Salutary Effect of *Cassia auriculata* L. Leaves on Hyperglycemia-Induced Atherosclerotic Environment in Streptozotocin Rats

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Abstract Diabetes mellitus is very often associated with dyslipidemia, increased oxidative stress and endothelial dysfunction that could develop atherosclerosis and consequently cardiovascular diseases. Medicinal plants with reputed traditional use to treat diabetes and cardiovascular diseases might provide valuable drugs. Therefore, the present study was designed to evaluate anti-atherosclerotic potential of aqueous extract of *Cassia auriculata* L. leaves in streptozotocin (STZ)-induced diabetic rats. The rats were rendered diabetic by STZ (45 mg/kg, ip). Diabetic rats were orally administered *C. auriculata* leaf extract at 400 mg/kg dose daily for 21 days. The supplementation of extract to the diabetic rats produced significant reduction in fasting blood glucose along with significant reversal in altered serum lipid profile and apolipoprotein B. Lipid peroxidation was found to be significantly suppressed in extract-fed diabetic rats. The significant reduction in serum levels of oxidized low-density lipoprotein, soluble vascular cell adhesion molecule and plasma fibrinogen with a concomitant elevation in serum nitric oxide was observed in diabetic rats following treatment with extract. Histopathological examination of heart myocardium of extract-treated diabetic rats revealed reversal of fatty change toward normal. These results suggest that *C. auriculata* aqueous leaf extract exhibits anti-atherosclerotic role in the diabetic state and it indicates toward the notion that extract may help to prevent the progression of cardiovascular diseases.

Keywords Atherosclerosis · *Cassia auriculata* · Diabetes mellitus · Endothelial dysfunction · Streptozotocin

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Introduction

The most common and life-threatening disorder that besets diabetic patients is cardiovascular diseases (CVD) [1]. Diabetic subjects have been shown to have high risk for CVD compared to non-diabetic population [2]. Atherosclerosis, a process which is exacerbated during diabetes, leads to the development of CVD. A number of factors have been attributed to the development of atherosclerosis in diabetes such as derangement in lipid and lipoprotein metabolism, oxidative stress, inflammation, endothelial dysfunction and coagulation abnormalities [3–5].

Several cardioprotective drugs are available in the pharmaceutical market, however, these drugs lag behind the desired properties such as efficacy and safety on long term use as well as cost. These factors do not fulfill the conditions for patient compliance [6]. Plants are the mines of bioactive phytochemicals that might serve as lead for the development of effective, safer and cheap novel drugs. A number of medicinal plants have shown their beneficial effect on CVD by virtue of lipid-lowering, antioxidant and anti-inflammatory effects [7].

Cassia auriculata (Caesalpiniacae) is a shrub characterized by the presence of leafy stipules, found in the regions of Asia. Indigenous people use different parts of

the plant for the treatment of various ailments [8, 9]. In Ayurvedic medicine, it is widely used as "Kalpa drug" or "Avarai Panchaga Choornam" for the control of sugar levels [10]. It is also one of the main constituents of many antidiabetic herbal formulations such as Diamed, Dianex and Diasulin [11–13].

The antidiabetic potential of *C. auriculata* flowers and leaves is well documented in the literature [10, 14]. Previously, we have also demonstrated the antidiabetic effect of aqueous extract of *C. auriculata* leaves in experimental diabetes [15]. The present study demonstrates the anti-atherosclerotic potential of *C. auriculata* aqueous leaf extract (CLEt) in streptozotocin (STZ) diabetic rat model. The study was designed to evaluate the effect of CLEt on lipid profile, apolipoprotein B (ApoB), lipid peroxidation and markers of endothelial function such as oxidized low-density lipoprotein (OxLDL), nitric oxide (NO), soluble vascular adhesion cell molecule-1 (sVCAM-1) and fibrinogen. Histopathological studies of heart tissue were also performed. The current study has first explored the effect of this plant on endothelial dysfunction and pathological changes of heart to establish its anti-atherosclerotic potential.

Materials and Methods

Plant Material and Preparation of Extract

The leaves of *C. auriculata* were collected from Nagercoil, Tamilnadu, India. The plant was authenticated by Dr. Sudhanshu Shekher Dash, a taxonomist in the Botanical Garden of Indian Republic, Noida, India, where a voucher specimen was lodged for further reference (Voucher specimen no. 1605).

The leaves were dried in the shade and ground in an electric grinder to a 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 brownish yellow 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 by Harborne [16] were used to screen the aqueous extract of *C. auriculata* leaves for its chemical constituents.

Experimental 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 polypropylene cages lined with husk under standard temperature conditions (22 ± 2°C). A 12 h light–dark cycle was maintained. The rats were fed on a standard pellet diet (Hindustan Liver Ltd., Mumbai) and water ad libitum. The animal 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 [17].

Experimental Induction of Diabetes

A freshly prepared solution of STZ (45 mg/kg body weight in 0.1 M citrate buffer, pH 4.5) was injected intra-peritoneally to overnight fasted rats [18]. The animals exhibited hyperglycemia within 48 h of STZ administration. The rats having fasting blood glucose (FBG) values of 250 mg/dl or above were considered for the study.

Experimental Design

Previously, we have shown 400 mg/kg body weight dose of CLEt as an effective dose in controlling hyperglycemia among the various doses tested i.e., 100, 200 and 400 mg/kg body weight [15]. Therefore, 400 mg/kg body weight dose of CLEt was used for the treatment of the animals to study its effect on hyperglycemia-induced atherosclerotic environment.

The rats were divided into four groups, with five rats in each group, as follows: (1) Group 1, normal control; (2) Group 2, diabetic control; (3) Group 3, diabetic rats treated with CLEt at a dose of 400 mg/kg body weight; (4) Group 4, diabetic rats treated with glibenclamide at a dose of 600 μg/kg body weight.

Control rats (groups 1 and 2) received vehicle i.e., distilled water. Group 3 received the extract dissolved in 1 ml of distilled water and group 4 received the standard antidiabetic drug, glibenclamide suspended in 1 ml of distilled water. The treatment was given daily for a period of 21 days using standard orogastric cannula. Blood samples were withdrawn from overnight fasted rats by retro-orbital venepuncture technique [19]. FBG was measured before the treatment (day 0) and on days 7, 14 and 21 after the treatment. All other parameters were analyzed at the end of the experiment period. After blood collection, the animals were euthanized through CO_2 inhalation to excise the heart for histopathological study. The heart was preserved in 10% neutral formalin and processed for paraffin embedding, sectioned at 5 μm and stained with hematoxylin and eosin (H&E) for the light microscopic observation of histomorphological changes.

Analytical Methods

Fasting blood glucose was determined by the method of Barham and Trinder [20]. Triglycerides (TG) were measured as per the method of Fossati and Lorenzo [21]. Total cholesterol (TC) was assayed as per the method of Allain et al. [22]. High-density lipoprotein (HDL) was determined by Burstein et al. [23] method. Low-density lipoprotein (LDL) was calculated by using the formula of Friedwald et al. [24]. ApoB was estimated by immunoturbidity method. All the lipid profile parameters were performed in serum. Lipid peroxides were measured in serum as thiobarbituric acid reactive substances (TBARS) by using the standard method of Satoh [25]. OxLDL in serum was estimated by the baseline levels of diene conjugates in lipid fraction of LDL as described by Ahotopa et al. [26]. The amount of serum NO was measured by the Greiss assay based on the method of Moshage et al. [27]. Serum sVCAM-1 and plasma fibrinogen levels were estimated by enzyme-linked immunosorbant assay (ELISA) using commercially available kits from Diaclone, Germany and Hiphen Biomed, France, respectively.

Statistical Analysis

Values are expressed as the mean ± SEM for five animals in each group. The data was analyzed by using repeated measure analysis of variance (ANOVA) followed by Dunnett's test and one-way ANOVA followed by Tukey's test using SPSS software version 17.0. The results were considered significant at $P < 0.05$.

Results

Phytochemical Screening

Preliminary phytochemical screening of the *C. auriculata* aqueous leaf extract revealed the presence of alkaloids, flavonoids, saponins, tannins, cardiac glycosides and phenols.

Antihyperglycemic Activity

Figure 1 demonstrates the levels of FBG in normal, diabetic control and diabetic treated rats at weekly intervals. Oral administration of CLEt and glibenclamide produced significant reduction ($P < 0.001$) in FBG levels from day 7 to day 21 in diabetic rats when compared with day 0. On the other hand, diabetic control rats maintained the marked hyperglycemia without any significant change throughout the experimental period.
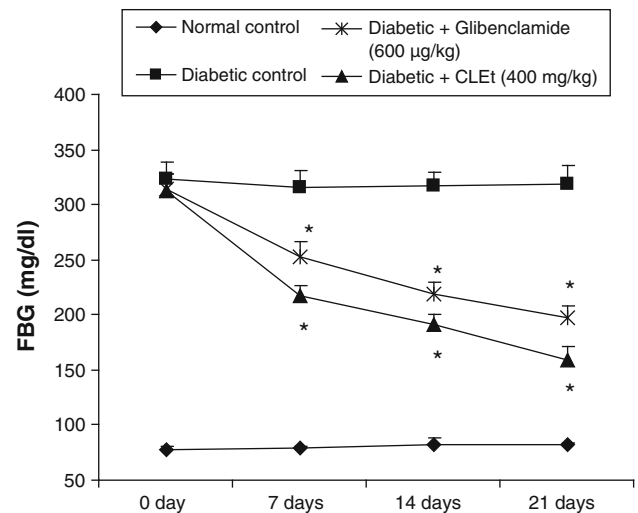
Fig. 1 Effect of *Cassia auriculata* aqueous leaf extract (*CLEt*) on fasting blood glucose (*FBG*) at weekly intervals in diabetic rats. Values are expressed as mean ± SEM ($n = 5$). * $P < 0.001$ compared to day 0

Effect on Lipid Profile, ApoB and Lipid Peroxidation

The effect of CLEt on serum lipids, lipoproteins, ApoB and lipid peroxidation is summarized in Table 1. The levels of TG, TC, LDL-C and ApoB were significantly increased, where as HDL-C was significantly decreased in diabetic control rats compared with normal rats. Following supplementation with CLEt for 3 weeks, these changes were significantly reversed. A significant elevation in the level of MDA was also observed in diabetic control rats as compared to healthy rats. Treatment of diabetic rats with CLEt reverted back the MDA level toward normal range. The effect of extract on these parameters was more prominent than that of glibenclamide, except TG which was significantly decreased at $P < 0.001$ in both extract and glibenclamide-treated rats.

Effect on Markers of Endothelial Function

Table 2 depicts the effect of CLEt on OxLDL, NO, sVCAM-1 and fibrinogen. The significant elevation in the levels of OxLDL, sVCAM-1 and fibrinogen was observed in diabetic control rats compared to normal. The supplementation of extract produced significant reversal in the levels of these parameters in the diabetic rats. NO, which is significantly decreased in diabetic control rats, was found to be markedly elevated in CLEt-treated diabetic rats. Glibenclamide-fed diabetic rats showed significant reduction in OxLDL, however, it did not exhibit significant effect on NO, sVCAM-1 and fibrinogen.

Table 1 Effect of *Cassia auriculata* aqueous leaf extract (CLEt) on lipid profile, apolipoprotein B and lipid peroxidation in diabetic rats after 3 weeks treatment

Groups	TG (mg/dl)	TC (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	ApoB (mg/dl)	MDA (nmole/ml)
Healthy control	61.8 ± 3.2	70.1 ± 2.2	34.0 ± 1.2	21.3 ± 1.6	70.8 ± 3.4	1.61 ± 0.05
Diabetic control	125.0 ± 7.3[+]	108.3 ± 4.9[+]	23.1 ± 0.9[+]	60.3 ± 4.7[+]	122.2 ± 5.0[+]	3.34 ± 0.19[+]
Diabetic + Glibenclamide	89.2 ± 5.2***	92.8 ± 3.0**	24.7 ± 0.7	49.8 ± 3.4	106.4 ± 5.6	2.62 ± 0.12*
Diabetic + CLEt	71.8 ± 5.3***	81.7 ± 2.5***	27.2 ± 0.6*	39.7 ± 2.2**	96.0 ± 5.0**	2.07 ± 0.08**

Values are expressed as mean ± SEM ($n = 5$)

MDA a byproduct of lipid peroxidation

TG triglycerides, *TC* total cholesterol, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *ApoB* apolipoprotein B, *MDA* malondialdehyde

[+] $P < 0.001$ versus healthy control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus diabetic control

Table 2 Effect of *Cassia auriculata* aqueous leaf extract (CLEt) on markers of endothelial function in diabetic rats after 3 weeks treatment

Groups	OxLDL (μmol/l)	NO (μmol/l)	sVCAM-1 (ng/ml)	Fibrinogen (ng/ml)
Healthy control	0.048 ± 0.003	20.9 ± 2.2	407 ± 34.9	1.93 ± 0.09
Diabetic control	0.137 ± 0.009[+]	9.3 ± 0.4[+]	1,124 ± 59.5[+]	2.70 ± 0.13[+]
Diabetic + glibenclamide	0.111 ± 0.005*	12.4 ± 0.7	932 ± 52.5	2.43 ± 0.11
Diabetic + CLEt	0.091 ± 0.004***	15.2 ± 0.5*	805 ± 61.7**	2.11 ± 0.09**

Values are expressed as mean ± SEM ($n = 5$)

OxLDL oxidized low-density lipoprotein, *NO* nitric oxide, *sVCAM-1* soluble vascular cell adhesion molecule-1

[+] $P < 0.001$ versus healthy control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus diabetic control

Histopathological Examination of Heart Tissue

Figure 2a showed normal architecture of myocardium in heart sections of healthy rats. Heart specimens from STZ-induced diabetic control rats (Fig. 2b) revealed fatty steatosis i.e., presence of fatty vacuoles in the cytoplasm of most of the myocytes with no sign of interstitial fibrosis or inflammation. Sections of heart of diabetic rats treated with CLEt (Fig. 2d) showed improvement in the myocardial morphology as reversal of fatty change was observed. Sections from glibenclamide-fed diabetic rats also demonstrated lesser vacuolation in the myocardium as compared to that of diabetic control rats (Fig. 2c).

Discussion

In the last few years, there has been an exponential growth in the field of herbal medicine because of their natural origin and less side effects. The plants synthesize a variety of secondary metabolites with antihyperglycemic and antiatherosclerotic potential which can play a major role in protection against hyperglycemia-induced atherosclerosis [28, 29]. In the present study, the phytochemical screening of *C. auriculata* aqueous leaf extract revealed the presence of alkaloids, flavonoids, saponins, tannins, phenols and cardiac glycosides. These phytochemicals have been shown to be endowed with antidiabetic and antiatherosclerotic activity [28, 29]. Flavonoids and saponins have lipid-lowering effect. Flavonoids have been shown to be capable of reducing the levels of LDL [30], while saponins exert hypocholesterolemic action [31]. Alkaloids, flavonoids, saponins, tannins etc. act as antioxidants through their ability to scavenge reactive oxygen species (ROS) and/or inhibit lipid peroxidation [32–34]. Alkaloids are also known to be the most potent anti-inflammatory agents of naturally occurring products of secondary metabolism, the same activity is shown to be attributed to flavonoids and saponins [29]. Therefore, the presence of these active constituents is speculated to account for the observed pharmacological effects of the extract.

Streptozotocin is a highly cytotoxic chemical of the β-cells of the pancreas [35] that causes reduction in insulin secretion and consequently hyperglycemia, which in turn leads to development of atherosclerosis [36]. Therefore, STZ rat model is being chosen to elucidate the role of aqueous leaf extract of *C. auriculata* in alleviating the atherosclerotic environment in diabetic condition.

The capacity of *C. auriculata* aqueous leaf extract to significantly bring down the elevated levels of blood

Fig. 2 Histopathological findings of myocardial tissue of diabetic rats treated with aqueous leaf extract of *Cassia auriculata* for 21 days (H&E, ×400). **a** Normal control, **b** diabetic control, **c** diabetic rats treated with glibenclamide and **d** diabetic rats treated with *C. auriculata* aqueous leaf extract
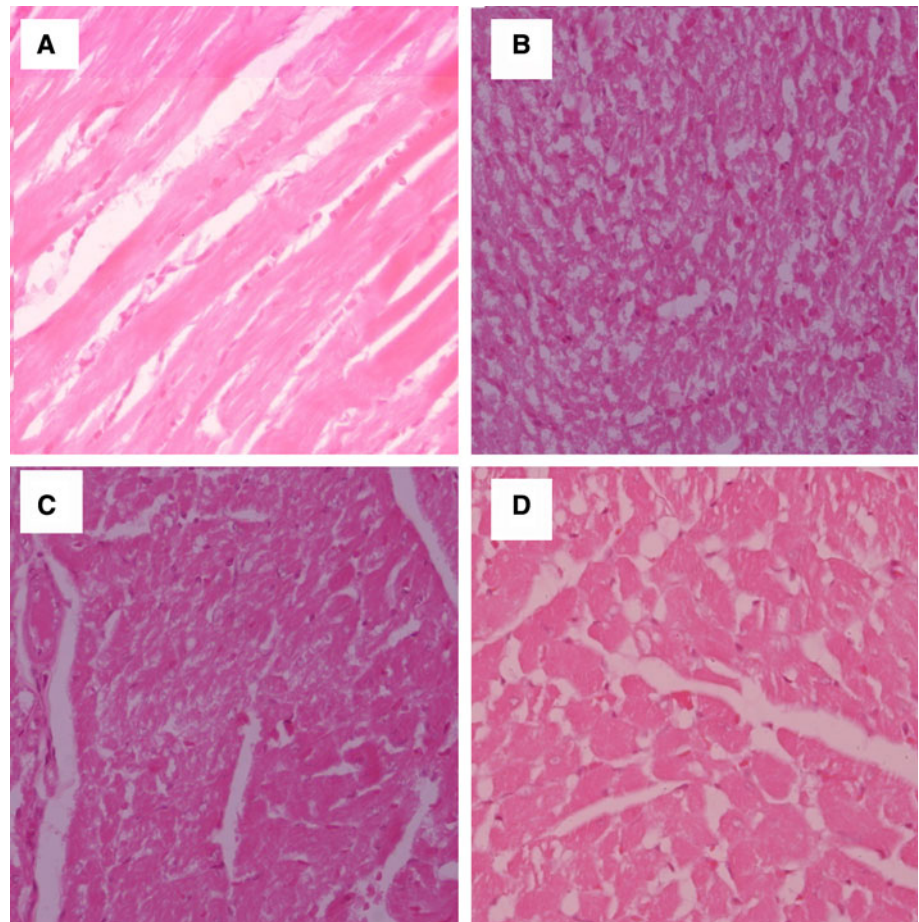
glucose in diabetic rats shows its antihyperglycemic activity, which is an essential trigger for the development of normal homeostasis during experimental diabetes. The antihyperglycemic effect shown by CLEt is consistent with our previous studies in experimentally induced diabetic rats and rabbits [15, 37]. Other researchers have also shown blood glucose lowering effect of *C. auriculata* leaves in hydro-alcoholic and ethanolic extracts [14, 38].

In diabetes, hyperglycemia is accompanied with dyslipidemia which is characterized by increase in TG, TC, and LDL-C, and fall in HDL-C [39]. The altered serum lipid profile was significantly improved in diabetic rats following treatment with the extract. The data showing favorable lipid profile exerted by *C. auriculata* leaf extract is in line with earlier reports [15, 40]. As insulin deficiency is responsible for the derangement of lipid metabolism in diabetes [41], the control in the levels of serum lipids might be due to improvement in insulin levels after administration of the extract to the diabetic rats. This hypothesis is supported by our previous report showing increased insulin levels in alloxan-induced diabetic rabbits treated with CLEt [37].

Diabetic dyslipidemia has long been shown to have a strong correlation with the development of atherosclerosis and coronary heart disease (CHD). The increased TG and TC levels, and decreased HDL level were known factors associated with CHD [39]. In the present study, the extract exhibited hypocholesterolemic and hypotriglyceridemic effects, while increased the level of HDL. Recent studies suggest that TG itself is independently related to CHD [42] and most of the antihypercholesterolemic drugs do not decrease TG levels. As the extract produced favorable effect on these factors, this suggests that the extract may help to prevent the progression of cardiovascular diseases. Furthermore, the role of ApoB as an important risk factor is biologically plausible, since ApoB concentration reflects the number of atherogenic lipoprotein particles. The risk of coronary artery diseases increases to 20-fold when ApoB and insulin levels are also raised [43]. Therefore, the reduction in the level of ApoB in CA_{100}-treated rats is an important determinant to show the antiatherosclerotic potential of CA_{100}.

Along with altered lipid metabolism, oxidative stress plays an important role in the pathogenesis of atherosclerosis [5, 44]. The elevated levels of blood glucose in

diabetes cause increased production of oxygen free radicals, which lead to membrane damage due to peroxidation of membrane lipids. MDA, which is a byproduct of lipid peroxidation, is considered to be a strong predictor of CVD. In the present study, the level of serum MDA was found to be decreased in extract-treated diabetic rats, indicating suppression of lipid peroxidation. The lowering of blood glucose levels following supplementation with the extract might be responsible for prevention of free radical generation and lipid peroxidation.

Impaired endothelial-dependant relaxation in diabetes mellitus is a consistent finding in animal models and human studies [45–47]. Oxidative stress is known to activate many of the cellular pathways that lead to endothelial dysfunction and remodeling. Endothelial dysfunction can promote both the formation of atherosclerotic plaques and the occurrence of acute events. The factors like oxidation of LDL, reduced NO level, overexpression of adhesion molecules and decreased fibrinolysis together contribute to endothelial dysfunction.

Oxidative modification of LDL plays a key role in the development of atherosclerosis and CHD. OxLDL is better able to enter the arterial wall and promotes atherosclerotic process by forming foam cells [48]. The present study demonstrates the beneficial effect of the extract on this atherogenic marker as extract-fed diabetic rats showed reduced level of OxLDL. Abnormalities in NO pathway during diabetes further favor the progression of atherosclerotic plaque formation [49]. Following treatment with extract to diabetic rats, a significant increase in the level of NO was observed. Improvement in the levels of OxLDL and NO following extract administration suggests its role in reducing the risk of atherosclerosis.

Adhesion molecules regulate the interaction of endothelium and leukocytes. An increase in their expression on the endothelial surface causes increased adhesion of leukocytes and this is one of the first steps leading to atheroma formation. Several studies have shown elevated levels of sVCAM-1, one of the major adhesion molecules, in clinical and experimental diabetes [50, 51]. It is also observed in the present study as STZ-induced diabetic rats showed increased level of sVCAM-1. Reduction in the expression of sVCAM-1 in extract-supplemented diabetic rats reflects the protective role of the extract against atheroma formation.

Moreover, diabetes is characterized by a variety of alterations in coagulation and fibrinolytic systems that combine to produce a prothrombotic state. The increased level of fibrinogen in diabetes is reported by several studies in humans as well as experimental animals [52, 53]. The enhanced fibrinogen level in diabetes is due to decreased fibrinolysis which leads to abnormal coagulation. Also in the present study, the altered level of fibrinogen was found

in diabetic control rats. However, administration of extract to diabetic rats restored the level of this prothrombotic factor toward normal. Thus, the extract may help in normalization of coagulation cascade and consequently prevent the thrombus formation.

The partial restoration of OxLDL, NO, sVCAM-1 and fibrinogen levels following supplementation with extract suggests its role in reducing the risk of developing cardiovascular diseases. It can also be suggested that the positive aspect of extract on endothelial function markers might be due to its antihyperglycemic and antioxidant activity as the hyperglycemia is a primary mediator of endothelial dysfunction [45], and subsequent ROS production plays a central role in inducing and maintaining the endothelial dysfunction in diabetes [44]. Moreover, the present study is the first attempt showing the alleviating effect of C. auriculata leaf extract on the functioning of the endothelium.

Histomorphological studies of heart myocardium further confirmed the antiatherosclerotic role of C. auriculata leaf extract as reversal of fatty change in the myocardium was observed in extract-treated rats, where as diabetic control rats showed prominent vacuolation (steatosis) in the myocytes. Furthermore, the current study provides first experimental evidence demonstrating retrieval of morphological changes in myocardial tissue following supplementation of C. auriculata leaf extract. Improvement in altered morphology of heart toward normal in extract-treated diabetic rats might be due to improved levels of lipids and lipoproteins. Impaired glucose disposal in diabetes leads to increased mobilization of fatty acids from the adipose tissue in the circulation. The utilization of these fatty acids by the myocytes for energy requirement results in increased production of acetyl CoA. Increased level of acetyl CoA is involved in lipid synthesis, which consequently leads to formation of fatty vacuoles in the myocytes. However, treatment with extract possibly sensitized myocytes for uptake of glucose and thus arrested lipid synthesis in these cells and protected vacuole formation.

The antihyperglycemic effect of CLEt is found to be comparable to that of glibenclamide. However, the data of the study revealed more pronounced effect of the extract in exhibiting favorable lipid profile, suppression of lipid peroxidation and improvement of endothelial function as compared to glibenclamide. Hence, with these findings it can be suggested that the extract may produce antihyperlipidemic, antioxidant and antithrombotic activity through additional mechanism(s) besides glycemic control. It is well known that increased activation of protein kinase C (PKC) during diabetes mediates proatherosclerotic processes via increased production of reactive oxygen species, decreased NO production and induction of adhesion molecules [5]. Thus, suppression of PKC activation can be

proposed as one of the possible mechanisms through which CLEt imparts anti-atherosclerotic activity.

In conclusion, it is evident from the data that *C. auriculata* aqueous leaf extract possesses antihyperlipidemic, antioxidant and antithrombotic potential. These activities indicate the role of *C. auriculata* leaf extract as a potent anti-atheroscleroctic agent. Thus, it can be suggested that aqueous leaf extract of *C. auriculata* has a potency to alleviate the progression of atherosclerosis and subsequent cardiovascular diseases.

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