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INFLUENCE OF CASSIA AURICULATA LEAVES ON PLASMA ANTIOXIDANTS IN STREPTOZOTOCIN- NICOTINAMIDE INDUCED EXPERIMENTAL DIABETES

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Abstract
Cassia auriculata is an evergreen shrub that grows in many parts of India and in other parts of Asia. The flower, leaves, stem, root, and unripe fruit are used for treatment, especially in Ayurvedic medicine. People *use Cassia auriculata* for diabetes, eye infections (conjunctivitis), joint and muscle pain (rheumatism), constipation, jaundice, liver disease, and urinary tract disorders. Oral administration of *Cassia auriculta* leaf extract (CLEt) of diabetic rats for 45 days resulted in significant reduction in blood glucose and significant increase in plasma insulin levels. A single dose of streptozotocin (65 mg/kg body weight) produced decrease in insulin, hyperglycemia, increased lipid peroxidation (thiobarbituric reactive substances [TBARS] and lipid hydroperoxides) and decreased antioxidant levels (vitamin C, vitamin E, reduced glutathione, ceruloplasmin). Oral administration of CLEt (0.45 g/kg body weight) and for 45 days to diabetic rats significantly increased the plasma insulin and plasma antioxidants and significantly decreased the lipid peroxidation. The effect of CLEt was better when compared with glibenclamide.

Key words *Cassia auriculata*, plasma lipid peroxidation, plasma antioxidants, diabetes mellitus

Introduction
Herbal medicine and omics systems science offer significant synergy to aid drug discovery and development. *Cassia auriculata*, a Caesalpiniaceae shrub, is native to India and Sri Lanka, present in Indo-Malaysia, and cultivated in Myanmar. In Ayurvedic medicine, *C. auriculata* is one of the notable medicinal herbs. The individual parts of the *C. auriculata* plant, including the flowers, flower buds, root, leaves, seeds, and bark, are used in traditional herbal medicine practices with various indications for each (Meena et al., 2019). On the other hand, while *C. auriculata* has been used as a medicinal herb in a context of diabetes, its mechanisms of action and the evidence base for its antidiabetic medicinal potentials and components need to be deciphered. Moreover, the phytocomposition of the various plant parts is not fully known (Murugan, 2015a).
Hyperglycemia promotes auto-oxidation of glucose to form free radicals. The generation of free radicals beyond the scavenging abilities of endogenous antioxidant defenses results in macro- and

microvascular dysfunction. Antioxidants such as N-acetylcysteine, vitamin C and α-lipoic acid are effective in reducing diabetic complications, indicating that it may be beneficial either by ingestion of natural antioxidants or through dietary supplementation (Murugan and Pari, 2007; Murugan, 2015b). However, while antioxidants are proving essential tools in the investigation of oxidant stress-related diabetic pathologies and despite the obvious potential merit of a replacement style therapy, the safety and efficacy of antioxidant supplementation in any future treatment, remains to be established (Murugan and Pari, 2006a; Murugan, 2015c)

Plants play a major role in the introduction of new therapeutic agents and have received much attention as sources of biologically active substances. *Cassia auriculata* L. (Ceasalpiniaceae) is a shrub that has attractive yellow flowers, commonly used for the treatment of skin disorders and body odour. It is a native plant present in different parts of India. Indigenous people use various parts of the plant for diabetes mellitus. It is widely used in Ayurvedic medicine as a "Kalpa drug" which contains five parts of the shrub (roots, leaves, flowers, bark and unripe fruits) which are taken in equal quantity, dried and then powdered to give "Avarai Panchaga Choornam", for the control of sugar levels and reduction of symptoms such as polyuria and thirst in diabetes (Shrotri and Aiman, 1960). A literature survey showed that a decoction of leaves, flowers, and seeds of the *Cassia auriculata* mediate an antidiabetic effect (Shrotri and Aiman, 1960). Thus, the available reports show that very little work has been done with respect to *Cassia auriculata* flowers, other than its hypoglycemic effects (Pari and Murugan, 2007; Murugan, 2010; Murugan, 2015a). In our previous study, we have demonstrated the antidiabetic effect of CFEt in STZ induced diabetic rats (Murugan, 2015b; Murugan, 2015c).

To our knowledge, so far no other biochemical investigations has been carried out on the effect of CLEt compared glibenclamide in plasma antioxidant status of experimental diabetic rats. The present investigation was carried out to study the effect of CLEt compared glibenclamide on plasma antioxidants in rats with STZ and nicotinamide induced diabetes.

Materials and methods

Chemicals

Stereptozotocin was obtained from Himedia Laboratory Limited, Mumbai, India. All other reagents used were of analytical grade.

Plant Material

Cassia auriculata leaves were collected freshly from Neyveli, Cuddalore District, Tamil Nadu, India. The plant was identified and authenticated at the Herbarium of Botany Directorate in Annamalai University.

Preparation of plant leaves extract

Five hundred g of *Cassia auriculata* leaves were extracted with 1,500 ml of water by the method of continuous hot extraction at 60ºC for six hours and evaporated. The residual extract was dissolved in water and used in the study (Jain, 1968).

Experimental procedure

In the experiment, a total of 24 rats (18 diabetic surviving rats, six normal rats) were used. The rats were divided in to six groups of six rats each.

Group 1: Normal untreated rats.

Group 2: Diabetic control rats given 1 ml of aqueous solution daily using an intragastric tube for 45 days.

Group 3: Diabetic rats given CLEt (0.45 g/kg body weight) in 1 ml of aqueous solution daily using an intragastric tube for 45 days.

Group 4: Diabetic rats given glibenclamide (600 µg/ kg body weight) in 1 ml of aqueous solution daily using an intragastric tube for 45days.

At the end of 45 days, the animals were deprived of food overnight and sacrificed by decapitation. Blood was collected in tubes containing potassium oxalate and sodium fluoride mixture for the estimation of blood glucose. Plasma was separated for the estimation of insulin and other biochemical parameters.

Analytical procedure

Measurement of blood glucose and plasma insulin

Blood glucose was estimated colorimetrically using commercial diagnostic kits (Sigma Diagnostics (I) Pvt Ltd, Baroda, India). Plasma insulin was assayed by the enzyme- linked immunosorbent assay method using a Boehringer-Mannheim kit with an ES300 Boehringer analyzer (Mannheim, Germany) Andersen et al., (Lott and Turner, 1975).

Estimation of Lipid peroxidation

Lipid peroxidation in plasma was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) and hydroperoxides using the methods of Fraga et al. (1988) and Jiang et al. (1992), respectively. In brief, 0.5 ml of plasma was treated with 2 ml of TBA-trichloroacetic acid (TCA)- HCl reagent (0.37% TBA, 0.25 N HCl and 15% TCA, 1:1:1 ratio), placed for 15 min in a water bath and then cooled and centrifuged for 10 min (1000 rpm) at room temperature, the clear supernatant was measured at 535 nm against a reference blank.

Hydroperoxides were expressed as mmoles/dl. Plasma (0.5 ml) was treated with 0.9 ml of Fox reagent (88 mg of BHT, 7.6 mg of xylenol orange and 0.8 mg of ammonium iron sulphate were added to 90 ml of methanol and 10 ml of 250 mmoles sulphuric acid) and incubated at 37°C for 30 min. The color development was read at 560 nm.

Estimation of ascorbic acid (Vitamin C)

Vitamin C was estimated by the method of Omaye et al. (1979). To 0.5 ml with 1.5 ml of 6% TCA and centrifuged for 20 minutes. To 0.5 ml of the supernatant, 0.5 ml of DNPH reagent was added and mixed well, allowed to stand at room temperature for an additional 3 hours and added 2.5 ml of 85% sulphuric acid and allowed to stand for 30 minutes. A set of standards containing 10-50µg of ascorbic acid were taken and processed similarly along with a blank, read at 530 nm. Ascorbic acid values were expressed as mg/dl plasma.

Estimation of Vitamin E

Vitamin E was determined by the method of Baker et al. (1951). To 0.1 ml of plasma, 1.5ml ethanol and 2.0 ml of petroleum ether were added, mixed and centrifuged. The supernatant was evaporated to dryness at 80°C. To this was added 0.2 ml of 2, 2′ -dipyridyl solutions and 0.2 ml of ferric chloride solution. Mixed well and kept in dark for 5 minutes and added 2 ml of butanol. The intense red colour developed was read at 520 nm. Standard tocopherol in the range of 10-100µg were taken and treated similarly along with blank containing only the reagent. The amount of α-tocopherol was expressed as mg/dl plasma**.** Protein was determined by the method of Lowry et al. (1951).

Estimation of Ceruloplasmin

Plasma ceruloplasmin was estimated by the method of Ravin (1961). 0.05 ml of plasma was added. To control, 1 ml of sodium azide was added and mixed. To both the tubes 1.0 ml of p-phenylenediamine was added, mixed and kept at 37°C for 1 h. 1 ml of sodium azide was then added to the test. All the tubes were kept at 4 to 10°C for 30 min. The colour developed was then read at 540nm with control as blank. Ceruloplasmin values were expressed as mg/dl.

Estimation of reduced glutathione

Reduced glutathione (GSH) was determined by the method of Ellman (1959). An aliquot (1.0 ml) of the supernatant was treated with 0.5 ml Ellman's reagent and 3.0 ml phosphate buffer (0.2M, pH 8.0) and the absorbance was read at 412 nm. GPx activity was expressed as µg GSH consumed/ min/mg protein and GSH as mg / dl plasma.

Statistical analysis

The data for various biochemical parameters were analyzed using analysis of variance (ANOVA), and the group means were compared by Duncan's multiple range test (DMRT). Values were considered statistically significant if $p < 0.05$ Duncan (1959).

Results

Table 1 shows the level of blood glucose, total haemoglobin, glycosylated haemoglobin and plasma insulin of different experimental groups. There was a significant elevation in blood glucose level, whereas plasma insulin levels decreased significantly in streptozotocin diabetic rats, compared with normal rats. The effect of CLEt was more prominent when compared with glibenclamide. The diabetic control rats showed a significant decrease in the level of total haemoglobin and significant increase in the level of glycosylated haemoglobin. Oral administration of CLEt to diabetic rats significantly restored total haemoglobin and glycosylated haemoglobin levels. In the case of normal rats, the level of haemoglobin and glycosylated haemoglobin remained unaltered.

Table 1. Effect of CLEt on the levels of blood glucose, plasma insulin, haemoglobin and glycosylated haemoglobin in normal and experimental rats

Groups	Fasting blood glucose (mg/dl)	Plasma insulin (µU/ml)	Total haemoglobin (g/dl)	Glycosylated haemoglobin (mg/g Hb)
Normal	98.32 ± 5.25^{a}	12.21 ± 0.42^{a}	12.47 ± 0.42^{a}	0.35 ± 0.02^{a}
Diabetic control	290.21 ± 7.89^{b}	3.99 ± 0.30^{b}	8.55 ± 0.30^{b}	0.77 ± 0.03^{b}
Diabetic+CLEt (0.45 g/kg)	117.02 ± 5.21^{c}	9.98 ± 0.65^{c}	11.32 ± 0.55^{c}	0.44 ± 0.02^{c}
Diabetic+ Glibencalamide (600 µg/ mg)	130.87 ± 7.21^{d}	9.67 ± 0.30^{d}	10.54 ± 0.45^{d}	0.51 ± 0.04^{d}

Values are given as mean ± S.D for 6 rats in each group.
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).

Untreated diabetic rats showed a significant increase in the levels of lipid peroxide and TBARS in plasma (Table 2). Rats treated with CLEt, however, had significantly lower plasma lipid peroxide and TBARS levels as compared with the untreated diabetic rats. The effect of CLEt was better than glibenclamide.

Table 2. Changes in the levels of plasma TBARS and hydroperoxides in normal and experimental animals

Groups	TBARS (mmoles/dl)	Hydroperoxides (x 10^{-5} mM/100ml)
Normal	0.22 ± 0.01^{a}	12.32 ± 0.46^{a}
Diabetic control	0.46 ± 0.02^{b}	22.65 ± 1.42^{b}
Diabetic + CLEt (0.45 g/kg)	0.24 ± 0.01^{c}	14.53 ± 0.66^{c}
Diabetic + Glibencalamide (600 µg/ mg)	0.30 ± 0.01^{d}	15.67 ± 0.55^{d}

Values are given as mean ± S.D for 6 rats in each group.
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).

Plasma GSH and vitamin C levels were significantly lower in diabetic rats than in normal rats and plasma ceruloplasmin and α-tocopherol levels were significantly higher in diabetic rats than in normal rats. In contrast, diabetic rats treated with CLEt had near normal levels of plasma antioxidants (Table 3). The effect of CLEt was more prominent compared with glibenclamide.

Table 3. Changes in levels of vitamin C, vitamin E, ceruloplasmin and reduced glutathione (GSH) in plasma of normal and experimental animals

Groups	Normal	Diabetic control	Diabetic+CLEt (0.45 g/kg)	Diabetic+ Glibencalamide (600 µg/ mg)
Vitamin C (mg/dl)	1.85 ± 0.10^{a}	0.78 ± 0.04^{b}	1.70 ± 0.11^{c}	1.65 ± 0.06^{d}
Vitamin E (mg/dl)	1.78 ± 0.05^{a}	0.64 ± 0.05^{b}	1.30 ± 0.07^{c}	1.27 ± 0.05^{d}
Ceruloplasmin (mg/dl)	20.41 ± 1.06^{a}	32.52 ± 2.35^{b}	23.30 ± 1.35^{c}	27.01 ± 1.30^{d}
Reduced Glutathione (mg / dl)	28.22 ± 1.25^{a}	12.31 ± 0.61^{b}	23.55 ± 1.35^{c}	20.75 ± 5.54^{d}

Values are given as mean ± S.D for 6 rats in each group.
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).

Discussion

Antioxidant activity is known to reflect the altered redox balance of affected fluids, tissues, or organs in several pathological states. Therefore, antioxidant concentrations or measures of their activity have been used to estimate the amount of oxidative stress. Also, measures of the total antioxidant potential of biological fluids have been developed, and they have proved to be useful tools for estimating the antioxidant activity in clinical settings (Wayner et al., 2019; Rice-Evans and Miller, 1994; Alho and Leinonen, 1998). During ischemia, cellular glutathione (GSH) is rapidly depleted, and this also impairs the regeneration of other antioxidants from their oxidized forms. Both blood plasma and cerebrospinal fluid (CSF) contain powerful chain-breaking antioxidants, such as α-tocopherol, ascorbic acid, uric acid, and protein-bound thiols. The concentrations and activities of these antioxidants may be important determinants of the IR-induced cerebral injury. The total antioxidant potential of CSF is substantially lower than that of plasma, and the major component of the potential in CSF seems to be ascorbic acid, while in plasma it is uric acid (Rice-Evans and Miller, 1994; Alho and Leinonen, 1998).

Free radical damage to cellular components and decomposition of hydroperoxide formed from oxidative breakdown of PUFAs are important factor in the development of cellular toxicity and pathology caused by lipid peroxidation. Lipid peroxide mediated tissue damages have been observed in the development of type I and type II diabetes mellitus (Feillet-Coudray et al. 1999). Diabetes mellitus is associated with generation of ROS leading to oxidative damage particularly in liver, kidney and brain (Mohamed et al. 1999). The elevated blood glucose levels in diabetes mellitus are thought to induce cell death through free radical formation that occurs as common sequel of diabetes-induced non-enzymatic modification of sugar moieties on proteins and lipids (Donnini et al. 1996). Oxidative stress in diabetes coexists with a decrease in the antioxidant status (Picton et al. 2001), which can increase the deleterious effects of free radicals. The Central Nervous System (CNS) is also susceptible to long term complications associated with diabetes (Aragno et al. 1997). Experimental models of diabetes in have provided evidence for functional and morphological alterations in the brain (Biessels et al. 1994). Free radicals are formed in the CNS as part of the normal metabolic processes (Wolff, 1993). High oxygen uptake and low antioxidant defenses increase the vulnerability of the CNS to oxidative damage (McCall, 1992).

Total peroxyl radical–trapping potential (TRAP) is a useful estimate of the total antioxidant activity of a given biological fluid. Changes in plasma TRAP have been observed in various clinical situations, including aging, lung cancer, acute infection, immobilization, diabetes mellitus, and coronary heart disease. Several modifications of the assay have been published, and they all share the same principle of producing peroxyl radicals at a steady rate (Rice-Evans and Miller, 1994; Alho and Leinonen, 1998). TBARS and hydroperoxides significantly increased in plasma and tissues (liver, kidney and brain) of diabetic control rats. Previous studies have also reported that there was an increased lipid peroxidation in plasma of diabetic rats (Murugan P and Pari, 2006a; Murugan and Pari, 2006b). The increase in oxygen free radicals in diabetes could be due to rise in blood glucose levels, which upon autoxidation generate free radicals.

Increased lipid peroxidation under diabetic conditions can be due to increased oxidative stress in the cell as a result of depletion of antioxidant scavenger systems. Aim our study, the diabetic tissues showed a decrease in the activities of key antioxidants like SOD, CAT, GSH, GPx, GST, GSH, vitamin C and vitamin E, which play an important role in scavenging the toxic intermediate of incomplete oxidation. SOD and CAT are the two major scavenging enzymes that remove toxic free radicals *in vivo*. Previous studies have reported that the activity of SOD is low in diabetes mellitus (Vucic et al. 1997). A decrease in the activity of these antioxidants can lead to an excess availability of superoxide anion $O_2^{\bullet-}$ and hydrogen peroxide in biological systems, which inturn generate hydroxyl radicals, resulting in initiation and propagation of lipid peroxidation (Kumuhekar and Katyane, 1992).

GSH is the most important biomolecule, which participates in the elimination of reactive intermediates by reducing hydroperoxides in the presence of GPx. GSH also functions as a free radical scavenger and in the repair of radical caused biological damage (Meister, 1984; Nicotera and Orrenius, 1986). Decreased glutathione levels in type 2 diabetes have been considered to be an indicator of increased oxidative stress (McLennan et al., 1991). The decrease in the GSH level represents the increased utilization in trapping the oxy radicals. GPx and GST catalyse the reduction of H_2O_2 and hydroperoxides to non-toxic products (Bruce et al., 1982). Previous studies reported by us as well as by others reveal that the activities of GPx and GST were significantly decreased in diabetic rat tissues. (Dias et al., 2005, Latha et al., 2004). The decreased activities of these enzymes result in the involvement of deleterious oxidative changes due to the accumulation of toxic products. Administration of CLEt and glibenclamide increased the content of GSH in the liver of diabetic rats. It is thought that damage to the body's cells and tissues caused by substances known as free radicals may lead to the development of type 2 diabetes. Vitamin C is an excellent hydrophilic antioxidant in plasma which is thought to have a protective role in diabetes by reducing the damage caused by free radicals (Frei et al., 1986). Studies involving different types of oxidative stress have shown that under all types of oxidative damage and therefore it would be helpful in prevention of diseases in which oxidative stress plays a causative or exacerbation role (Zhang and Omaye, 2001). The observed significant decrease in the level of plasma vitamin C could be due to the increased utilisation of vitamin C as an antioxidant defense against ROS or to a decrease in the GSH level, since GSH is required for the recycling of vitamin C (Inefers and Sies, 1988).

Diabetes is accompanied by an increased oxidative damage to all the bimolecular. Enhanced oxidative stress contributes to the development of the diabetic complications. The key lipid soluble chain breaking antioxidant,-tocopherol, is known to be deficient in diabetes (Scholz et al., 1997). Human intervention studies have indicated the role of vitamin E in improving the endothelial function, the retinal blood flow and the renal dysfunction. Low levels of plasma antioxidants have been implicated as a risk factor for the development of diabetes (Vatassery et al. 1983). This decrease could have been due to increased utilization of vitamin C as an antioxidant defense against increased ROS or to a decrease in the GSH level, since GSH is required for the recycling of vitamin

C (Inefers and Sies, 1988). Both vitamin C and vitamin E are known to prevent detectable lipid peroxidation, and under physiological conditions, it has been suggested that vitamin C helps to recycle vitamin E from its radical form (Garg and Bansal, 2000). Oral administration of CLEt to diabetic rats restored the level of vitamin E to near normal. It is likely that increased level of vitamin E may be due to increased levels of GSH.

Elevated serum ceruloplasmin is a biomarker for oxidative stress. Diabetes mellitus is known to be a state of oxidative stress which causes complications of Diabetes mellitus including diabetic retinopathy. Ceruloplasmin forms a major part of the extracellular antioxidant defense. It inhibits iron and copper dependent lipid peroxidation and also has a $O_2^{\bullet -}$ scavenging activity (Halliwell and Gutteridge, 1990). The level of ceruloplasmin has been reported to increase under disease condition that leads to the generation of oxygen products such as $O_2^{\bullet -}$ and H_2O_2 (Dormandy, 1980). Further, increase in ceruloplasmin is an indication of increase antioxidant defense to compensate the loss of other antioxidant enzymes. Administration of CLEt to diabetic rats restored the level of ceruloplasmin. The observed increase in the level of plasma ceruloplasmin in diabetic rats may be a protective response to an increase in circulating unbound Fe^{2+}, which would act as a catalyst for further free radical induced lipid peroxidation.

Administration of CLEt reduced the lipid peroxidation and increased the activity of antioxidants, which could help to control free radical, as CLEt offered protection to cells against oxidative stress by scavenging free radicals (Murugan, 2015a; Murugan, 2015b; Murugan, 2015c) generated during diabetes. The increased levels of free radical scavenging enzymes may act as an added compensation mechanism to maintain the cell integrity and protection against free radical damage. It is well known that *Cassia auriculata* induce antioxidant enzymes, such as GPx, GST and NADPH: quinone reductase. Our study shows that administration of CLEt significantly decreased the plasma TBARS and hydroperoxides. It has been also supported by previous report of CLEt increase hepatic GSH levels and induce certain forms of GSH transferase important in preventing lipid peroxidation and detoxification of toxic lipid aldehydes in diabetic cataract rats (Murugan, 2010). This indicates the antiperoxidative effect of CLEt.

In conclusion, the present investigation shows that CLEt possesses antioxidant effect that may contribute to its protective action against lipid peroxidation and enhancing effect on cellular antioxidant defense. This activity contributes to the protection against oxidative damage in STZ and nicotinamide induced diabetes. The effect of CLEt was more prominent compared with glibenclamide.

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