MD and SD rats. The activity of these enzymes was partially restored,
following supplementation with the extract in both models of diabetic rats. The increase in SOD and CAT activities by treatment with CLEt evidently showed its antioxidant property against oxygen free radicals.

In addition to reduced activity of antioxidant enzymes, depletion in GSH levels of erythrocytes was also observed in both MD and SD rats. GSH, an important antioxidant is involved in the removal of hydro- and organic-peroxides that are formed as products of normal cellular processes or toxic insults. In diabetes, it is found to be decreased remarkably. Treatment with the extract increased the erythrocyte’s GSH levels in both MD and SD rats, which is essential to maintain the structural and functional integrity of the erythrocytes.

The restoration of altered lipid peroxidation and antioxidant defense system, following treatment with the extract could be primarily due to the subsequent lowering of blood glucose levels. It has been postulated that free circulating glucose is the proximal source of increased oxidative stress in hyperglycemic condition. Glucose has been demonstrated to undergo autoxidation generating oxygen free radicals. Autoxidation of glucose is directly linked to protein glycation, which is another source of free radicals. Thus, the lowering of blood glucose levels would prevent the formation of ROS and oxidative stress.

In MD as well as SD rats, the antihyperglycemic activity of CLEt was comparable to that of glibenclamide, a second generation sulphonylurea. However, the antioxidant effect of the extract was significantly better than that of glibenclamide in both MD and SD rats. This suggested that the CLEt decreased oxidative stress not only by controlling the blood glucose, but it might also have direct impact on antioxidant defense system and pathways, leading to free radical generation. A number of other plant extracts have been reported to have antioxidant activity. A polyphenol-rich extract from seeds of Fenugreek is reported to protect diabetic human erythrocytes against hydrogen peroxide-induced oxidative stress.

The rats treated with up to 2 g/kg dose of the extract for 21 days survived and appeared active and healthy. No signs of abnormal behavior, hypoglycemic episodes, adverse toxicological modification in liver and kidney and/or mortality were observed. The decreased levels of liver and kidney function parameters in extract-treated animals compared to control animals indicated the protective role of the extract in diabetes associated complications. Also, no significant change was observed in the body weight gained between control and treated rats. Therefore, the extract appeared to be relatively safe up to the dose of 2 g/kg.

In conclusion, the present study revealed that CLEt decreased oxidative stress in both MD and SD rats, which might be due to its antioxidant action. The antidiabetic action of CLEt may be attributed to free radical scavenging and antioxidant activities of the extract. The relatively non-toxic nature of extract suggests it a potential therapeutic candidate for more detailed investigations.

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