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PROTECTIVE EFFECT OF ETHANOLIC EXTRACT OF *COMMIPHORA MUKUL* GUM RESIN AGAINST OXIDATIVE STRESS IN THE BRAIN OF STREPTOZOTOCIN INDUCED DIABETIC WISTAR MALE RATS

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ABSTRACT

The objective of the study was to investigate the possible neuroprotective effect of ethanolic extract of *Commiphora mukul* gum resin (EtCMGR) against oxidative stress in the brain of streptozotocin (STZ) induced diabetic Wistar rats. The experimental animals were divided into four groups: control (C), control treated with EtCMGR (C+CM), diabetic (D) and diabetic treated with EtCMGR (D+CM). Diabetes was induced by a single intraperitoneal injection of STZ (55 mg/kg body weight). Plant extract treated groups (C+CM and D+CM) were administered EtCMGR at a dose of 200 mg/kg body weight/day by gavage for 60 days. Diabetic rats showed hyperglycemia, hypoinsulinemia with impaired insulin sensitivity. EtCMGR treatment to diabetic (D+CM group) rats prevented the rise in glucose level by 96.7 %, while enhancing insulin level (77.7 %) and improving insulin sensitivity (27.3 %) compared to D group. The brain antioxidant status of D group rats showed higher levels of lipid peroxidation (77.9 %), protein glycation (100 %), and increased activities of xanthine oxidase (47.1 %) and sorbitol dehydrogenase (101.9 %) and lowered concentration of reduced glutathione (38.2 %) and decreased activities of antioxidant enzymes i.e., glutathione reductase (24 %), glutathione peroxidase (24.4 %) and superoxide dismutase (42.1 %) and increased activities of catalase (87.4 %) and glutathione-S-transferase (45.3 %) compared to control group. While EtCMGR treatment for 60 days in D+CM group prevented the observed abnormalities of antioxidant status of D group. This study demonstrates that EtCMGR is a potent neuroprotective agent against oxidative damage induced under diabetes.

Keywords: *Commiphora mukul*, streptozotocin, antioxidant enzymes, rat brain

INTRODUCTION

Diabetes is characterized by hyperglycemia and metabolic abnormalities due to decreased insulin level/action, causing metabolic and physiological changes in various organs including brain (Genet et al., 2002). The brain was considered as an insulin-insensitive tissue, however, recent molecular studies indicated that insulin is present in several regions of the central nervous system acting as a neuromodulator, inhibiting food intake and stimulating fat oxida-

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tion (Zhao and Alkon, 2002). Hyperglycemia is the most important factor responsible for the onset and progression of diabetic complication by producing oxidative stress (Giugliano et al., 1996). Diabetes also contributes to cerebrovascular complication, reduction in cerebral blood flow, disturbance of blood brain barrier and cerebral edema (Aragno et al., 1997). Cognitive deficits, along with morphological and neurochemical alterations illustrate that the neurological complications of diabetes are not limited to the peripheral neuropathies (Biessels et al., 1994). The neurological consequences of diabetes in the central nervous system are now receiving greater attention. Glucose uptake or utilization is decreased in the brain during diabetes providing a potential mechanism for increased vulnerability to pathological events (McCall, 1992). Many studies showed hyperglycemia through excessive production of reactive oxygen species, glucose induced oxidative-nitrosative stress and increased operation of polyol pathway, which are critical pathogenic mechanisms that initiate a cascade of downstream metabolic and a neurovascular perturbation (Hounsom et al., 2001). Concomitant with this new understanding of the injurious role of free radical oxidants in neuropathology, there is increasing appreciation for the need of both fundamental and clinical research in the development of the potential preventive and therapeutic benefits for a variety of antioxidant nutritional and pharmacological interventions. Treatment strategies that focus on decreasing oxidative stress as well as enhancing antioxidant defense systems might present important options for the treatment of diabetic complications. Additionally, free radical scavengers have been shown to protect neurons against a variety of experimental neurodegenerative conditions (Mosmann and Behl, 2002) as well as for attenuating the oxidative stress in STZ-induced diabetes (Agustin et al., 1993; Montilla et al., 1998). Recently there has been a growing interest in anti-diabetic and antioxidant agents from natural products especially those derived from the plants, because plant sources are usually considered to be less toxic and more free from side effects than synthetic drugs.

Commiphora mukul (C. mukul) belonging to the family of Burseraceae, commonly known as guggul, a herbal resin of the *C. mukul* tree, also known as gum guggul or oleoresin, has been used in Indian ayurvedic medicine for centuries to treat a variety of ailments, including obesity, bone fractures, arthritis, inflammation, cardiovascular diseases and lipid disorders (Satyavathi, 1991; Sinal and Gonzalez, 2002; Urizar and Moore, 2003). Traditional uses of guggul include its anti-inflammatory, antispasmodic, carminative, emmenagogue, hypoglycemic, alterative, antiseptic, astringent, a thyroid stimulant, anthelminitic and antihyperlipidemic properties. It also increases fibrinolytic activity and decreases platelet adhesiveness and scavenges free radicals in patients with atherosclerotic ischemic disease, thus reducing the cellular damage from ischemia (Sheela and Augusti, 1995; Gaur et al., 1997; Mary et al., 2003). Guggulipid and ethyl extract of guggul are established hypolipidemic agents in clinical practice. Guggul and guggulsterone were found to be effective antioxidants against LDL oxidation (Wang et al., 2004). Indeed several studies revealed cardio and neuronal protective activity of guggulsterone in animal models (Chander et al., 2003). However, little is known about the possible attenuating effect of EtCMGR on diabetic induced oxidative stress in rat brain. In addition, our earlier studies proved hepatoprotective activity of *C. mukul* against oxidative stress seen in insulin deficient (Bellamkonda et al., 2011) and insulin resistant (Ramesh and Saralakumari, 2012) diabetic Wistar rats promoted us to design the present study. Hence, our major goal is to characterize oxidative stress in the brain of STZ-induced diabetic rats and to evaluate the neuroprotective effect of EtCMGR against diabetic induced oxidative stress in the brain.

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MATERIALS AND METHODS

Chemicals

The chemicals used in the current study were procured from Sigma Chemical Company (St. Louis, MO, USA), and SISCO Research Laboratory Pvt. Ltd, Mumbai, India).

Plant extract

Ethanolic extract of *C. mukul* gum resin (brown, dry powder with lot No. L6211038) was obtained from the manufacturers and exporters of herbal extracts, Plantex Pvt. Ltd., Vijayawada, Andhra Pradesh, India. The procedure followed by the firm for the preparation of extract is as follows: The plant was identified by Dr. K. Narasimha Reddy, taxonomist, Laila impex R and D Center, Vijayawada. The collected plant sample (resin) was washed thoroughly with tap water, dried at room temperature away from sun light, cut into small pieces and then powdered. Ethanolic extract was prepared by cold maceration of gum resin powder in ethanol for 7 days. The extract was filtered, concentrated under reduced pressure and finally dried in a vacuum desiccator. Herb to product ratio was 8:1. A voucher specimen has been deposited in the Department of Biochemistry, Sri Krishnadevaraya University, Anantapur, under number DSK-CM-2010. The extract was stored at 0–4 °C. The extract was suspended in 5 % Tween-80 in distilled water prior to use.

Induction of diabetes mellitus

Male Wistar rats of 2-3 month old weighing (125-150 g) were procured from Sri Venkateswara enterprises (Bangalore, India). They were acclimatized for 7 days in our animal house (Regd. No. 470/01/a/CPCSEA, dt. 24th Aug, 2001) and maintained under standard conditions of temperature and relative humidity with 12 h light/dark cycles. Diabetes was induced in overnight fasted rats by a single intraperitoneal injection of freshly prepared STZ (55 mg/kg body weight) in 0.05 M ice-cold citrate buffer (pH 4.5) in a volume of 0.1 ml per rat. After 72 hours of STZ administration, the plasma glucose level of each rat was determined for confirmation of diabetic induction. Rats with plasma glucose level above 230 mg/dL were considered as diabetic and used subsequently.

Experimental design

In the present experiment, a total of 32 rats (16 control and 16 diabetic surviving rats) were used. The rats were divided into four groups of eight each: control (C); control treated with EtCMGR (C+CM); diabetic (D); diabetic treated EtCMGR (D+CM). The dose of EtCMGR in the current study is based on the earlier reports on the extract (Panda and Kar, 1999). The extract was administered through oral intubation to C+CM and D+CM groups at a dose of 200 mg/kg body weight per day for 60 days.

Initially (0 day) and at the end of experimental period (60 days), blood was collected from 12 h fasted rats with capillary tube from retro-orbital sinus from experimental animals into fresh vials containing EDTA (10 mg/ml) as anti-coagulant. The plasma obtained after centrifugation was used for glucose and insulin assay. Plasma glucose level was estimated by glucose oxidase-peroxidase enzymatic method using span diagnostic kit as per the manufacturers instructions and plasma insulin level was estimated by radioimmuno assay method (Bhabha Atomic Research Centre, Mumbai). Homeostasis Model Assessment (HOMA), used as an index to measure the degree of insulin resistance (IR), was calculated by the formula: fasting plasma insulin (µU/ml) × fasting glucose (mmol/L) / 22.5 (Pickavance et al., 1999).

Animal sacrifice and organ collection

After the experimental period of 60 days, the animals from each experimental group were starved for 12 h and sacrificed by cervical dislocation and immediately the whole brain was dissected out and washed with ice-cold saline and used for analysis. Ten percent homogenate of brain was pre-

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pared by using Potter-elvehjem homogenizer with Teflon pestle at a 4 °C in 0.15 M KCl. The whole homogenate was used for estimation of reduced glutathione (GSH) and lipid peroxidation (LPO). The homogenate was centrifuged in cooling centrifuge (12000 rpm for 45 min at 0-4 ^{o}C) to remove the debris and supernatant was used for enzyme assays. The total protein content of the brain in the whole homogenate was estimated by the method of Lowry et al. (1951).

Estimation of lipid peroxidation

Lipid peroxidation in brain was estimated colorimetrically by measuring the thiobarbituric acid reactive substances (TBARS) by the method of Utley et al. (1967). To 0.1 ml of 10 % tissue homogenate, 2 ml of 10 % TCA and 4 ml of 0.67 % TBA were added and heated in a water bath for 30 min and cooled. Absorbance of the supernatant was read at 535 nm. The extent of LPO was expressed as nmoles of malondialdehyde (MDA) formed/g tissue, using a molar extinction co-efficient of MDA as 1.56×10^5 M^{-1} cm^{-1}.

Estimation of reduced glutathione

Reduced glutathione was estimated in brain homogenate (10 %) using a disulfide compound, 5,5'-dithio-bis-nitrobenzoic acid (DTNB), which readily gets reduced by sulfhydryl compounds forming a highly colored anion having maximum absorbance at 412 nm, as outlined by the method of Ellman's (1959).

Estimation of protein glycation

Protein glycation in brain was measured by quantification of amadori product via Borohydride-Periodate assay as modified by Zhang and Swann (1999).

Assay of enzymes in brain homogenate

Sorbitol dehydrogenase

Sorbitol dehydrogenase (SDH) activity was measured by the method of Gerlach and Hiby as described by Bergmeyer and Bernt (1974). To 1.6 ml of triethanolamine (107 mM, pH 7.4), 0.1 ml of NADH (0.4 mM) and 0.1 ml of supernatant of brain homogenate were added and incubated at 25 ^{0}C for 30 min. The reaction was initiated by the addition of 0.3 ml of fructose (4 M). Decrease in absorbance was monitored at 1 min interval for 5 min at 340 nm. The activity was expressed as nmoles of NADH oxidized/min/mg protein by using millimolar extinction coefficient of NADH as 6.22.

Xanthine oxidase

Xanthine oxidase (XO) catalyses the oxidation of xanthine to uric acid. The activity of enzyme in brain was assayed by the increase in uric acid concentration by the method of Roussos (1967). Briefly, the assay mixture consists of 0.3 ml of Tris-HCl buffer (50 mM, pH 7.4), 0.3 ml of $CuSO_4$ (10 mM), 0.05 ml of xanthine (2.6 mM in 0.05 M glycine buffer, pH 7.4) and 0.25 ml of distilled water. The reaction was initiated by the addition of 0.05 ml of brain homogenate and increase in absorbance was monitored at 290 nm at 15 sec interval for 2 min. The activity was calculated by using a molar extinction co-efficient of uric acid as 1.22.

Glutathione reductase

Glutathione reductase (GR) catalyses the reduction of oxidized glutathione (GSSG) by NADPH to GSH. The activity of GR in brain was measured by the method of Pinto and Bartley (1969). In brief, the assay mixture consists of 0.5 ml of phosphate buffer (0.25 M, pH 7.4), 1.0 ml of EDTA (25 mM), 0.1 ml of NADPH (1 mM), 0.96 ml of distilled water and 0.1 ml of brain homogenate. The reaction was initiated by the addition of 0.024 ml of GSSG (50 mM). The activity was expressed as µmoles of NADPH oxidized/min/mg protein by using millimolar extinction coefficient of NADPH as 6.22.

Glutathione peroxidase

Glutathione peroxidase (GPx) in brain was measured by the method of Rotruck et al. (1973). Briefly, the reaction mixture

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consists of 0.5 ml of phosphate buffer (0.2 M, pH 7.0 containing 0.4 mM EDTA, and 10 mM sodium azide), 0.2 ml of brain homogenate, 0.2 ml of GSH (2 mM) and 0.1 ml of H_2O_2 (0.2 mM) and incubated for 10 min at room temperature along with blank containing all reagents expect tissue homogenate. The reaction was arrested by the addition of 0.5 ml of TCA (10 %), centrifuged at 4000 rpm for 5 min and GSH content in 0.5 ml of supernatant was estimated by following the method of Ellman's (1959). The activity was expressed as µg of GSH consumed/min/mg protein.

Glutathione-S-transferase

Glutathione-S-transferase (GST) in brain was measured by the method of Habig et al. (1974). The assay mixture consists of 1.7 ml of sodium phosphate buffer (0.14 M, pH 6.5), 0.2 ml of GSH (30 mM) and 0.04 ml of homogenate. The reaction was initiated by addition of 0.06 ml of 1-chloro-2,4-dinitrobenzene (CDNB) (0.01 M, dissolved in 50 % ethanol). The activity was calculated by using millimolar extinction co-efficient of CDNB-GSH conjugate as 9.6. The activity was expressed as µmoles of CDNB-GSH conjugate formed/min/mg protein.

Catalase

Catalase (CAT) activity was assayed by the method of Beers and Sizer (1952). The assay mixture consists of 1.9 ml of sodium phosphate buffer (0.05 M, pH 7.0), 1.0 ml of H_2O_2 (0.059 M) and 0.1 ml of brain homogenate. The activity was expressed as nmoles of H_2O_2 decomposed/min/mg protein.

Superoxide dismutase

Superoxide dismutase (SOD) activity in brain was determined according to a modified procedure adopted by Soon and Tan (2000). Superoxide dismutase activity was measured based on the ability of the enzyme to inhibit the oxidation of pyrogallol. The assay mixture consists of 0.5 ml of phosphate buffer (50 mM, pH 7.8 containing 1 mM EDTA), 0.02 ml of brain homogenate and 0.86 ml of distilled water. The reaction was initiated by the addition of 0.02 ml of pyrogallol (10 mM, in 0.01 N HCl). One unit of SOD was defined as the amount of enzyme required to inhibit the oxidation of pyrogallol by 50 % in standard assay system of 3 ml. The activity was expressed as Units/min/mg of protein.

RESULTS

General observation and characteristics

No visible side effects and variations in animal behavior (respiratory distress, abnormal locomotion and cathelepsy) were observed in C+CM group of rats, indicating the non-toxic nature of *C. mukul* administration.

Body weight, fasting plasma glucose, insulin and HOMA

Table 1 represents body weight, fasting plasma glucose and insulin and HOMA values of four experimental groups at 0 (initial) and 60 (final) days of experimental period. Characteristic symptoms of diabetes such as polyphagia, polydipsia, polyuria and loss of body weight observed in the D group were rectified in D+CM group. Groups D and D+CM showed a slight but significantly lesser initial body weight compared to C and C+CM groups. During the experimental period, D group showed a decrease in the body weight whereas C, C+CM and D+CM groups showed a trend of increase in body weight. At the end of the experimental period, D group showed (20 %) reduction in body weight while C, C+CM and D+CM groups showed an increase in the body weight by 54 %, 50 %, and 29 % respectively. By the end of the experimental period, the body weight of D+CM group was 25 % lower than C group but 49 % greater than D group.

Table 1: Effect of EtCMGR on body weight, fasting plasma glucose, insulin level and HOMA values in streptozotocin-induced diabetic rats

Groups↓ Parameter→	Period of experimentation	Body weight (gm)	Plasma Glucose (mg/dL)	Plasma Insulin (μU/mL)	HOMA
C	Initial (0 day)	182.5 ± 1.99[a]	74.5 ± 1.10[a]	42.0 ± 0.65[a]	7.7 ± 0.16[a]
	Final (60 day)	282.0 ± 1.99[e]	76.8 ± 1.59[a]	46.0 ± 1.25[a]	8.7 ± 0.30[b]
C+CM	Initial (0 day)	184.3 ± 2.31[a]	75.1 ± 0.91[a]	41.1 ± 0.39[a]	7.6 ± 0.18[a]
	Final (60 day)	276.5 ± 1.47[e]	74.3 ± 1.32[a]	44.7 ± 1.22[a]	8.2 ± 0.32[b]
D	Initial (0 day)	168.9 ± 1.87[b]	388.3 ± 4.63[c]	12.3 ± 1.06[b]	11.8 ± 0.44[c]
	Final (60 day)	134.6 ± 1.63[c]	425.2 ± 3.72[d]	1.6 ± 0.65[b]	12.2 ± 0.73[c]
D+CM	Initial (0 day)	166.2 ± 1.33[b]	397.6 ± 4.69[c]	12.8 ± 0.51[b]	12.6 ± 0.44[c]
	Final (60 day)	214.6 ± 2.45[d]	95.3 ± 1.48[b]	29.3 ± 0.93[c]	6.9 ± 0.18[d]

Values are expressed as mean ± S.E.M (n=8). Means with different superscripts within the column are significantly different at P<0.05 (Duncan's multiple range test).
C: control, C+CM: control treated with *Commiphora mukul*, D: diabetic, D+CM: diabetic treated with *Commiphora mukul,* HOMA: Homeostasis Model Assessment, EtCMGR: ethanolic extract of *Commiphora mukul* gum resin

At 0 days, plasma glucose levels of D and D+CM groups were significantly higher than corresponding value of C group. This indicates the induction of hyperglycemia in D and D+CM groups. Plasma glucose level of C group and C+CM group rats at 60 days were not deviated from their corresponding initial values. Group D rats showed 10 % increase in plasma glucose level at 60 days compared to its initial value. A significant antihyperglycemic effect of EtCMGR administration was evident in D+CM group by 76 % reduction in the plasma glucose level at 60 days compared to initial day and bringing the value to near control level.

The initial fasting plasma insulin levels of D and D+CM groups were around 4-fold lower than C and C+CM groups. By the end of the experimental period, plasma insulin levels showed no variation in C and C+CM groups and 5.7 % decrease in D group and 129 % increase in D+CM group compared to their respective initial values. Thus, at the end of the experimentation, the plasma insulin level of D+CM group was 152 % higher than D group, but still 36 % lower than C group.

HOMA approach has been widely used in clinical research to assess insulin sensitivity. There was no deviation in the initial HOMA values between C and C+CM groups. Significantly higher initial HOMA values were observed in D and D+CM groups compared to C group. At the end of the experimental period, C and C+CM groups showed 13 % and 7.9 % increase in their HOMA values when compared to their respective initial values. HOMA value of D group at 60 days was significantly higher than C and C+CM groups with no significant variation from its initial HOMA value, whereas D+CM group showed 45.1 % decrease in its HOMA value compared to its initial value. Thus, by the end of experimentation, the HOMA value of D+CM group was significantly lower than the remaining three groups.

Brain oxidative stress markers and antioxidants

Table 2 illustrates the levels of markers of oxidative stress viz. GSH, LPO, protein glycation, and activities of XO and SDH in the brain of four experimental groups. Group D showed depleted brain GSH levels (38 %) as compared to C group but treatment with EtCMGR (D+CM) limited the depletion to 26 % only. C+CM group showed no variation in the levels of oxidative stress markers from C group. Group D showed significantly higher levels of LPO (78 %), protein glycation (101 %), XO (47 %) and SDH (34 %) as compared to C-group. D+CM group showed significantly lower LPO (29 %), protein glycation

(34 %), and decreased activities of XO (19 %) and SDH (46 %), when compared with D group but they are still significantly higher than C group (26 %, 32 %, 9.5 % and 20 % respectively).

Table 3 indicates the activities of antioxidant enzymes in the brain of four experimental groups. Group D showed significantly higher activities of GST (45 %) and CAT (87 %) and significantly lower activities of GR (24 %), GPx (24 %) and SOD (42 %) as compared to C group. Group D+CM showed significantly lower activities of GST (21 %) and CAT (21 %) and significantly higher activities of GR (25 %), GPx (13 %) and SOD (45 %), when compared with D group.

Table 2: Effect of EtCMGR administration on brain oxidative stress markers in streptozotocin-induced diabetic rats

Experimental groups	C	C+CM	D	D+CM
Reduced glutathione (µg/mg protein)	7.41 ± 0.58[c]	7.99 ± 0.34[c,a]	4.58 ± 0.70[a]	5.52 ± 0.34[a]
Lipid peroxidation (nmols of MDA/mg protein)	0.190 ± 0.022[a]	0.164 ± 0.009[a]	0.338 ± 0.021[b]	0.240 ± 0.018[a]
Protein glycation (nmols of fructose/mg protein)	30.86 ± 0.47[a]	34.25 ± 0.61[b]	62.01 ± 1.35[d]	40.79 ± 1.07[c]
Xanthine oxidase (µmoles of NADH formed/min/mg protein)	24.52 ± 1.27[a]	19.28 ± 1.33[b]	36.07 ± 1.36[d]	29.33 ± 1.09[c]
Sorbitol dehydrogenase (nmols of NADH consumed/min/mg protein)	0.106 ± 0.010[a]	0.134 ± 0.004[a]	0.214 ± 0.011[b]	0.116 ± 0.006[a]

Values are expressed as mean ± S.E.M (n=8). Means with different superscripts within the row are significantly different at P<0.05 (Duncan's multiple range test). C: control, C+CM: control treated with Commiphora mukul, D: diabetic, D+CM: diabetic treated with Commiphora mukul, EtCMGR: ethanolic extract of Commiphora mukul gum resin

Table 3: Effect of EtCMGR administration on the activities of antioxidant enzymes in the brain of streptozotocin-induced diabetic rat

Experimental groups	C	C+CM	D	D+CM
Glutathione reductase (µmols of NADPH oxidized/ min/mg protein)	31.41 ± 1.65[a]	32.88 ± 1.17[a]	23.88 ± 0.75[b]	29.94 ± 0.66[a]
Glutathione peroxidase (µg of GSH consumed/min/ mg protein)	12.27 ± 0.29[c]	12.10 ± 0.33[c]	9.28 ± 0.36[a]	10.45 ± 0.25[b]
Glutathione-S-transferase (µmols of GSH-CDNB conjugate formed/min/ mg protein)	0.150 ± 0.007[a]	0.158 ± 0.005[a]	0.218 ± 0.009[b]	0.172 ± 0.009[a]
Superoxide dismutase (U/min/mg protein)	8.97 ± 0.07[d]	8.70 ± 0.23[b]	5.19 ± 0.13[a]	7.53 ± 0.19[c]
Catalase (mmols of H_2O_2 decomposed/min/mg protein)	2.62 ± 0.24[c]	1.88 ± 0.14[d]	4.91 ± 0.20[a]	3.8 ± 0.19[b]

Values are expressed as mean ± S.E.M (n=8). Means with different superscripts within the row are significantly different at P<0.05 (Duncan's multiple range test). C: control, C+CM: control treated with *Commiphora mukul*, D: diabetic, D+CM: diabetic treated with *Commiphora mukul,* EtCMGR: ethanolic extract of *Commiphora mukul* gum resin

Statistical analysis

The results were expressed as mean ± S.E.M. Data were analyzed for significant difference using Duncan's Multiple Range (DMR) test ($P < 0.05$) (Duncan, 1955).

DISCUSSION

Streptozotocin induced hyperglycemia in rodents is considered to be a good model for diabetes mellitus with insulin deficiency condition and a relevant example of endogenous chronic oxidative stress. The development of hyperglycemia in rats following STZ injection is primarily due to direct pancreatic β-cell dysfunction. STZ [(2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose)] is a potent DNA methylating agent and acts as a donor of nitric oxide in pancreatic cells. β-cells are particularly sensitive to damage by nitric oxide and free radicals because of their low levels of antioxidant potential (Spinas, 1999). Further it causes DNA strand breaks in pancreatic islets stimulating nuclear poly (ADP-ribose) synthetase and thus depleting the intracellular NAD^+ levels, which in turn inhibits pro-insulin synthesis and induces diabetes (Wilson et al., 1988). In the present study, treatment with EtCMGR showed significant antihyperglycemic activity in D+CM group rats. The antihyperglycemic activity of this plant may be, at least in part, through release of insulin from the pancreas in the view of measured plasma insulin concentration and increased insulin sensitivity as an evident by decreased HOMA values in D+CM group rats compared to D group.

The significant increase in plasma insulin levels of D+CM group compared to D group may be attributed to the regeneration the STZ-destructed β-cells which is probably due to the fact that pancreas contains stable (quiescent) cells that have the capacity to regenerate (Banerjee et al., 2005; Cano et al., 2008). Therefore, the surviving cells can proliferate to replace the lost cells. Phytochemicals such as flavonoids and alkaloids present in the EtCMGR may have protected the intact functional β-cells from further deterioration through oxidative stress. Hence, β-cells remain active and continue to produce insulin. It is also claimed that antioxidants such as flavonoids are possibly beneficial in preventing STZ-induced diabetes by stopping oxidative damage of the pancreas, and increasing insulin secretion by the regeneration of pancreatic β-cells (Bravi et al., 2006).

The observed significant increase in the HOMA values by the end of experimental period in C and C+CM groups indicates a gradual progression of insulin resistance with aging. However, this increase is less in C+CM group compared to C group indicating the protective effect of *C. mukul* against progression of insulin resistance with aging. Significantly higher initial and final HOMA values of D group compared to corresponding values of C group indicate existence of decreased insulin sensitivity in STZ-induced diabetic rats in addition to insulin deficiency. Insulin sensitizing activity of *C. mukul* is further evident from the decreased final HOMA values of D+CM group compared to its initial as well as remaining group's final HOMA values.

Our earlier studies demonstrated the existence of oxidative stress in liver (Bellamkonda et al., 2011), kidney (Rasineni et al., 2010), pancreas and heart (Ramesh et al., 2012) in STZ-induced diabetic rats and in erythrocytes and reticulocytes of diabetic patients (Sailaja et al., 2003). In agreement with earlier reports (Montilla et al., 1998), the present study demonstrates that STZ-induced diabetes mellitus causes oxidative stress in the brain. The present study is the first to show the neuroprotective effect of *C. mukul* against diabetes induced alterations in the brain of rats.

Streptozotocin induced diabetes serves as an excellent model to study the cellular, biochemical, molecular and morphological changes in brain induced by stress during diabetes (Aragno et al., 2000). Hyperglycemia is the most important factor in the onset and progress of diabetic complications mainly by producing oxidative stress. Hyperglycemia induced tissue damage favours some particular subset of cell types,

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which are involved in diabetic complication (capillary endothelial cells of retina, mesangial cells of glomerulus, neurons and Schwann cells in peripheral nerves) due to the fact that most cells are able to reduce the transport of glucose inside the cell when they are exposed to hyperglycemia, so that internal glucose concentration remains constant. Brain is an insulin independent tissue and leads to high exposure to glucose under hyperglycemic conditions. Cerebral glucose increased after the onset of diabetes in rats (Nayeemunnisa, 2009). This increased intracellular glucose load leads to auto-oxidation of glucose, generation of free radicals, enhanced lipid peroxidation and non-enzymic glycation proteins, increased polyol pathway. The central nervous system is highly susceptible to oxidative stress. Vulnerability of brain to oxidative stress induced by oxygen free radicals seems to be due to the fact that on one hand, the brain utilizes about one fifth of total oxygen demand of body and on other because of its relatively small antioxidant capacity. Further, the brain contains relatively high concentration of easily peroxidiable fatty acids (Carney et al., 1991) and it is known that certain regions of brain are highly enriched in iron, a metal that in its free form is catalytically involved in production of damaging oxygen free radical species. Antioxidant therapy is proved to be remarkably beneficial to combat reactive oxygen species induced injury in CNS. Our results showed that lipid peroxidative damage of the brain increased in D group of rats. This is associated with significant increase in brain protein glycation and enhanced activities of sorbitol dehydrogenase and xanthine oxidase in diabetic rats. Lipid peroxidation is a free radical related process which is potentially harmful because its uncontrolled self enhancing process causes disruption of membranes, lipids and other cell components. Reactive oxygen species can increase the permeability of blood brain barrier and inhibits the mitochondrial respiration leading to geometrical progression of lipid peroxidation (Gilman et al., 1993). Our reports of increase in extent of lipid peroxidation in the brain of diabetic rats are in agreement with earlier studies (Kumar and Menon, 1993; Pari and Murugan, 2007; Rastogi et al., 2008) suggesting that the increased lipid peroxidation plays a major role in the development of enhanced oxidative stress.

One of the consequences of chronic hyperglycemia is the excessive non-enzymic glycation of proteins known as Millard reaction and leads to alter protein confirmation and function and decreased its half life period, resulting in complication of diabetes. Enhanced glycated protein concentration observed in the brain of D group of rats may induce the formation of oxygen derived free radicals contributing to enhancement of oxidative stress. Our report of increase in extent of protein glycation in diabetic rat brain is in consistence with previous reports in literature (Brownlee et al., 1988; Das Evcimen et al., 2004). In addition, the enhanced protein glycation in D group rats may also be due to increased operation of polyol pathway as reflected by increased activity of sorbitol dehydrogenase in diabetic rat, thus leading to the increased production of sorbitol and fructose. Under hyperglycemic conditions, high glucose flux through sorbitol pathway accounts for one third of glucose metabolism (Gonzalez et al., 1984). This has important implication in terms of redox changes of $NADP^+$ and NAD^+ couples and metabolism of glucose by alterative pathways (Jeffrey and Jornvall, 1983). Conversion of glucose to sorbitol by aldose reductase requires NADPH and forms $NADP^+$ thereby competing with other NADPH requiring reactions. Conversion of sorbitol to fructose by sorbitol dehydrogenase is coupled to the reduction of NAD^+ to NADH and this competes with glycolysis at the glyceraldehyde dehydrogenase step for NAD^+ (Gonzalez et al., 1986). It reduces the proportion of NADPH by NAD^+ and increases the proportion of NADH by NAD^+. The trouble in the oxidation of NADH in the respiratory chain is indicated as hyperglycemia “pseudohypox-

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ia" and leads to increasing the quantity of reactive oxygen species in the cells.

Further increased activity of XO, an important source of oxygen free radicals in the brain of D group rats may contribute to the free radical mediated damage and development of diabetic complication of brain. Xanthine oxidoreductase under normal condition exists in dehydrogenase form and uses NAD^+ and there is very little production of superoxide anions. Under NAD^+ deficient and/or ischemic condition, there is depletion of ATP leading to subsequent loss of membrane Ca^{2+} gradient. Increased calcium levels activate Ca^{2+} dependent proteases which cause selective proteolysis of the dehydrogenases to convert it into xanthine oxidase which acts both on hypoxanthine and xanthine at the expense of molecular oxygen to produce superoxide ions. Increased activity of XO in the diabetic rats in the present study is in agreement with earlier studies in rat brain (Ates et al., 2007).

Reduced glutathione is a major endogenous antioxidant which counter balances free radical mediated damage (Mazumder et al., 2005). It is well known that GSH is involved in the protection of normal cell structure and function by maintaining the redox homeostasis, quenching free radicals and by participating in detoxification reactions (Matcovis et al., 1982). A significant decrease in the level of GSH observed in the brain of D group rats compared to C group rats could be the result of decreased synthesis or increased degradation/utilization of GSH by increased oxidative stress. The decreased GR activity, the enzyme responsible for regeneration of GSH from GSSG may also contribute to the observed depleted levels of GSH in the brain of D group rats. The significant decrease in GR activity in the brain of D group rats may be due to less availability of NADPH, the cofactor required for this enzyme because diversion of NADPH by enhanced operation of polyol pathway as reflected by significantly increased activity of SDH in D group rats. Besides, the reported reduced ATP levels (Mastrocola et al., 2005) as a result of change in the activities of mitochondrial respiratory chain enzymes in the brain of diabetic rats are also closely related to the decreased de novo synthesis of GSH. Our reports of decreased GSH and GR activity in D group rats were supported by earlier studies (Montilla et al., 2005; Pari and Murugan, 2007) suggesting that the decreased GSH are sensitive and potentially relevant indicators of neurotoxicity and play a role in the development of diabetic complications in brain. Superoxide dismutase protects the cell against the toxic effect of superoxide radicals by converting it to less toxic H_2O_2. Glutathione peroxidase is responsible both for metabolizing lipid peroxides and for the decomposes of H_2O_2. The significant decrease in the activities of SOD and GPx in the brain of D group rats compared to C group rats implied weak antioxidant status through these enzyme activities. The increase in activity of XO in D group is important since XO is recognized as one of the extra mitochondrial sites of superoxide generation. This problem gets further aggravated by the decreased activity of SOD in diabetic rats. The depletion in GSH level of D group rats may be responsible for the decreased activity of GPx since GSH is the substrate for the GPx activity. However, surprisingly there was an increased CAT and GST activities in diabetic rat brain compared to control in the study. A survey of literature revealed that activities of antioxidant enzymes varied in the different organs and different diabetic phases (Huang et al., 2006). Glutathione-S-transferase is a multifunctional protein found in many tissues and shows a broad specificity for organic hydroperoxides but not for H_2O_2 (Freeman and Crapo, 1982) and it has an important role in the central nervous system including a line of defense in astrocytes against potentially toxic xenobiotics and in protecting the myelin sheath (Cooper and Meister, 1993). Our reports of increased GST in diabetic rats suggest enhanced utilization of GSH for detoxification of toxic products. Increased CAT activity decomposes H_2O_2 without generation of free radicals by minimizing one electron transfer (Corrocher et al., 1986; Mueller et al., 1997) of diabetic rat which indicates a compensatory adaptive mechanism against oxidative stress to maintain homeostasis. However, enhanced activities of CAT and GST are not sufficient to overcome the oxidative stress as reflected by enhanced oxidative stress markers in the present study.

Antioxidant potential of *C. mukul* against oxidative stress is evident with lower levels of LPO, protein glycation, activities of XO and SDH, higher levels of GSH and increased activities of antioxidant enzymes as seen in the brain of D+CM group when compared with D group. The antioxidant potential of this plant was well documented in literature (Bellamkonda et al., 2011; Ramesh and Saralakumari, 2012). The antihyperglycemic effect with increased insulin sensitivity of *C. mukul* is evident from the decreased plasma glucose level of D+CM group which further resulted in decreased flux of glucose through polyol pathway by decreasing the SDH activity indicating a consequent increase in the ratio of NADPH/NADP$^+$. Thus increased availability of NADPH may be responsible for increased GR activity, restoring it to control values in D+CM group rats. This in turn elevates the availability of GSH, the substrate for GPx, resulting in increased activity of GPx, which further scavenges lipid peroxides, hydrogen peroxides and hydroxyl radicals resulting in normalization of LPO in D+CM group. Further, the significant increase in the activity of SOD of D+CM rats compared to D group rats bringing it to near control values reflects the protection against superoxide radicals and thus oxidative stress. The imbalance in the activities of antioxidant enzymes in the D group of rats as reflected by enhanced activities of CAT and GST was corrected by restoring these enzyme activities to near control and control values respectively in D+CM group. Earlier studies showed wild type diabetic mice are susceptible to develop diabetic neuropathy by reduced nerve conduction velocity, structural abnormalities in the nervous tissue (Yagihashi et al., 2001) with decreased GSH level in their sciatic nerve whereas Chung et al. (2003) reported that polyol pathway was the major source of diabetes induced oxidative stress from their observation of no change in GSH levels with unaltered nerve conduction velocity in aldose reductase gene knockout diabetic mice. The entire process of antioxidant potential of *C. mukul* can be explained by a mechanism whereby decreased flux through the polyol pathway increases the effectiveness of GSH redox cycle in scavenging free radicals in addition to "*C. mukul*" antihyperglycemic, insulinogenic and enhanced insulin sensitizing activities.

Till date, several chemical components such as diterpenes, sterols, steroids, esters and higher alcohols have been identified in *C. mukul* (Zhu et al., 2001; Wang et al., 2004). Oleoresin of *C. mukul* is a mixture of several steroid lipids. Of these steroids, Z-guggulsterone and E-guggulsterone are the most effective (Patil et al*.*, 1972; Sukh, 1987). Guggulsterones in the body are easily reduced to guggulsterols which behave as powerful antioxidants. The antioxidant properties of guggulsterols could be explained by the fact that their hydroxyl groups are present at α-positions of double bonds, similar to antioxidant vitamins, and are soluble in lipids. The steroid structure also contains H, CH_3 and O bond, which indicates that the drug, like other herbs may also quench free radicals such as hydroxyl and singlet oxygen due to its antioxidant effect thus causing a decrease in lipid peroxides similar to the action of probucol (Anderson et al*.*, 1991). The guggulsterols, appear to be non-toxic, because they have no highly reactive groups in any position of the isomer structure, similar to tocopherols. Guggulipid, the resin of *C. mukul* was reported to decline LPO in hypercholesterolemic humans (Singh et al., 1994) and Cu^{+2} mediated oxidative stress (Chander et al., 2003). Guggulipid and guggulsterone have been demonstrated to reduce the risk of cardiac events and improved cardiac function in experimental and clinical studies (Nityanand et al., 1989; Sheela and Augusti, 1995; Gaur et al., 1997; Szapary et al., 2003; Ulbricht et al., 2005). The protective action of guggulsterone is due to antioxidant property because it inhibits the generation of oxygen free radicals (Chander et al., 2002). Guggulsterone was also reported to have protection against oxidative modifications of lipid and protein components of LDL induced by Cu^{2+} *in vitro* (Singh et al., 1977). A standard alcoholic fraction from guggulipid containing E-guggulsterone was already studied and proved for its protection against free radical damage in the skin and scavenging of superoxide anions and hydroxyl radicals in non-enzymatic test systems (McCord and Fridovich, 1969). Studies of Saxena et al. (2007) demonstrated that the protective effect of gugulipid isolated from *Commmiphora whighitii* against STZ-induced memory deficits model of dementia in mice was due to antioxidant potential. Thus, the antioxidant potential of *C. mukul* observed in the present study may be due to the guggulsterone component of the resin.

CONCLUSION

The results from the present study suggest that *C. mukul* provides significant protection to brain against the consequence of hyperglycemia and diabetes induced oxidative stress by scavenging reactive oxygen species and blocking free radical interactions. Thus, *C. mukul* may be potentially beneficial in prevention of diabetic induced neuropathy. The beneficial effects of *C. mukul* on brain oxidative stress is important in view of the evidences linking increased oxidative stress to a number of neurodegenerative diseases like Parkinson’s disease, Alzheimer disease, in addition to diabetic neuropathy.

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