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RESEARCH ARTICLE

EFFECT OF *CASSIA AURICULATA* L ON ERYTHROCYTE MEMBRANE BOUND ENZYMES AND ANTIOXIDANT STATUS IN EXPERIMENTAL DIABETES

***Pidaran Murugan**

Department of Biochemistry, Faculty of Science, Bharathidhasn University, constituent College, peramablure-621107, Tamil Nadu, India

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ABSTRACT

Cassia auriculata L. (Ceasalpiniaceae) has been used traditionally as antidiabetic and has been proven scientifically to possess high antioxidant activity and anticancer properties. In the present study the effect of *Cassia auriculata L* (CFEt) and glibenclamide on erythrocyte membrane bound enzymes and antioxidants activity in streptozotocin (STZ) and nicotinamide induced type 2 diabetic model was investigated. Oral administration of CFEt at 0.45 mg/kg body weight to diabetic rats for 45 days. The effect of CFEt and glibenclamide on thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxide (Gpx), glutathione-S-transferase (GST), reduced glutathione (GSH) and membrane bound enzymes were studied. The effect of CFEt was compared with glibenclamide. The levels of erythrocyte TBARS, were increased significantly whereas the level of plasma insulin and haemoglobin, erythrocyte antioxidants (SOD, CAT, GPx, GST and GSH), membrane bound total ATPase, Na^{+}/K^{+}-ATPase, Ca^{2+}- ATPase, Mg^{2+}- ATPase were decreased significantly in diabetic rats. Administration of CFEt to diabetic rats showed erythrocyte TBARS. In addition the levels of erythrocyte antioxidants and the activities of membrane bound enzymes also were increased in CFEt treated diabetic rats. The present study indicates that the CFEt possesses a significant beneficial effect on erythrocyte membrane bound enzymes and antioxidants defense in addition to its antidiabetic effect.

INTRODUCTION

Diabetes mellitus is the most common human metabolic disease affecting about 200 million people in World. Changes in the concentrations of lipids including cholesterol, triglycerides are complications frequently observed with diabetes mellitus and certainly contribute to the development of vascular disease (Howard et al. 1978). Diabetes mellitus is a multifactorial disease, which is also characterized by elevated production of reactive oxygen species (ROS). This generation of free radicals during cellular metabolism, and by certain environmental factors including lifestyle appears to play a critical role in the pathogenesis of diabetes. Diabetic patients have an increased incidence of vascular diseases, and it has been suggested that free radical activity is increased in diabetes (Oberley 1998). Glucose itself and hyperglycemia related increased protein glycosylation are important source of free radicals. *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.

Corresponding author: Pidaran Murugan,
Department of Biochemistry, Faculty of Science, Bharathidhasn University, constituent College, peramablure-621107, Tamil Nadu, India.

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. In our previous study, we have demonstrated the antidiabetic effect of *Cassia auriculata* flower extract (CFEt) in streptozotocin (STZ) induced diabetic rats (Pari and Latha, 2002). To our knowledge, so far no other biochemical investigations have been carried out on the effect of CFEt in erythrocyte antioxidants and the activities of membrane bound enzymes of experimental diabetic rats. The present investigation was carried out to study the effect of CFEt on erythrocyte antioxidants and the activities of membrane bound enzymes in rats with STZ and nicotinamide induced diabetes.

MATERIALS AND METHODS

Animals: Studies were performed on adult male albino rats of Wistar strain weighing 180-220g. According to the experimental protocol approved by the Committee for

Research and Animal Ethics of Annamalai University, animals were housed in cages and maintained in 24 ± 2; ° C normal temperature and a 12 hour light/dark cycle. The animals were fed on pellet diet (Lipton India Ltd., Mumbai) and water *ad libitum*.

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

Plant Material: Tanner's cassia flowers 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. A voucher specimen (No.231) was deposited in the Botany Department of Annamalai University.

Preparation of plant extract: 500 g of Tanner's cassia flowers 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.

Induction of diabetes: Non-Insulin dependent diabetes mellitus was induced (Masiello et al., 1998) in overnight fasted rats by a single intraperitonial injection (i.p) of STZ (65 mg/kg body weight), 15 min after the i.p administration of nicotinamide (110 mg/kg body weight). STZ was dissolved in citrate buffer (pH 4.5) and nicotinamide was dissolved in normal saline. Hyperglycemia was confirmed by the elevated glucose levels in blood, determined at 72 h and then on day 7 after injection. The animals with blood glucose concentration more than 200 mg/dl was used for the study (Pari and Murugan, 2005). To our knowledge, so far no other biochemical investigations has been carried out on the effect of CFEt in plasma and tissue glycoproteins of experimental diabetic rats. The present investigation was carried out to study the effect of CFEt on plasma and tissue glycoproteins in rats with STZ induced diabetes.

Induction of experimental diabetes: A freshly prepared solution of STZ (45 mg/kg i.p) in 0.1 M citrate buffer, pH 4.5 was injected intraperitoneally in a volume of 1 ml/kg. After 48 hours of STZ administration, rats with moderate diabetes having glycosuira and hyperglycaemia (i.e. with a blood glucose of 200- 300 mg/dl) were taken for the experiment.

Experimental procedure: In the experiment, a total of 36 rats (30 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 CFEt (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 experimental period, the rats were deprived of food overnight and blood was collected in a tube containing potassium oxalate and sodium fluoride for the estimation of blood glucose, haemoglobin and glycosylated haemoglobin. Plasma was separated for the assay of insulin. The pancreas were also dissected out and placed into ice-cold containers for histopathology examination.

Analytical procedure

Preparation of haemolysate: From 2 ml of blood, erythrocytes were separated by centrifugation at 1000×*g* for 10 min at 4 °C. The erythrocyte layer was washed three times with 10 volumes of 10 mmol/L PBS. The washed erythrocytes were suspended in phosphate buffer saline (PBS) and adjusted to a hematocrit (HCT) of 5 or 10%. An aliquot of 0.5 ml washed RBC was lysed with 4.5 ml of ice cooled distilled water to prepare haemolysate.

Preparation of erythrocyte membrane: The erythrocyte membrane (haemoglobin free ghost erythrocyte) was isolated according to the procedure of Dodge et al., (1963) with a change in buffer according to Quist (1980). The packed cells were washed three times with isotonic 310 mM Tris–HCl buffer, pH 7.4. Haemolysis was prepared from red blood cells into 20mM hypotonic Tris–HCl buffer, pH 7.2. Ghosts were sedimented by using a high-speed refrigerated centrifuge at 20,000×g for 40 min. The haemolysate was decanted carefully and the ghost button was resuspended and the buffer of the same strength was added to reconstitute the original volume. The ratio of cells to the washing solution was approximately 1:3 by volume. The procedure was repeated thrice, till the membrane became colourless. The pellets were resuspended in 100mM Tris–HCl buffers, pH 7.2. Aliquots from this were used for the estimations.

Determination of erythrocyte membrane bound enzymes: Total ATPase was assayed by the method of Evans (1986) with modification, the activity of Na^+/K^+-dependent ATPase and Mg^{2+}-ATPase were assayed according to the procedure of Bonting (1970) and Ohinishi et al., (1982) respectively. The incubation mixture in a total volume of 2.0 ml contained 0.1ml buffer 1.5 ml KCl, 0.1 ml NaCl, 0.1 ml $MgCl_2$, 0.1 ml ATP and 0.1 ml RBC membrane. The mixture was incubated at 370 for 20 min. the reaction was arrested by 1.ml 10% TCA and then centrifuged. The phasphorus liberated was estimated by the method Fiske and Subbarow (1925) supernatants with 1.0 ml ammonium molybdate and 0.4 ml of ANSA were added the blue colure developed was read at 640 nm after 30 minutes. AT Pases activity expressed as µ mole of phosphorus liberated/min/mg protein at 37°C. The incubation mixture after incubation at 37^0 C for 10 min. the reaction was initiated by 0.2 ml of RBC membrane. The contents were incubated at 37^0 C for 15 minuted.1.0 ml of 10% TCA was added at the end of 15 min to arrest the reaction. The incubation mixture contained 1.0 ml of buffer 0.2 ml of magnesium sulphate, 0.2 ml of NaCl, 0.2ml of EDTA and 0.2 ml of ATP. After incubation at 37° C for 10 min, the 0.2ml RBC membrane initiated the reaction. The contents were incubated at 37^0C for 15 minuted.1.0 ml of 10% TCA was added at the end of 15 min to arrest the reaction. The phasphorus liberated. Mg^{2+}- ATPase activity expressed as µg of phosphorus liberated/min/mg protein. The activity of Ca^{2+}- ATPase was assayed according to the method of Hjerken and Pan (1983). The reaction mixture contained 0.1 ml of buffer 0.1ml of $CaCl_2$, o.1 ml of ATP and 0.1 ml of RBC membrane, the contents were incubated at 37^0 C for 15 min. the reaction was then arrested by 0.5 ml 10%

TCA. The phasphorus was liberated. Ca^{2+}-ATPase activity expressed as μg of phosphorus liberated/min/mg protein.

Determination of lipid peroxidation and antioxidants: The activity of TBARS was estimated by the method of Donnan (1950). Superoxide dismutase (SOD) was assayed by the method of Kakkar et al. (1984). Catalase (CAT) was estimated by the method of Sinha (1972). Glutathione peroxides (GPx) activity was measured by the method described by Rotruck et al. (1973). Glutathione-S-transferase (GST) activity was determined spectrophotometrically by the method of Habig et al. (1974). Erythrocyte reduced glutathione (GSH) was determined by the method of Beutler et al. (1963).

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, 1957).

RESULTS

The effect of CFEt on changes in the activities of erythrocyte membrane bound total ATPase, Na^+/K^+ ATPase, Ca^{2+}-ATPase and Mg^{2+}- ATPase of normal and experimental rats are shown in table 1. In diabetic rats, the activities of total ATPase, Na^+/K^+ ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase were significantly lowered when compared to normal rats. Administration of CFEt and glibenclmide to diabetic rats significantly elevated the activities of total ATPase, Na^+/k^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase as compared to diabetic control rats. Table 2 shows the activities of TBARS, CAT, SOD, GPx, GST, and GSH in the erythrocytes of diabetic and normal rats. Rats treated with CFEt and glibenclmide, however, had significantly lower erythrocytes TBARS levels as compared with the untreated diabetic rats. Diabetic rats had decreased activities of CAT, SOD, GPx, GST and GSH in the erythrocytes as compared with normal rats. Diabetic rats treated with CFEt and glibenclmide showed reversal of these parameters to near normal.

DISCUSSION

Diabetes mellitus is often associated with the development of vascular degenerative complications affecting both large vessels and the microvasculature (Hanssen, 1997). Several studies have emphasized the multiplicity of disturbances affecting the metabolism of carbohydrates, proteins and lipids in diabetes (Vlassara, 1997). Carbohydrates seem to play a central role in the development of chronic diabetic complications. Indeed, glucose and other reducing sugars participate in one of the major pathogenic mechanisms, i.e., nonenzymatic glycosylation or glycation. Glycation is a non-enzymatic modification of macromolecules induced by the hyperglycemic state during diabetes mellitus. Several studies have shown increased lipid peroxidation in clinical and experimental diabetes (Sundaram et al., 1996). The results showed elevation of lipid peroxidation in the tissues of diabetic group. The increase in oxygen free radicals in diabetes could be due to rise in blood glucose levels, which upon autoxidation generate free radicals. STZ has been shown to produce oxygen free radicals (Ivorra a et al., 1989). Previous studies have reported that the activity of SOD is low in diabetes mellitus (Feillet-Coudray et al., 1999).

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). The result of increased activities of SOD and CAT suggest that CFEt contains a free radical scavenging activity, which could exert a beneficial effect against pathological alterations caused by the presence of $O_2^{\bullet-}$ and $OH^{\bullet}$. The increased activity of SOD accelerates dismutaion of $O_2^{\bullet-}$ to hydrogen peroxide, which is removed by CAT (Aebi, 1984). This action could involve mechanisms related to scavenging activity of CFEt. 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. Associated with the changes in lipid peroxidation the diabetic tissues showed decreased activities of key antioxidants 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 and Feillet-Coudray et al., 1999). 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). The result of increased activities of SOD and CAT suggest that CFEt contains a free radical scavenging activity, which could exert a beneficial effect against pathological alterations caused by the presence of $O_2^{\bullet-}$ and $OH^{\bullet}$. The increased activity of SOD accelerates dismutaion of $O_2^{\bullet-}$ to hydrogen peroxide, which is removed by CAT (Aebi, 1984). This action could involve mechanisms related to scavenging activity of CFEt.

GSH is an important inhibitor of free radical mediated lipid peroxidation (Meister and Anderson,1987). The decreased levels of plasma GSH in diabetes may be due to increased utilization in trapping the oxyradicals. Several workers have also reported decreased levels of plasma GSH and in experimental diabetic rats. GSH is the first line of defense against proxidant status (Ahmed et al.,2000) and GSH was evaluated after CFEt administration. GSH systems may have the ability to manage oxidative stress with adaptional changes in enzymes regulating GSH metabolism. In the present study, treatment with CFEt significantly increased the GSH levels. Increase in GSH level may inturn activates the GSH dependent enzymes such as glutathione peroxidase and glutathione-S-transferase. Changes in membrane lipid composition and enzymatic properties of membrane bound enzymes are shown to occur in diabetes. In this investigation the activities of erythrocyte membrane bound enzymes such as Na^+/K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase were significantly inhibited in STZ diabetic rats. Similarly, the reduced activities of ATPase in erythrocytes and other tissues of STZ induced diabetic rats have been already reported (Mamta and Surendra, 1992). STZ induced diabetes is characterized by a severe rearrangement of sub cellular metabolism and structural alterations of cell membrane. Thus the observed significant aberrations in the activity of erythrocyte membrane bound ATPases indicates the structure and function of the cell membranes are severely altered in diabetes, which may in turn, play an important role in the development of diabetic vascular complications.

Table.1 Changes in the levels of total ATPase, Na^+ /K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase normal and experimental rats

Groups	Total ATPase	Na^+ /K^+ ATPase	Ca^{2+}ATPase	Mg^{2+}ATPase
Normal	1.88 ± 0.10^a	0.87 ± 0.03^a	0.37 ± 0.02^a	2.40 ± 0.02^a
Diabetic control	0.79 ± 0.04^b	0.35 ± 0.02^b	0.22 ± 0.01^b	1.70 ± 0.02^b
Diabetic + CFEt (0.45 mg/kg)	1.50 ± 0.09^c	0.71 ± 0.03^c	0.31 ± 0.02^c	2.30 ± 0.02^c
Diabetic + Glibnclamide (600 □g/kg)	1.31 ± 0.05^d	0.62 ± 0.02^d	0.29 ± 0.01^d	1.40 ± 0.04^d

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

Table 2. Changes in activities of TBARS, catalase, superoxide dismutase, glutathione peroxidase, glutathione–S–transferase and reduced glutathione in erythrocytes of normal and experimental rats

Groups	Normal	Diabetic control	Diabetic + CFEt (0.45 mg/kg)	Diabetic + Glibnclamide (600 µg/kg)
TBARS (pM/mgHb)	1.75 ± 0.10^a	3.16 ± 0.22^b	2.05 ± 0.14^c	2.335 ± 0.11^d
CAT (Units[A] / mg Hb)	165.41 ± 7.55^a	90.54 ± 5.46^b	140.21 ± 7.10^c	120.02 ± 5.78^d
SOD (Units[B] / mg Hb)	6.75 ± 0.31^a	3.39 ± 0.23^b	5.34 ± 0.33^c	5.31 ± 0.20^d
GPx (Units[C] / mg Hb)	14.53 ± 0.58^a	9.09 ± 0.58^b	13.48 ± 0.60^c	12.09 ± 0.58^d
GST(Units[D] / mg Hb)	6.59 ± 0.35^a	4.14 ± 0.21^b	5.59 ± 0.32^c	5.23 ± 0.20^d
GSH (mM/g Hb)	13.90 ± 0.63^a	9.81 ± 0.58^b	11.79 ± 0.60^c	11.21 ± 0.39^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.
A - µ mole of H_2O_2 consumed / minute.
B – One unit of activity was taken as the enzyme reaction which gave 50% inhibition of NBT reduction in one minute.
C - µ g of GSH consumed / min
D - µ moles of CDNB – GSH conjugate formed / min.

The activity of Na^+/K^+-ATPase was found to be diminished in diabetic erythrocytes (Mamta and Surendra, 1992). The membrane Na^+/K^+-ATPase is concerned with the maintenance of a low intracellular concentration of Na^+. Decreases activity of Na^+/K^+-ATPase can lead to a decrease in Na^+ efflux and thereby alter the membrane permeability. The functional state of red blood cells depends on their active Na^+/K^+ sequestering property. The alter membrane structure and function of erythrocytes in diabetes may in turn affect the membrane microenvironment responsible for variations in the activity of membrane bound enzymes. *In vivo* insulin treatment restoring all the altered membrane bound ATPases activities to near normal and insulin also is responsible for the regulations of membrane bound ATPases activities. The lack of insulin reduces the units of Na^+/K^+- ATPase in the membrane. Insufficient Na^+/K^+-ATPase activity in diabetes leads to a consequently increases the amount of activator Ca^{2+} accessible for contraction the Na^+/K^+-ATPase in several tissues (Ver et al.,1999). These finding were accompanied by raise in intracellular Ca^{2+} concentrations, a fact that occurs when the Na^+/K^+-ATPase activity is reduced. The Ca^{2+}-ATPase is the major active Ca^{2+} transport protein responsible for maintenance of normal intracellular calcium level in variety of cell types. The homeostasis of intracellular calcium is mainly regulated by a balance between membrane influx and efflux, the influx is due both to voltage-dependent and receptor operated channels, while the main mechanisms of efflux is the active transport by the Ca^{2+} -ATPase. Mg^{2+}-ATPase is involved in energy requiring processes in the cell whereas Ca^{2+}-ATPase is responsible for the signal transduction pathways and membrane fluidity. Moreover Ca^{2+} transport is strictly integrated with the regulation of sodium transport as the Na^+-Ca^{2+} exchange pathway is driven by the electrochemical potential of sodium and maintained by the activity of the Na^+-pump.

The intracellular concentration of calcium regulates the activities of Mg^{2+}- ATPase (involved in energy requiring process in the cell) and Na^+/K^+-ATPase and therefore, Ca^{2+} may play role in the regulations of sodium reabsorption. The inhibition of these transport systems in the cell may result in a sustained increase in cytosolic Ca^{2+} concentrations producing over stimulation of cellular processes leading ultimately to cell death (Fairhurst et al., 1982). Defective increase of cytoplasmic concentration of Ca^{2+} is assumed to account for the impaired glucose tolerance in various peripheral and central diabetic patients. The activity of Ca^{2+}-ATPase has been shown to be decrease in erythrocyte membrane of diabetic rats, which results in the increased erythrocyte total Ca^{2+} levels as a result of the permeability alterations (Ramana devi et al., 1997). In conclusion lipid peroxidation and glycosylation of proteins can cause reduction in the activities of enzymes and alteration in the structure and function of membranes (Flecha et al., 1990). A reduction in the lipid peroxidation and glycosylation of proteins can prevent diminution in the activities of ATPases, which is beneficial because any reduction in ATPases activity can affect the intracellular concentrations of Na^+, K^+ and Ca^{2+}, alter the signal transduction pathway, and affect contractility, which in turn leads to cellular dysfunction. Administration of CFEt and glibenclamide to diabetic rats showed significant elevation in the activities of total ATPase, Na^+/K^+-ATPase, Ca^{2+}- ATPase and Mg^{2+}- ATPase in erythrocyte membrane when compared with diabetic control rats. The reversal of erythrocyte membrane bound ATPases activity in diabetic rats by CFEt and glibenclamide could be due to increase in metabolism of glucose, and thus the lowering of the glucose concentration in diabetic rats, would result in the activate antioxidant defense, reduction of free radical production, lipid peroxidation and the glycosylation of haemoglobin observed in this study.

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