Research Article

Protective Effect of Ethanol Extract of Leaves of *Caesalpinia bonduc* on Oxidative States Associated with Streptozotocin Induced Diabetic Rats

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ABSTRACT

The ethanol extract of leaves of *Caesalpinia bonduc* (EECB) was studied for its antidiabetic activity in Streptozotocin induced diabetic rats. It was principally aimed to correlate the efficacious role of EECB on reduction of oxidative state associated with diabetes. The extract was found to be potent antidiabetic evidenced by significant reduction of blood glucose level in diabetic rats (58.14% reduction of blood glucose level at 300 mg/kg on day 10; P< 0.01). The level of peroxidised lipid in terms of TBARS was increased markedly while the level of antioxidant enzymes namely SOD, CAT and GSH were noticeably decreased in both liver and kidney tissues in diabetic rats. In the study EECB at 300 mg/kg was found to significantly decrease the TBARS (1.03±0.034 and 1.54±0.034 mM/100 g tissue; P<0.01) and increase the GSH (32±1.414 and 24.66±0.988 mg/100 g tissue; P<01), SOD (5.61±0.197 and 9.16±0.158 U/mg protein; P< 0.01) and CAT (62.66±1.116 and 25.66±1.282 U/mg protein; P<0.01) levels in liver and kidney respectively in diabetic rats. Results were found to be comparable to that obtained with standard antidiabetic drug Glibenclamide.

Keywords: *Caesalpinia bonduc*; Leaves; Antioxidant; Diabetes; Streptozotocin.

INTRODUCTION

There are reports of toxic effects by oxygen free radicals (Hughes *et al*., 1998), especially in diabetic condition (Feillet-Coudray *et al*., 1998). Diabetes mellitus is a multifunctional disease characterized by hyperglycemia and lipoprotein abnormalities (Scoppola *et al*., 2001). It causes the damage of cell membrane and in turn production of the oxygen centered free radicals, called reactive oxygen species (ROS). ROS includes superoxide anion radical (O^{-}_{2}), hydroxyl radical (OH·), nitric oxide (NO·), hydrogen peroxide (H_2O_2) and other molecules (Huang & Manton, 2004). In hyperglycemia, there is not only an increase in the production of ROS but also it affects antioxidant reaction catalyzed by ROS scavenging enzymes (Uchimara *et al*., 1999). All organisms possess antioxidant enzymes like reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT), which are responsible for scavenging ROS (Ugochukwu *et al*., 2003). A number of research observations have indicated that the risk of chronic diseases like diabetes in human is markedly reduced by dietary antioxidants chiefly present in foodstuff. Thus the aim of the present investigation was to investigate the protective role of a traditional antidiabetic herb on augmented oxidative states associated with diabetes mellitus.

Caesalpinia bonduc. (L.) Roxb.(Family: Caesalpinioideae) is a perennial plant producing annual stems up to several meters long and it is distributed throughout India, near the sea-coasts, especially Bengal, Bihar, Mumbai and whole of the southern India. The leaves of the plants have liver and gastric tonic,

fungicidal and anticonvulsant properties (Kirtikar and Basu, 1975).
The plant contains a number of secondary metabolites, namely sitosterol, caesalpins, bonducin, caesane and flavonoids (Chopra *et al.,* 1994).
The rural people of Purnia, Bihar, India, also use the aqueous extract of the leaves of the plant for the treatment of diabetes. The aim of the present investigation was to evaluate the antidiabetic activity of ethanol extract of leaves of *Caesalpinia bonduc* on Streptozotocin (STZ) induced diabetic rats, as well the establishment of efficacious role of the extract to alleviate the augmented oxidative state associated with diabetes mellitus in term of thiobarbituric acid reactive species (TBARS), GSH, SOD and CAT activities in liver and kidney.

Experimental

Plant material

Leaves of *Caesalpinia bonduc.* (L.) Roxb. (Family: Caesalpinioideae) were collected in the month of April and May from Purnia district, Bihar, India. The plant was authenticated by the Taxonomist of the Central National Herbarium, Botanical Survey of India, Shibpur, Howrah, India with reference number CNH/1-I (44)/2006/ Tech II/996 dated 07.08.2006. A voucher specimen of the herbarium had been preserved in the laboratory for future reference.

Preparation of ethanol extract

Ethanol extract of the leaves was prepared in accordance to the method of National Institute of Health and Family Welfare (NIHFW), New Delhi, India. Dried matured leaves of *Caesalpinia bonduc* were crushed in an electrical grinder to fine powder of mesh 40. 500 g of powder was then extracted with 2.5 L of 95% ethanol in a soxhlet apparatus until the powder became completely exhausted. The resulting extract was filtered by course sieve filter paper. The filtrate was dried under reduced pressure with the help of rotary vacuum evaporator and finally lyophilized to give an extract sample. The extract was stored in a desiccator for use in subsequent experiments.

Phytochemical studies

The extract was subjected to preliminary phytochemical study using standard methods. (Khandelwal, 2005).

Animals

Healthy adult Wister strain albino rats of both sex between 2-3 months of age and weighing 180-240 g procured from the M/S B. M. Ghosh Enterprises, Kolkata, India, were used for the study. Animals were acclimatized for a period of 2 weeks in the laboratory environment prior to the study. The rats were housed in polypropylene cages (4 animals per cage), maintained under standard laboratory conditions (i.e., 12:12 hour light and dark sequence; at an ambient temperature of 25±2^{o}C; 35-60% humidity); the animals were fed with standard rat pellet diet (Hindustan Liver Ltd. Mumbai, India) and water *ad libitum*. The principles of Laboratory Animals Care (NIII, 1985) were followed. The study was approved by the institutional animal ethical committee, Jadavpur University, Kolkata, India (R. No. 367/01/C/CPCSEA) and was performed as per the guidelines of Care Prevention Control Supervision of Experimental Animals (CPCSEA), Nee Delhi, India.

Chemicals

Thiobarbituric acid, nitroblue tetrazolium (NBT) and nicotinamide adenine dinucleotide (NAD) were purchased from Loba Chemie, Mumbai, India; 5, 5-dithio bis-2-nitro benzoic acid (DTNB), reduced glutathione (GSH) and STZ were procured from SISCO Research Lab, Bombay, India; Glibenclamide (Daonil^{TM}, Hoechst, India) tablets were purchased from local medical store, Jadavpur, India. All chemicals and reagents used were of analytical grade.

Acute toxicity studies

Twenty animals were used in each group, which received different doses of extract in oral route. Different doses of each material ranging between 0.05 to 1.0 g/kg were administered to the animals. Then doses were extended up to the result was observed. Control animals received aqueous tween 80 solution 2% (v/v). After administration of the extract by the oral route the animals were observed under open field condition for 72 hours and the number of deaths and signs of clinical toxicity were recorded. Finally the median lethal dose (MLD) and 95% confidence limits were calculated (Litchfield and Wilcoxin, 1949).

Induction of diabetes

Hyperglycaemia was induced in overnight fasted rats by a single intraperitoneal injection of 65 mg/kg STZ in a volume 1ml/kg body weight (Siddque *et al.,* 1987). Owing to the instability of STZ in aqueous media, the solution was made in citrate buffer (pH 4.5) immediately before injection (Karunanayake *et al.*, 1974). The elevated glucose level in plasma, determined at 48 hrs after injection, confirmed hyperglycaemia. The rats found hyperglycaemic were screened for the experiment.

Experimental design

In the experiment a total of 30 rats (24 diabetic rats, 6 normal rats) were used. One group of six normal rats was kept as normal control and treated with distilled water. Diabetic animals were divided into four groups of six rats in each group. Two groups of diabetic animals were treated with EECB at doses of 150 and 300 mg/kg body weight respectively by oral route; one group of diabetic animals was treated with standard drug Glibenclamide at a dose of 10 mg/kg body weight orally (Mandal *et al.*, 1997) while the remaining group was kept as diabetic control only treated with equal volume of distilled water in place of the extract. All doses were started forty-eight hours after STZ injection. Fasting blood glucose levels were estimated on overnight fasted rats on day 1 and 10.

After 10 days of treatment, all the rats were sacrificed by decapitation; livers and kidneys were separated and washed thoroughly with sterile isotonic saline buffer to clear off blood. The tissues were immediately transferred to ice-cold containers containing 0.9% sodium chloride and homogenized in 0.1N Tris-HCl buffer (pH 7.4). This homogenized mass was used for the estimation of TBARS activity. For the assay of SOD, CAT and GSH 10% homogenate in 0.2 M phosphate buffer pH 8.0 was centrifuged and the clear supernatant was used for the subsequent assays of the enzymes.

Analytical procedure

Fasting blood glucose was estimated with the help of single touch glucometer (Ascensia Entrust, Bayer Health Care, USA). TBARS in tissues was estimated according to the methods of Fraga *et al*., 1988, with little modification. GSH (Ellman, 1959), SOD (Kakkar *et al.,* 1984) and CAT (Sinha, 1972) activities in selected tissues viz. liver and kidney were estimated by standard assay procedures.

Statistical analysis

Statistical analysis was performed using one way analysis of variance ANOVA followed by post hoc Dunnett's *t*-test using computerized GraphPad InStat version 3.05 software, U.S.A. Results were expressed as mean±standard error of mean (SEM) from six rats in each group. Differences between groups were considered significant at $p< 0.05$ levels.

Results and Discussion

The preliminary phytochemical analysis showed the presence of triterpenoids and flavonoids in EECB.

From the acute toxicity study it has been observed that the LD_{50} value of EECB by oral route was 4.5 g/kg.

Before treatment schedule, fasting blood glucose level in all the animals was normal. After 48 h treatment with STZ, the fasting blood glucose level was

significantly elevated in the range of 300-350 mg/dl and it was significantly (P<0.01) reduced by 10 days treatment with EECB. Thus the ethanol extract of leaves of *Caesalpinia bonduc* was found to be potent in reducing the elevated blood glucose level of diabetic rats in a dose dependent manner (Table 1). The reduction of blood glucose level was found to be significant (P<0.01) at both 150 and 300 mg/kg doses, on the 10^{th} day, while maximum effect of reduction of blood glucose level was found on day 10, p<0.01 in Glibenclamide treated diabetic rats.

A significant elevation of lipid peroxides in terms of TBARS in liver and kidney was found in diabetic rats (1.52±0.025 mM/100 g tissue and 2.09±0.050 mM/100 g tissue, respectively, in liver and kidney; P<0.01, when compared to the normal group). Administration of EECB significantly lowered TBARS level in diabetic rats particularly at the dose of 300 mg/kg. The reduction of TBARS level to 1.03±0.034 mM/100 g tissue (P < 0.01) and 1.54±0.034 mM/100 g tissue (P<0.05), respectively, in liver and kidney were observed at 300 mg/kg dose of EECB, which was comparable to that of standard glibenclamide (10 mg/kg) showing reduction up to 1.06±0.007 mM/100 g tissue and 1.54±0.007 mM/100 g tissue levels (P < 0.01), respectively, in liver and kidney (Table 2).

The GSH level was significantly lowered in diabetic rats than in normal rats as indicated in Table 3. Administration of EECB at 300 mg/kg body weight and Glibenclamide (10 mg/kg body weight) increased significantly the GSH levels in liver and kidney (32±1.414 and 24.66±0.988 mg/100 g tissue, respectively; p<0.01), as compared with the levels in diabetic rats.

Tables 4 and 5 show the activities of SOD and CAT respectively. The levels of all the enzymatic antioxidants were significantly lowered in diabetic rats as compared with their values in normal rats. Administration of EECB significantly increased the activities of all the enzymatic antioxidants. For SOD, maximum increase (5.71±0.242 and 10.96±0.290 U/mg protein) was observed at the dose of 300 mg/kg in liver and kidney tissues, respectively (p<0.01), while for CAT, maximum enhancement of level (62.66±1.116 and 25.66±1.282 U/mg protein) was observed at the same dose in liver and kidney tissues respectively (p<0.01). All results were very much comparable to that of standard glibenclamide.

Streptozotocin produces diabetes by liberating oxygen free radicals, which cause lipid peroxide mediated pancreatic injury (Rakieten *et al.,* 1963; Palmer *et al.*, 1988). At diabetic state, oxidative free radicals (ROS) are generated which cause oxidative stress due to impaired glucose metabolism and protein glycation (Ceriello *et al.,* 1992; Wolff *et al.,* 1991). ROS induced oxidative tissue damage plays an important role in many clinical disorders such as heart disease, diabetes, gout and cancer (Slater, 1984; Meneghini, 1988).

Elevated level of lipid peroxidation in tissues and plasma of STZ induced diabetic rats is one of the characteristic features of chronic diabetes (Maxwell *et al.,* 1997). Oxidative stress associated with peroxidation of cellular lipids is determined by TBARS assay. The results show that the administration of EECB reduced liver and kidney peroxide, which is an indication of inhibition of oxidative damage of hepatic and renal tissues.

GSH is a major endogenous antioxidant which counters the balance of free radical mediated damage. The decrease in liver GSH levels represents increased utilization due to oxidative stress (Anuradha & Selvam, 1993). Impairment of the GSH redox cycle is an important mechanism of cell protection against ROS (Inouye *et al.*, 1998). Increase in GSH content in liver and kidney after EECB treatment may be a function of lipid peroxidation inhibition.

Reduced activities of SOD and CAT in liver and kidney were observed during diabetes and this might result in a number of deleterious effects due to accumulation of super oxide radicals and hydrogen

peroxide (Searle & Wilson, 1980). After treatment of EECB there was increase in SOD and CAT levels in liver and kidney. The above observations indicated that the EECB seems to be effective for reducing oxidative stress associated with diabetes. The leaves of *Caesalpinia bonduc* have been reported to be rich in flavonoids (Kundu *et al*., 2011, communicated article) which are well known antioxidants that might scavenge the free radicals generated during diabetes. To isolate bioactive flavonoid from the extract and to substantiate its effectiveness against oxidation dependant diabetic pathogenesis will be the future steps of the present work.

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Table I: Effect of EECB on fasting blood glucose level of STZ induced diabetic rats

Groups	Blood Glucose levels (mg/dl)	
	1st day	10th day
Normal (Distilled water)	72.33±1.706	83±1.528
STZ induced diabetic control	306.66±2.472[a]**	328.5±1.648[a]**
STZ + EECB (150 mg/kg)	271.66±3.333[b]**	245±1.461[b]**
STZ + EECB (300 mg/kg)	252.5±3.819[b]**	191±1.880[b]**
STZ + Glibenclamide (10 mg/kg)	212.5±3.819[b]**	127.83±2.358[b]**

Each value is the mean ± SEM, 6 rats in each group; STZ (65 mg/kg) was injected to control and all other extract and drug treated groups;
[a]STZ induced diabetic group vs normal group, **P< 0.01;
[b]extract treated group vs STZ induced diabetic group, **P< 0.01

Table II: Effect of EECB on TBARS level in liver and kidney of STZ induced diabetic rats

Group	TBARS (mM/100 g tissue)	
	Liver	Kidney
Normal (Distilled water)	0.86±0.026	1.35±0.026
STZ induced diabetic control	1.52±0.025[a]**	2.09±0.050[a]**
STZ + EECB (150 mg/kg)	1.17±0.044[b]**	1.75±0.043[b]**
STZ + EECB (300 mg/kg)	1.03±0.034[b]**	1.54±0.034[b]**
STZ + Glibenclamide (10 mg/kg)	1.06±0.007[b]**	1.54±0.007[b]**

Each value is the mean ± SEM, 6 rats in each group;
STZ (65 mg/kg) was injected to control and all other extract and drug treated groups;
[a]STZ induced diabetic group vs normal group, **P< 0.01;
[b]extract treated group vs STZ induced diabetic group, **P< 0.01

Table III: Effect of EECB on GSH level in liver and kidney of STZ induced diabetic rats

Group	GSH (mg/100 g tissue)	
	Liver	Kidney
Normal (Distilled water)	40±2.295	18.66±1.476
STZ induced diabetic control	22.16±1.167[a]**	3.06±0.249[a]**
STZ + EECB (150 mg/kg)	30.33±1.687[b]**	8.81±0.353[b]**
STZ + EECB (300 mg/kg)	32±1.414[b]**	24.66±0.988[b]**
STZ + Glibenclamide (10 mg/kg)	32±0.9661[b]**	25±1.693[b]**

Each value is the mean ± SEM, 6 rats in each group;
STZ (65 mg/kg) was injected to control and all other extract and drug treated groups;
[a]STZ induced diabetic group vs normal group, **P< 0.01;
[b]extract treated group vs STZ induced diabetic group, **P< 0.01.

Table IV: Effect of EECB on SOD level in liver and kidney of STZ induced diabetic rats

Group	SOD (U/mg protein)	
	Liver	Kidney
Normal (Distilled water)	6.76±0.147	11.86±0.143
STZ induced diabetic control	2.76±0.145[a]**	5.83±0.170[a]**
STZ + EECB (150 mg/kg)	4.78±0.224[b]**	8.4±0.136[b]**
STZ + EECB (300 mg/kg)	5.61±0.197[b]**	9.16±0.158[b]**
STZ + Glibenclamide (10 mg/kg)	5.71±0.242[b]**	10.96±0.290[b]**

U- One unit of activity was taken as the enzyme reaction which gave 50% inhibition of NBT reduction in 1 minute.
Each value is the mean ± SEM, 6 rats in each group; STZ (65 mg/kg) was injected to control and all other extract and drug treated groups; [a]STZ induced diabetic group vs normal group, **P< 0.01; [b]extract treated group vs STZ induced diabetic group, **P< 0.01

Table V: Effect of EECB on CAT level in liver and kidney of STZ induced diabetic rats

Group	CAT (U/mg protein)	
	Liver	Kidney
Normal (Distilled water)	82.16±3.060	32.16±1.352
Diabetic control (Distilled water)	39.33±1.256[a]**	18±1.461[a]**
Diabetic + EECB (150mg/kg)	57±1.390[b]**	25.33±1.282[b]**
Diabetic + EECB (300 mg/kg)	62.66±1.116[b]**	25.66±1.282[b]**
Diabetic + Glibenclamide (10 mg/kg)	72.16±1.701[b]**	26.66±1.282[b]*

CAT-m moles of hydrogen peroxide consumed/minute.
Each value is the mean ± SEM, 6 rats in each group;
STZ (65 mg/kg) was injected to control and all other extract and drug treated groups;
[a]STZ induced diabetic group vs normal group, **P< 0.01;
[b]extract treated group vs STZ induced diabetic group, *P< 0.05, **P< 0.01

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