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Erythrocyte redox status in streptozotocin diabetic rats: effect of *Casearia esculenta* root extract

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The present study was aimed to investigate the effect of Casearia esculenta root extract on erythrocyte lipid peroxidation and to assess the status of antioxidants in red blood cells of streptozotocin (STZ) diabetic rats. The study showed a significant elevation ($p < 0.05$) of erythrocyte thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation and significant reduction ($p < 0.05$) in reduced glutathione (GSH), ascorbic acid (vitamin C), α-tocopherol (vitamin E), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in the STZ diabetic rats. The study also observed significant reduction in membrane cholesterol and phospholipid content in STZ diabetic rats. By oral administration of *C. esculenta* (200 and 300 mg/kg body wt.) for 45 days to the diabetic rats these values approached almost normal levels. A dose of 300 mg/kg body weight *C. esculenta* extract showed better antioxidant effects than 200 mg/kg body weight.

1. Introduction

Reactive oxygen species (ROS) are generated in biological systems through metabolic processes and exogenous sources such as food components, drugs, UV light, ionizing radiation and air pollution [1]. The uncontrolled production of ROS has been implicated in the pathophysiology of many diseases including diabetes mellitus. Elevated levels of blood glucose (hyperglycaemia) [2] is the hallmark of diabetes mellitus, which has been reported to generate, ROS such as free hydroxyl radicals ($^{\bullet}OH$) and superoxides ($O_2^{\bullet -}$) that can cause lipid peroxidation [3]. Moreover, ROS have also been implicated in the mechanism of the damage to the red blood cells (RBC) [4–6]. This was suggested to be due to the non-enzymatic glycosylation of various tissue proteins [7].

Many defense mechanisms are present in the living organism to limit the level of ROS and the damage they inflict. Included among them are endogenous enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [8].

In addition to these endogenous mechanisms, much attention has been paid to the antioxidant role of some dietary components from natural resources which could efficiently quench the elevated oxygen derived free radicals. Nowadays, many plant resources are used by natural healers all over the world to treat the symptoms and complications of diabetes mellitus [9–11].

Casearia esculenta Roxb. (Flacourtiaceae) popularly known as Kadala-Zhinjill, ''Kottarkovai'' in Tamil ''Wild cowrie fruit'' in English and ''Saptarangi'' in Sanskrit, is a shrub richly distributed in the Konkan plateau, South India.

In Indian traditional medicine, the plant has been a popular remedy for the treatment of diabetes mellitus [12–14] and our study plant is one of the major ingredients of D-400, a large selling antidiabetic drug in India (Himalaya drug Co. Bangalore) [15]. The first scientific study was undertaken by Gupta et al. [16] and they reported the hypoglycemic effect of this plant in rat and rabbits. Choudhury and Basu [17] then reported that *C. esculenta* root extract contained uncharacterized hypoglycemic factor(s) which reduced blood sugar levels in experimental animals.

Our own preliminary research was highly encouraging which revealed that blood glucose levels were significantly lowered after oral administration of *C. esculenta* root extract and no harmful side effects were observed throughout the study [18, 19]. To our knowledge, no detailed investigations had been carried out to shed light on the erythrocyte antioxidant property of *C. esculenta.*

The increased production of free radicals may cause disruption of the cell membrane [20]. Therefore, the present study was planned to investigate the extent of lipid peroxidation and antioxidant's status in the erythrocytes of normal and STZ-diabetes rats and also to study the possible influence of *C. esculenta* root extract on membrane lipid peroxidation antioxidant status and erythrocyte membrane lipids.

2. Investigations and results

Table 1 depicts the levels of thiobarbituric acid reactive substances (TBARS) (an index of tissue injury) and non-enzymatic antioxidants such as reduced glutathione (GSH), ascorbic acid (vitamin C) and α-tocopherol (vitamin E) in the red blood cells of control and STZ diabetic rats. The extent of erythrocyte lipid peroxidation measured as the degree of thiobarbituric acid reactive substances (TBARS) produced in the cell significantly increased in

Table 1: Erythrocyte concentrations of TBARS, GSH, ascorbic acid and α-tocopherol in normal and STZ diabetic animals

Groups	Treatment (dose/kg body wt)	TBARS (ηmol/mg protein)	GSH (mg/dL)	Ascorbic acid (mg/dL)	α-Tocopherol (μg/mg protein)
I	Control (2% gum acacia)	1.80 ± 0.15^a	71.10 ± 4.03^a	1.84 ± 0.13^a	1.09 ± 0.06^a
II	Diabetic control	4.14 ± 0.72^b	47.21 ± 4.90^b	0.98 ± 0.51^b	0.48 ± 0.03^b
III	Diabetic + *C. esculenta* (200 mg/kg body wt.)	2.71 ± 0.37^c	59.43 ± 3.89^c	1.61 ± 0.18^c	0.82 ± 0.05^c
IV	Diabetic + *C. esculenta* (300 mg/kg body wt.)	2.07 ± 0.23^d	65.55 ± 2.71^d	1.83 ± 0.22^a	0.98 ± 0.08^d
V	Diabetic + glibenclamide (600 μg/kg body wt.)	1.96 ± 0.22^a	66.10 ± 4.90^d	1.83 ± 0.28^a	1.08 ± 0.07^a

Values are means ± S.D for six animals in each group
Values not sharing a common superscript differ significantly at $p < 0.05$, Duncan's Multiple Range Test (DMRT)

the STZ diabetic rats by about two fold (130%) and the non enzymatic antioxidants such as GSH, ascorbic acid and α-tocopherol significantly decreased in STZ diabetic rats when compared with control rats. Oral administration of *C. esculenta* (200 and 300 mg/kg body wt.) and glibenclamide for 45 days significantly brought these values close to normal.

Table 2 shows the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in control and STZ diabetic rats. The activities of SOD, CAT and GPx significantly decreased in STZ rats when compared with controls. Oral administration of *C. esculenta* (200 and 300 mg/kg body wt.) significantly increased the activities to near normal when compared with untreated diabetic rats.

Table 3 presents the changes in the erythrocyte membrane cholesterol and phospholipid content of the control and STZ diabetic rats. There was a significant reduction in erythrocyte membrane cholesterol and phospholipids content when compared to control rats. Oral administration of *C. esculenta* extract (200 and 300 mg/kg body wt.) for 45 days of treatment reversed these changes towards normal.

3. Discussion

The increased lipid peroxidation observed in erythrocytes of the STZ diabetic rats was in agreement with the finding of Kumar and Menon [21]. Such alteration in the lipid peroxide metabolism has been suggested to be due to the nonenzymatically glycosylated proteins which have been shown to be a source of free radicals including superoxide and hydrogen peroxide [22]. Glycosylation of proteins can also occur in several tissues including alpha-crystalline, making them vulnerable to increased lipid peroxidation. This possibly explains the biochemical basis of complications which occur in such tissues of the diabetes [22]. To our knowledge, no study has been carried out to investigate the extent of lipid peroxidation and antioxidant status in erythrocyte membranes of STZ diabetic rats after treatment with *C. esculenta*.

The elevated cellular lipid peroxidation in STZ diabetic rats was accompanied by depletion of cellular GSH. The reduced glutathione is the cellular first line defense mechanism against lipid peroxidation, which is known to be present at high concentrations in the red blood cells of normal individuals [23]. The reduction in cellular GSH and erythrocyte α-tocopherol levels associated with the increased lipid peroxidation in the STZ diabetes was in congruence with the results of Bono et al. [24]. A similar reduction in the GSH level and the activity of superoxide dismutase has been reported in the present communication. The present study also agrees with Murakami et al. [25] who reported a significant reduction in the activities of γ-glutamylcysteine synthetase and glutathione reductase in the diabetic patients. This report indicates that both the synthesis and maintenance of GSH are altered in diabetics.

A significant decrease in SOD, CAT and GPx activities are observed in the erythrocytes of diabetic rats when compared with control animals, which may result in a number of deleterious effects due to the accumulation of superoxide radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) [26]. Administration of *C. esculenta* root extract resulted in the elevation of SOD to near normal values. $O_2^{\bullet -}$ and hydroxyl radicals ($^{\bullet}OH$) induce various injuries in surrounding tissues and play an important role in several clinical disorders. Any compound, with rich antioxidant properties, might contribute towards the partial or total alleviation of

Table 2: Erythrocyte activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in normal and STZ diabetic animals

Groups	Treatment (dose/kg body wt)	SOD (U^a/mg Hb)	CAT (U^b/mg Hb)	GPx (U^c/mg Hb)
I	Control (2% gum acacia)	6.93 ± 0.97^a	174.23 ± 10.14^a	14.00 ± 1.66^a
II	Diabetic control	3.58 ± 0.83^b	98.59 ± 11.13^b	8.79 ± 1.05^b
III	Diabetic + *C. esculenta* (200 mg/kg body wt.)	4.88 ± 0.512^c	138.45 ± 11.29^c	10.93 ± 0.74^c
IV	Diabetic + *C. esculenta* (300 mg/kg body wt.)	5.52 ± 0.42^d	161.56 ± 14.43^a	13.06 ± 0.74^a
V	Diabetic + glibenclamide (600 μg/kg body wt.)	6.62 ± 0.22^a	171.90 ± 8.11^a	13.17 ± 1.106^a

U^a = enzyme concentration required to inhibits the NBT 50%
U^b = μmol of H_2O_2 consumed/min
U^c = μg of GSH utilized/min
Values not sharing a common superscript differ significantly at $p < 0.05$, Duncan's Multiple Range Test (DMRT)

Table 3: Erythrocyte content of cholesterol (C) and phospholipid (P) and C/P ratio in normal and STZ experimental animals

Groups	Treatment (dose/kg body wt)	Cholesterol (μg/mg protein)	Phospholipids (μg/mg protein)	C/P ratio
I	Control (2% gum acacia)	146.05 ± 7.02^{a}	286.20 ± 26.96^{a}	0.50 ± 0.04
II	Diabetic control	107.79 ± 9.63^{b}	212.29 ± 24.67^{b}	0.53 ± 0.15
III	Diabetic + *C. esculenta* (200 mg/kg body wt.)	114.54 ± 19.57^{c}	238.41 ± 16.02^{c}	0.48 ± 0.10
IV	Diabetic + *C. esculenta* *(300 mg/kg body wt.)*	127.38 ± 8.10^{d}	267.01 ± 23.08^{a}	0.47 ± 0.06
V	Diabetic + glibenclamide (600 μg/kg body wt.)	133.77 ± 7.00^{d}	270.50 ± 15.34^{a}	0.49 ± 0.04

Values not sharing a common superscript differ significantly at $p < 0.05$, Duncan's Multiple Range Test (DMRT)

organ damage. Therefore, removal of $(O_2^{\bullet -})$ and $^{\bullet}OH$ is probably one of the effective defenses of a living body against diseases. The elevated levels of SOD clearly show that *C. esculenta* extract contains a free radicals scavenging activity which could exert a beneficial action against pathological alteration caused by the presence of $(O_2^{\bullet -})$ and $^{\bullet}OH$. The decreased activity of catalase in erythrocytes of STZ diabetic rats could be due to the increased endogenous production of superoxide anions. H_2O_2 is toxic itself and can be a precursor of other toxic species. It can react with $(O_2^{\bullet -})$ to form $^{\bullet}OH$ leading to increased lipid peroxidation and hence higher TBARS levels.

Interestingly, several reports suggested that the increase in lipid peroxidation was associated with an increase in the activity of erythrocyte glucose-6-phosphate dehydrogenase (G6PD) [27]. The result indicated a significant correlation between the lipid peroxide content and the extent of enzyme activity. The erythrocyte G6PD is the major producer of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) required for the recycling of GSH and ascorbate [28]. Since under the conditions of increased free radicals production, the ratio of the oxidized to reduced form of the coenzyme ($NADP^+$/NADPH) is increased, a condition known to activate the G6PD [29], thus, an increase in enzyme activity with an increase in lipid peroxidation is justified. Membrane ascorbic acid levels also decreased in diabetic subjects. Some investigators have shown a decrease in the ascorbate with an increase in dehydroascorbate levels in the diabetic patients [30, 31]. This was suggested to be due to a slower rate of the reduction of dehydroascorbate back into ascorbate [32]. Johnston et al. [33] demonstrated that by raising the base line plasma ascorbate level from 37.5 μm mol/L to 53.8 μm mol/L it was possible to increase the erythrocyte GSH by 46.7% which indicates an intimate relationship between the plasma ascorbate level and the erythrocyte content of GSH. The present study also observed a significant elevation in the erythrocyte content of GSH to about 25.88% and 38.84% respectively after *C. esculenta* (200 and 300 mg/kg body wt.) treatment for 45 days.

The overall effects of the increased rate of cellular lipid peroxidation and reduced defense mechanisms were reflected in the erythrocyte membrane composition.

The presented results show that the total phospholipid content of the erythrocyte membrane was reduced in STZ diabetic rats. This was apparently a consequence of the increased cellular lipid peroxidation, Jain et al. [20] reported that the choline containing phospholipids (phosphatidyl choline and sphingomyelin) which exist at the outer side of the membrane were not altered, whereas the phosphatidylethanolamine and phosphatidylserine fractions, existing at the inner side of the erythrocyte membrane were reduced. This supports the hypothesis that the phospholipid fractions closer to the site of peroxidation reaction were affected. Our findings were in agreement with the report of Jain [20] who demonstrated that incubation of human red blood cells in elevated glucose concentrations caused peroxides of the membrane lipids and the formation of phospholilpid-malondialdehyde adducts. The adduct was found to be formed by crosslinking between phosphatidylserine and malondialdehyde. Similarly, a reduction in the cholesterol content of the erythrocyte membrane was also observed in STZ diabetic rats [34]. Reduction in the membrane cholesterol content is known to increase the disordering and hence alter the fluidity of membrane [35, 36].

In conclusion, the present study highlights a possible role of lipid peroxidation in the contribution to the diabetes-induced complications, involving alterations in the cell membrane composition. The results indicate that treatment of rats with *C. esculenta* for 45 days prevents the occurrence of lipid peroxidation and thus alleviates the adverse complications of diabetes mellitus.

4. Experimental

4.1. Plant material

Roots of *Casearia esculenta* were collected from Western ghats of Tamil Nadu and the plant was botanically authenticated by Dr. C. Chelladurai, Research Officer, Survey Medicinal Plant Unit (S.M.P.U.), Central Council for Research in Siddha and Ayurvedic, Siddha Medical College, Palayamkottai, Tamilnadu. A voucher specimen was deposited in the (AU2145) Department of Botany, Annamalai University, Annamalainagar, Tamilnadu. The plant root was air dried at 25 °C in the room and the dried root was ground into fine powder with an auto-mix blender and the powdered part was kept in a deep freezer until the time of use.

4.2. Preparation of aqueous extract

100 g of dry fine powder was suspended in 250 ml water for 2 h and then boiled at 60–65 °C for 30 min (since a boiled decoction of root of this plant has been used as remedy for diabetes). The extract was preserved and the processes were repeated for three times with the residual powder, each time collecting the extract. The collected extract was pooled and passed through a fine cotton cloth. The filtrate upon evaporation at 40 °C yielded 12% semi-solid extract.

4.3. Drugs and chemicals

Streptozotocin (STZ) was obtained form Sigma chemical company. All other chemicals used were of analytical grade.

4.4. Animals

Male Wistar albino rats (weighing 140–160 g) were procured from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University, Annamalai Nagar. Animals were maintained at Central Animal House and the animals were fed on standard diet (Hindustan Lever, Bangalore) and water *ad libitum.* All studies were conducted in accordance with the National Institute Health "Guide for the Care and Use of Laboratory Animals" [37] and the study was approved by the Ethical Committee of Rajah Muthaih Medical College and Hospital, Annamalai University, Annamalainagar.

4.5. Experimental induction of diabetes

Adult (9 weeks old) male Wistar rats were made diabetic with an intraperitoneal injection of streptozotocin (STZ, 50 mg/kg body weight) dissolved in citrate buffer (0.1 M, pH 4.5). Streptozotocin injected animals exhibited massive glycosuria and hyperglycemia within a few days. Diabetes was confirmed in STZ rats by measuring the fasting blood glucose concentration, 96 h after injection with STZ. Albino rats with a blood glucose level above 240 mg/dl were considered to be diabetic and were used in the experiment. Six rats were injected with 2% gum acacia alone that served as control.

4.6. Animals

After the induction of diabetes the rats were divided into five groups of six animals each.
Group I: Control rats receiving vehicle solution (2% gum acacia). Group II: Diabetic control. Group III: Diabetic rats receiving *Casearia esculenta* root extract (200 mg/kg body weight) in 2% gum acacia using an intragastric tube daily for 45 days. Group IV: Diabetic rats given *C. esculenta* root extract (300 mg/kg body weight) in 2% gum acacia using an intragastric tube daily for 45 days. Group V: Diabetic rats receiving glibenclamide orally (600 μg/kg body weight) as aqueous solution using an intragastric tube daily for 45 days.
After 45 days of treatment, the animals were sacrificed by cervical decapitation. The blood was collected in heparinised centrifuge tubes and the plasma was separated by centrifugation at 1200 × g for 15 min. The puffy coat was removed and the erythrocytes were washed three times with physiological saline. Aliquots of erythrocytes were kept at 4 °C until analysis.

4.7. Biochemical analysis

4.7.1. Estimation of lipid peroxidation products

Thiobarbituric acid was added to erythrocyte samples under acidic conditions and the absorbance of colour that developed after heating was estimated spectrophotometrically at 535 nm [38]. 1,1′,3,3′-tetramethoxy propane was used as an internal standard and the erythrocyte concentration was expressed as nmol/mg protein.

4.7.2. Estimation of erythrocyte GSH

GSH in erythrocytes was measured according to the method of Ellman [39]. This method is based on the development of a yellow colour when 5,5′-dithio-bis-2-nitro benzoic acid (DTNB) is added to compounds containing sulfhydryl groups.

4.7.3. Estimation of erythrocyte ascorbic acid

Erythrocyte ascorbic acid was estimated by the method of Roe and Kuether [40]. The ascorbic acid was converted to dehydroascorbic acid by mixing with acid washed norit and was then coupled with 2,4-dinitrophenyl hydrazine (DNPH) in the presence of thiourea, a mild reducing agent. The coupled dinitrophenyl hydrazine was converted into an orange red coloured complex when treated with sulphuric acid which was read colorimetrically at 520 nm.

4.7.4. Estimation of erythrocyte α-tocopherol

Erythrocyte α-tocopherol was estimated by the method of Baker and Frank [41]. This method involves the reduction of ferric ions to ferrous ions by α-tocopherol and the formation of a red coloured complex with 2,2′-dipyridyl. Absorbance of the chromophore was measured at 520 nm.

4.7.5. Preparation of erythrocyte haemolysate

A known volume of RBC was lysed with hypotonic phosphate buffer at pH 7.4. The haemolysate was separated by centrifugation at 10,000 rpm for 15 min at 20 °C and the supernatant was used for the estimations of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

4.7.6. Assay of superoxide dismutase (SOD, EC 1.15.1.1)

SOD was assayed by the inhibition of formation of NADH-phenazine methosulphate nitroblue tetrazolium formazan [42]. The reaction was initiated by the addition of NADH after incubation for 90 s the reaction was stopped by the addition of glacial acetic acid. The colour formed at the end of the reaction was extracted into the butanol layer and measured at 520 nm.

4.7.7. Assay of catalase (CAT, EC 1.11.1.6)

CAT was assayed colorimetrically by the method of Sinha [43]. Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate when heated in the presence of H_2O_2. The chromic acetate formed was measured at 620 nm. The catalase preparation was allowed to split H_2O_2 for different periods of time. The reaction was stopped at different time intervals by the addition of a dichromate – acetic acid mixture and the remaining H_2O_2 was determined colorimetrically as chromic acetate.

4.7.8. Assay of glutathione peroxidase (GPx, EC 1.11.1.9)

GPx was estimated by the method of Rotruck et al. [44]. A known amount of haemolysate was allowed to react with H_2O_2 in the presence of GSH for a specified time period, then the remaining GSH content was allowed to react with DTNB and the yellow colour developed was measured at 412 nm.

4.7.9. Isolation of RBC membrane

The erythrocyte membrane was isolated by the method of Dodge et al. [45]. The packed cells remaining after the removal of plasma were washed three times with isotonic Tris-HCl buffer (pH 7.4). Haemolysis was performed by pipetting out the washed red blood cell suspension into a polypropylene centrifuge tube, which contained hypotonic Tris-HCl buffer (pH 7.2). Ghosts were sedimented in a high speed refrigerated centrifuge at 20,000 × g for 40 min. The supernatant was decanted and the ghost button was resuspended by swirling. Sufficient buffer of the same strength was added to reconstitute the original volume. The ratio of cells to washing solution was approximately 1 : 3 by volume. The membrane was made up to a known volume with Tris-HCl buffer (0.1 M, pH 7.2).

4.7.10. Estimation of erythrocyte cholesterol

The lipid was extracted from the erythrocytes by the method of Folch et al. [46] using a chloroform – methanol mixture (2 : 1 v/v) for the estimation of cholesterol and phospholipid. Erythrocyte cholesterol was assayed by an autoanalyser (Boehringer Mannheim, Germany).

4.7.11. Estimation of erythrocyte phospholipid

Phospholipids were estimated by the method of Zilversmit and Davis [47]. The organic phosphorus in the lipid was converted to inorganic phosphorus by digesting with 1 ml of concentrated nitric acid and 1 ml of 5 N sulfuric acid to a colourless solution. 1 ml of water was added, and the mixture was boiled for 15 s, followed by the addition of 1 ml ammonium molybdate and 0.4 ml 1-amino-2-napthol-4-sulfonic acid. The colour developed was read at 680 nm.

4.8. Statistical analysis

Values were represented as means ± S.D., Data were analysed using Analysis of Variance (ANOVA) and group mean were compared with Duncan's Multiple Range Rest (DMRT).

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