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Evaluation of *in-vivo* antioxidant and oral glucose tolerance test of ethanolic extract of *Calotropis gigantea* Linn. against streptozotocin-induced diabetic rats

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ABSTRACT

Antioxidant compounds play an important role in preventing or delaying the onset of major degenerative diseases. The physiological role of antioxidant compounds is to scavenge for free radicals. Active oxygen (hydroxyl, peroxy radicals and single oxygen) is highly toxic and an important causative agent of many diseases including cancer, heart disease, cataract and congestive disorders. Antioxidant compounds block the oxidation processes that produce free radicals which contribute towards these chronic diseases and aging. The purpose of this study was to evaluate the *in-vivo* antioxidant activity of *Calotropis gigantea* Linn. against streptozotocin-induced diabetic rats.

Keywords: *Calotropis gigantea* Linn., Streptozotocin, *In-vivo* antioxidant, Oral glucose tolerance test (OGTT), Histopathology.

1. Introduction

Calotropis gigantea Linn. (Asclepiadaceae) commonly known as milkweed or swallow-wort, is a common wasteland weed. *Calotropis* belongs to Asclepiadaceae or Milkweed or Ak family which includes 280 genera and 2,000 species of world-wide distribution but most abundant in the sub-tropics and tropics, and rare in cold countries. Different parts of this plant such as the leaves, stem, flowers, and root bark are prescribed by traditional healer in Asia in a variety of disorders of gastrointestinal, cardiovascular, biliary, hepatic, urinary and respiratory origin^[1,2]. Type 2 diabetes is associated with increased oxidative stress. Free radicals are continuously produced in the body as the result of normal metabolic processes and interaction with environmental stimuli. Under physiological conditions, a wide range of antioxidant defenses protect against adverse effects of free radical production *in vivo*^[3]. Oxidative stress results from an imbalance between radical production and reduced activity of antioxidant defenses or both these phenomena. Hyperglycemia causes release of tissue damaging reactive oxygen species (ROS) that disturbs balance between radical production and protective antioxidant defense^[4]. It has been proposed that streptozotocin (STZ) acts as a diabetogenic agent owing to its ability to destroy pancreatic β -cells, possibly by a free radical mechanism^[5, 6]. The level of lipid peroxidation in cell is controlled by various cellular defense mechanisms consisting of enzymatic and nonenzymatic scavenging systems which are altered in diabetes^[7]. Moreover, disturbances of antioxidant defense systems in diabetes showed alteration in antioxidant enzyme levels, such as superoxide dismutase (SOD) and catalase (CAT), along with impaired glutathione (GSH) metabolism^[8]. Chemical with antioxidant properties and free radical scavengers may help in the regeneration of β -cell and protect pancreatic islets against cytotoxic effect of STZ^[9]. Antioxidants provide protection in living organisms from damage caused by uncontrolled production of ROS concomitant lipid peroxidation, protein damage and DNA strand breaking. Ethno medical literature contains a large number of plants including, *Calotropis gigantea* (Asclepiadaceae) that can be used against diseases, like diabetes, atherosclerosis, ischemic heart disease, disorders induced by free radicals and other reactive oxygen species^[10].

Hence, the present study was under taken to explore *Calotropis gigantea* (Asclepiadaceae) free radical scavenging activity in STZ-induced diabetic rats.

2. Materials and method

2.1 Chemicals and reagents

Streptozotocin was purchased from Aldrich, Nitro blue tetrazolium (NBT), dimethyl sulphoxide, potassium chloride and sodium chloride from Ranbaxy Laboratories Ltd., Mohali, India. Sulphanilic acids and sodium bicarbonate from E-Merck (India) Ltd, Mumbai, India. Hydrogen peroxide was obtained from Hi-Media Lab Pvt. Ltd, Mumbai. All other chemicals used in the studies were analytical /laboratory grades procured from the following manufacturers, Loba chemie, ACROS Organics, Merck lab, S.D. Fine chemicals, Fluka.

2.2 Collection and Authentication

The fresh leaves (whole plant) of *Calotropis gigantea* was collected during June 2011, from the ABS Botanical gardens karpatti Salem district, Tamilnadu. The plant species was identified and authenticated by taxonomist Dr. A. Balasubramanian. A voucher specimen was retained in the department for future reference.

2.3 Preparation of the Extract

The collected fresh plant materials of *C. gigantea* were successively extracted with 95% ethanol by continuous hot percolation method using soxhlet apparatus. The solvent was removed under reduced pressure. The extract obtained was kept for drying and stored in vacuum desiccator.

2.4 Animals

Healthy, adult Wistar rats of both sexes (180-220 gm) were obtained from the Institutional Animal Ethics Committee, Mahatma Gandhi Institute of Pharmacy Uttar Pradesh. The animals were kept in a well-ventilated room and the animals had exposed to 12 hrs day and night cycle with a temperature between 20 ± 3 °C. The animals were housed in large spacious, hygienic polypropylene cages during the course of the experimental period. The animals were fed with water and rat feed ad libitum, supplied by this institution. All the experiments were performed after obtaining prior approval from IAEC Number 160/PO/a/12/CPSCA (30.4.2011).

2.5 Induction of Diabetes

2.5.1 Preparation of Streptozotocin:

The Streptozotocin was made at a final concentration of 50 mg/kg by dissolving in citrate buffer (pH 4.5) the solution was then kept refrigerated overnight to facilitate its dissolution. Streptozotocin was administered i.p to the rats.

Non-Insulin dependent diabetes mellitus (NIDDM) was induced in overnight fasted rats by a single intraperitoneal injection (i.p.) of 50 mg/kg streptozotocin. Hyperglycemia was confirmed by the elevated glucose levels in plasma, determined at 72 hrs. The rats with permanent NIDDM (250-350 mg/dL) were used for the study.

2.6 Experimental Design

All the procedures for antidiabetic activity were performed in accordance with the Institutional Animal Ethical Committee constituted as per the norms of CPCSEA, under the Ministry of Animal Welfare, Govt. of India, New Delhi, India. In order to

assess the anti-diabetic activity, the animals were divided in five

groups of six animals in each group^[11,12].

Group 1: Untreated Control

Group 2: Diabetic control

Group 3: Positive control (glibenclamide 10 mg/kg b.w i.p)

Group 4: EtOH extract of *Calotropis gigantea* (100 mg/kg, orally)

Group 5: EtOH extract of *Calotropis gigantea* (200 mg/kg, orally)

Group 6: ChF of *Calotropis gigantea* (100 mg/kg, orally)

Group 7: BtF of *Calotropis gigantea* (100 mg/kg, orally)

The test drug was administered for 21 days at a four different dose level 100, 200 mg/kg for ethanolic extract and 100, 100 mg/kg each of two successive fractions made in aqueous and given by orally. The blood was collected by retro-orbital sinus under light diethyl ether anesthesia. The blood was centrifuged at 3000 rpm for 10 minutes. Oral glucose tolerance test (OGTT) was also performed. Pancreas was used for *in vivo* antioxidant. On the day of termination of the study, the animals were sacrificed, liver were excised and stored in 10% buffered neutral formalin for histopathological studies^[13-15].

2.7 *In-vivo* antioxidant by rat pancreas homogenate

Tissue homogenization

Pancreas was collected in ice-cold container weighed accurately and mixed with Triss buffer solution (0.1 M) in tissue homogenizer. Homogenate was centrifuged and Supernatant was used for antioxidant enzyme (SOD, CAT, GSH, and MDA) level.

2.7.1 SOD (Superoxide dismutase) estimation:

Assay mixture contained 0.1 ml of supernatant, 1.2 ml of buffer, 0.1 ml of PMS, 0.3 ml of NBT, 0.2 ml of NADH. Reaction was started b addition of NADH. After incubation at 30 °C for 90 s, the reaction was stopped by addition of 0.1 ml of Glacial acetic acid reaction mixture was stirred vigorously with 4.0 ml of n-butanol. Color intensity of the chromogen in the butanol was measured spectrophotometrically at 560 nm.

2.7.2 Catalase estimation:

Buffer and H₂O₂ were incubated and kept at 25 °C for 30 min prior to initiation of assays. Samples (0.5 mg per 10 µl) and sucrose kept on ice throughout procedure. Pipette the following into 3 ml quartz cuvette. Buffer 2.250 ml, Sample or sucrose 0.100 ml, H₂O₂ 0.650 ml to initialize reaction. Measure and record change in absorbance at 240 nm for 2-3 min.

2.7.3 Estimation of glutathione reductase activity:

The assay system consisted of 1.65 mL of buffer, 0.1 mL of EDTA, 0.05 mL of oxidized glutathione, 0.1 mL of NADPH and 0.05 mL of supernatant in a total mixture of 2 ml. The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm at 30 s intervals for 3 min.

2.7.4 Estimation of Thiobarbituric acid reaction substance (TBARS)

Incubation mixture was made 5.0 ml with double distilled water and then heated in boiling water bath for 30 min. After cooling the red chromogen was extracted into 5 ml of the mixture of n- butanol and pyridine (15:1 v/v) centrifuged at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. 1, 1, 3, 3- tetra ethoxy propane (TEP) was used as an external standard and the level of lipid peroxides.

2.8 Oral glucose tolerance test (OGTT)

The oral glucose tolerance test was performed in overnight fasted

(18 h) normal animals. Rats divided in to six group (n-6) were administered 10 mg/kg Glibenclamide, 100 mg/kg, 200 mg/kg ethanolic extract, 100 mg/kg chloroform fraction, and 100 mg/kg n-butanol fraction, respectively. Glucose (2 g/kg) was fed 30 min. after the administration of extracts. Blood was withdrawn from the retro orbital sinus under ether inhalation (to minimize the distress) at 0, 30, 60, 90, and 120 min. of extract administration. The fasting blood glucose levels were estimated by glucose oxidase-peroxidase method.

2.9 Histopathological study

The liver samples fixed were fixed for 48 h in 10% formal saline were dehydrated by passing successfully in different mixture of ethyl alcohol-water, cleaned in xylene and embedded in paraffin. Sections of liver were prepared and, then stained with hematoxylin and eosin dye, which mounted in neutral deparaffinated xylene

(DPX) medium for microscopic observations.

2.10 Statistical Analysis

All the data are expressed as mean±SEM and analysed statistically using ANOVA followed Dunnett's test and compare with respective control group. A value of $P<0.001$ was considered as statistically significant.

3 Result

3.1 Effect of pancreatic tissue GSH, SOD, Catalase and MDA activities in control, diabetes and treatment groups

As shown in Table 1 there was increase significantly differences in SOD, MDA enzyme ($P<0.001$, $P<0.05$) activities but GSH, CAT shown significantly decrease when compared to control and diabetic group ($P<0.001$, $P<0.01$). GSH, MDA and Catalase shown significantly increase and SOD shown moderate action after 21 days treatment in diabetic group ($P<0.001$, $P<0.01$, $P<0.05$).

Table 1: Effect of *Calotropis gigantea* leaves of extract/fractions on *in vivo* antioxidant parameter by pancreas homogenate

S. No	Group	GSH ($\mu\text{g}/\text{mg}$ of protein)	SOD (unit/min/gm tissue)	Catalase (μmol of $\text{H}_2\text{O}_2/\text{min}/\text{gm}$ tissue)	MDA ($\mu\text{g}/\text{mg}$ of protein)
1	Untreated control	19.30±0.720	0.657±0.158	3.686±0.104	3.816±0.043
2	Diabetic control	10.54±0.383 ^{##}	0.866±0.271 [#]	1.474±0.076 ^{###}	8.692±0.216 ^{###}
3	Diabetic + Glibenclamide (10mg/kg)	15.05±1.350 ^{**}	0.500±0.111 [*]	3.785±0.088 ^{***}	4.15±0.18 ^{***}
4	Diabetic +EtOH(100mg/kg)	15.88±0.114 ^{**}	0.237±0.038 [*]	2.702±0.104 ^{***}	4.70±0.092 ^{***}
5	Diabetic + EtOH(200mg/kg)	18.192±0.283 ^{**}	0.273±0.037 [*]	3.318±0.045 ^{***}	3.97±0.05 ^{***}
6	Diabetic + ChF(100mg/kg)	15.29±0.319 ^{**}	0.350±0.087 [*]	2.715±0.051 ^{***}	4.79±0.05 ^{***}
7	Diabetic + BtF(100mg/kg)	14.58±0.1037 ^{**}	0.22±0.033 [*]	2.158±0.082 ^{***}	6.63±0.07 ^{***}

All value are expressed as mean ± SEM (n=6). *** $P<0.001$, ** $P<0.01$, * $P<0.05$ as compared to diabetic control

^{##} $P<0.001$, [#] $P<0.01$, [#] $P<0.05$ as compared to untreated control. One-way ANOVA followed by Bonferroni multiple comparison tests

3.2 Effect of ethanolic extract/fractions on Oral glucose tolerance test

The ethanolic extract/fractions showed a significant reduction in

blood glucose levels from 30 min onwards when compared to control group of animals. The changes in glucose reduction in blood in all groups of animals were given in Table 2.

Table 2: Effect of *Calotropis gigantea* leaves on OGTT

S. No	GROUP	Blood glucose concentration (mg/dL)				
		0 min	30 min	60 min	90 min	120 min
1.	Untreated Control	88.16±1.24	110.5±1.33	103±0.73	102.5±0.76	96.33±0.98
2.	Diabetic + Glibenclamide (10mg/kg)	82.5±0.76	94.2±4.01 ^{###}	80.5±2.18 ^{###}	78±2.12 ^{###}	66.5±0.76 ^{###}
3.	Diabetic + EtOH(100mg/kg)	83.33±0.88	98±2.78 ^{**}	86.16±5.06 ^{**}	89.5±2.86 ^{***}	75.66±1.33 ^{***}
4.	Diabetic + EtOH(200mg/kg)	87.83±2.15	99.66±3.11 ^{**}	84.5±4.42 ^{**}	85.83±2.77 ^{***}	84±1.39 ^{***}
5.	Diabetic + ChF(100mg/kg)	88.66±2.01	98.5±1.99 ^{**}	84.83±3.30 ^{**}	89.33±4.11 ^{***}	83.66±2.87 ^{***}
6.	Diabetic + BtF(100mg/kg)	89.16±2.33	96.16±1.81 ^{**}	86.66±2.87 ^{**}	90.6±3.13 ^{***}	83.1±2.73 ^{***}

All value are expressed as mean ± SEM (n=6). *** $P<0.001$, ** $P<0.01$, as compared to control value

^{###} $P<0.001$, ^{##} $P<0.01$ as compared to positive control. One-way ANOVA followed by Bonferroni multiple comparison tests

3.3 Histopathological Examination of Liver

Histopathological examination of Liver

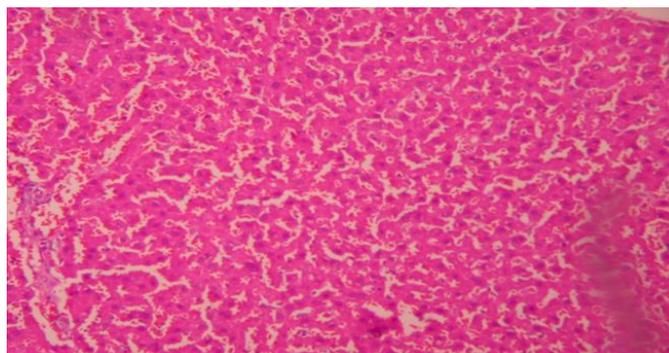


Fig 1a: Photomicrograph of normal rat liver showing no pathological changes

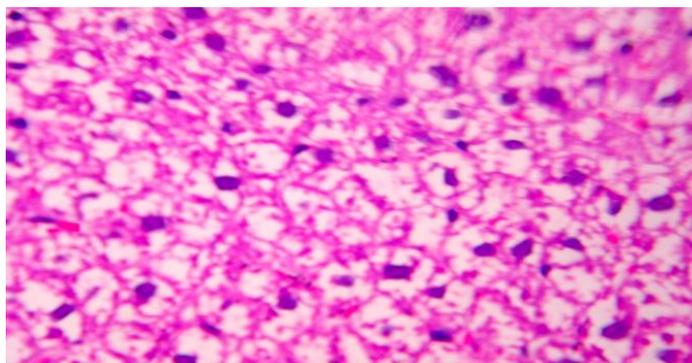


Fig 1b: Photomicrograph of diabetic rat liver showing lesions and inflammation

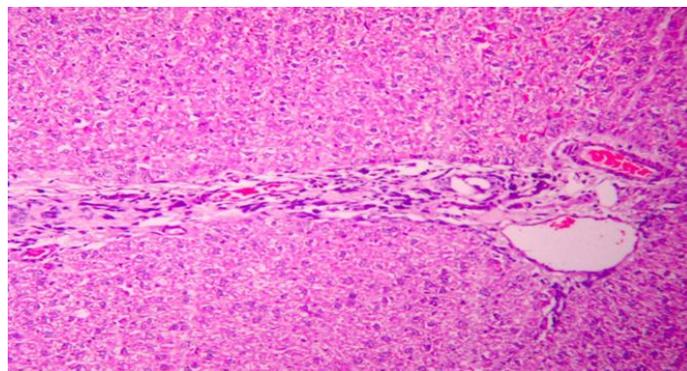


Fig 1c: Photomicrograph of glibenclamide treated rat liver showing near normal morphology

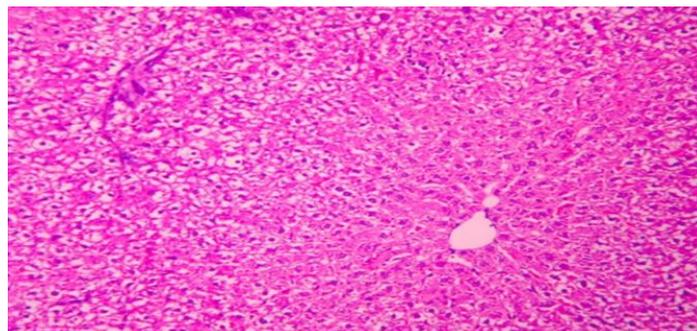


Fig 1d: Photomicrograph of EtOH 100 mg/kg treated rat liver showing Congestion of portal triad with mild inflammation

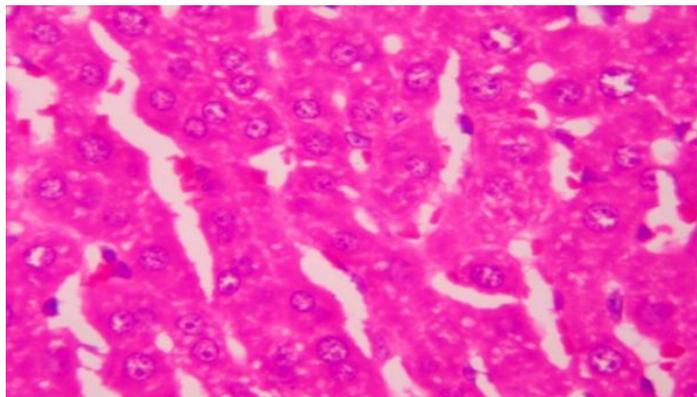


Fig 1e: Photomicrograph of EtOH 200 mg/kg treated rat liver showing Near normal with mild congestion

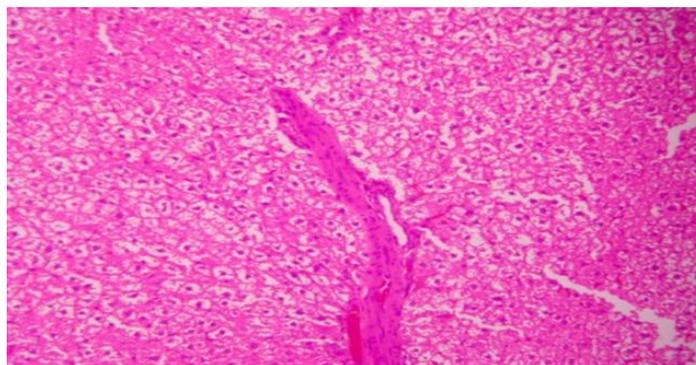


Fig 1f: Photomicrograph of ChF 100 mg/kg treated rat liver showing Sinusoidal congestion and fatty degeneration in the form fatty flake

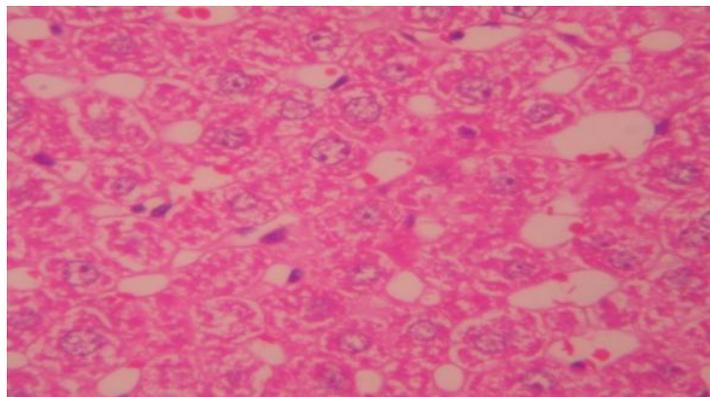


Fig 1g: Photomicrograph of BtF 100 mg/kg treated rat liver showing less inflammation only around central vein

Fig 1: Photomicrographs of rat liver stained by haematoxylin and eosin Microscope magnification (100 X)

4. Discussion

The increase in oxygen free radicals in diabetes could be due to rise in blood glucose levels which upon generate free radicals. Lipid peroxide mediated damage has been observed in the development of diabetes mellitus. An elevated level of lipid peroxides in the plasma of STZ diabetic rats and lipid peroxidation is one of the characteristic features of chronic diabetes. The increased levels of thiobarbituric acid reactive substances (TBARS), MDA and hydroperoxides are the indices of lipid peroxidation.

The antioxidant enzymes include superoxide dismutase (SOD), catalase, (CAT) and glutathione reductase (GSH) and malonaldehyde (MDA) play a role as protective enzyme in the defense system. Increased the level of MDA, SOD and decreased levels of GSH and CAT in the diabetic state may be due to inactivation caused by reactive oxygen species. In present study the levels of both GSH and CAT were significantly increase and MDA, SOD were significantly decrease the action when compared to diabetic control after 21 days treatment of CG extract/fractions^[16]. The fundamental mechanism underlying hyperglycemia involves over production and decrease utilization of glucose by tissue. Persistent hyperglycemia the common characteristic of diabetes can cause most diabetic complication. In all patients treatment should aim to lower blood glucose to near normal level^[17]. In our investigation the oral glucose tolerance test study revealed that the CG extract/fractions have the capacity to lower blood glucose levels.

Histopathology reports of liver gave additional support to the study. Liver sections of normal animals showed the normal architecture with well brought out central vein, well preserved

cytoplasm and prominent nucleolus whereas the diabetic group section showed the presence of feathery degeneration, micro and macro cellular fatty changes and inflammatory cells around portal tract. The other groups showed good protection from STZ induced changes in the liver.

From the data of antioxidant profile it was found that the ethanolic extract/fractions of *Calotropis gigantea* leaves effectively maintain and regulate the antioxidant system.

5. Conclusion

It suggests that administration of extract of *Calotropis gigantea* may be helpful in the prevention of oxidative stress associated with diabetic complications. In the present study, it is concluded that *Calotropis gigantea* extract has free radical scavenging activity and improved antioxidant effect.

6. Conflict of interest

The authors declare that they have no conflict of interest.

7. Acknowledgement

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