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Antioxidant effect of *Lagerstroemia speciosa* Pers (Banaba) leaf extract in streptozotocin-induced diabetic mice

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Aqueous leaf extract of *L. speciosa* (banaba) effectively decreased the blood glucose in streptozotocin-induced diabetic mice after 15th day of banaba exposure. Further, banaba leaf extract have the potential to inhibit lipid peroxidation and effectively intercept/neutralize reactive oxygen species such as super oxide, H_2O_2 and NO based free radicals. The aqueous banaba leaf extract (150 mg/kg bodyweight) duly reduced STZ generated reactive intermediates and radical species helping to regulate normal levels of antioxidative markers like superoxide dismutase, catalase, glutathione-S-transferase and reduced glutathione.

Keywords: Diabetes, Free radicals, *Lagerstroemia*, Oxidative stress

Diabetes mellitus (DM) is characterized by abnormal insulin secretion, derangements in carbohydrate/lipid metabolism and is diagnosed by hyperglycemia. The prevalence of diabetes is increasing worldwide, with an approximate doubling of new cases predicted to occur by the year 2025[1,2]. This multi-factorial disease is hypothesized to damage cell membranes resulting in elevated production of reactive oxygen species (ROS) creating oxidative stress. An increase in ROS, an impairment of antioxidant defence systems or an insufficient capacity to repair oxidative damage, the three factors contributing to oxidative stress, appears to play a critical role in pathogenesis. The major concern with diabetes clearly relates to marked increase of neuropathy and series morbidity and mortality related to the development of other complications[3].

Tropical plant *Lagerstroemia speciosa* Pers., (Lythraceae) also called banaba and found in India, Philippines, southern China, Malaya and tropical Australia, has been used as a folk medicine for the treatment of diabetes and kidney diseases[5]. Hypoglycemic activity of banaba extract was studied in genetically induced diabetic type II KK-Ay mice[6]. Originally, because of its hypoglycemic activity, banaba leaf was thought to contain "insulin-like principle", a type of plant – derived peptide hormone. However, there is a dearth of literature supporting the antioxidant potential and oxidative status on consumption of the banaba leaf extract. In light of this, the study is designed to evaluate the *in vitro* antioxidant activity of banaba leaf extract as well as *in vivo* antioxidant status in the liver and brain tissue samples following the administration of graded doses of banaba extract in STZ-diabetic mice.

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Materials and Methods

Plant material—Standardized aqueous leaf extract of *Lagerstroemia speciosa* (banaba), having 1% Corosolic acid fraction, was procured from Changsha Botaniex Inc, China.

Chemicals—Streptozotocin (STZ), 2, 2′-azinobis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carbonic acid (TROLOX) were obtained from Sigma Aldrich. Butylated hydroxy toluene (BHT) was purchased from Merck India Ltd. All other chemicals were of analytical grade.

Animals—Adult (3- months-old) albino mice, *Mus musculus,* weighing 30±5g were procured from Sri Raghavendra Enterprises, Bangalore and acclimatized to laboratory conditions (12:12h dark/light, 25° ± 2°C). Standard mice pellet diet was given *ad libitum;* the animals were maintained in accordance with the guidelines of National Institute of Nutrition, ICMR Hyderabad and approved under Institutional Animal Ethical Committee Bangalore University, Bangalore.

Induction of experimental diabetes—A freshly prepared solution of streptozotocin (50 mg/kg body weight) in 0.1 mol/L citrate buffer, *p*H 4.5 was injected intraperitoneally in a volume of 1 mL/kg body weight[7]. Five days after streptozotocin administration, mice with moderate diabetes having hyperglycemia (110-120 mg/dL) were used for the experiment[8,9].

Experimental design—To check the dose response on hyperglycemia, five grades of aqueous banaba leaf extract (BLE) in 0.1 mL having 50, 100, 150, 250, 500 mg/kg body weight/day for two months were given in the pilot study to diabetic animals using intra-gastric tube on 5th day of STZ administration respectively. To assess the antihyperglycemic effect of BLE, blood was collected by tail vein puncture from overnight fasted animals and the blood glucose levels were measured on 5th, 10th, 15th, 30th, 45th and 60th day of banaba exposures.

In the second set, to check the dose-response efficacy of grades of BLE (50-500 mg/kg body weight/day) on oxidative stress indices in liver and brain tissues, the animals were sacrificed after 15 days of BLE exposure as the extract has shown antihyperglycemic effect on the 15th day of BLE exposure[10]. Dissected liver and brain tissues were washed in ice-cold saline, patted dry and used for biochemical assays.

In vitro antioxidant activity

Trolox equivalent antioxidant capacity (TEAC assay—The antioxidant activity by the TEAC assay was determined as per Re *et al.*[11], using the radical cation $ABTS^{+\bullet}$. The $ABTS^{+\bullet}$ radical cation was pregenerated by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) while incubating for 12–16 h in dark at room temperature until the reaction was complete and the absorbance was stable. The working solution of $ABTS^{+\bullet}$ was obtained by diluting the stock solution in phosphate buffer saline (PBS, *p*H 7.4) to give an absorption of 0.70±0.02 at λ = 734 nm. ABTS working solution (about 3 mL) was mixed with 10 µL of the Trolox standard (50 mM) or the test samples (0.05-10 mg/mL) and the absorbance was measured at every 10 sec for 6 min at 734 nm. All experiments were repeated six times.

The TEAC value was calculated by measuring the area under curve, derived from plotting the percentage inhibition of the absorbance as a function of time. The calculation of the area under the curve was performed for one sample dilution which had a final percentage inhibition between 20 and 80%. Each extract was analysed in triplicate. The antioxidant activity of the plant extracts was expressed as µmol of TE/ g weight of the extract[12].

Nitric oxide scavenging assay—The activity was measured according to the modified method of Sreejayan and Rao[13]. To 4mL of the extract having different concentrations of banaba (1-50 µg/mL) were added 1 ml of sodium nitroprusside (SNP) solution (5 mM) and incubated for 2 h at 27°C. An aliquot (2 mL) of the incubation solution was removed and diluted with 1.2 mL of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylene diamine dihydrochloride). The absorbance of the chromophore was read immediately at 550 nm and compared with standard, BHT.

$$\text{Scavenging activity } (\%) = \frac{[\text{Abs (control)} - \text{Abs(test)}]}{\text{Abs(control)}} \times 100$$

where, Abs (control): Absorbance of the control and Abs (test) : Absorbance of the extract/standard.

Superoxide scavenging assay—The activity was evaluated using nitro blue tetrazolium (NBT) reduction method[14].

H_2O_2 radical scavenging assay—The ability of the extract to scavenge H_2O_2 was determined according to the method by Ruch *et al.*[15].

Reducing power assay—The reducing power of the BLE was determined according to the method of Oyaizu *et al.*[16].

Determination of plasma glucose level—Fasting blood glucose was estimated by O-toluidine method[17].

In vivo antioxidant assays—Oxidative stress markers viz lipid peroxidation (LPO)[18], superoxide dismutase (SOD)[19], catalase (CAT)[20], reduced glutathione (GSH)[21] and glutathione-S-transferase (GST)[22] were assayed. Protein was estimated by the method of Lowry *et al.*[23], using bovine serum albumin as standard.

Statistical analysis—Results are expressed as mean ± SD of 6 observations. Data compilation was carried out using SPSS 15.0 software. Under *in vitro* assays, linear regression analysis was used to calculate the IC_{50} values. Data from dose-response study of BLE on hyperglycemia were analysed using two way univariate ANOVA by Duncan multiple range test (DMRT) post hoc to examine

the effective day and dose of BLE exposure as well as Oneway Analysis of Variance (ANOVA) to examine effective dose on each day examined and the data from *in vivo* biochemical studies by employing one-way ANOVA followed by Bonferroni post hoc.

Results

The total antioxidant activity of the extract was calculated from the decolorization of $ABTS^{+\bullet}$ which was measured spectrophotometrically at 734 nm. The TEAC assay results, using the standard curve of Trolox standard on ABTS (R^2 = 0.865), showed the total antioxidant activity of BLE (1% corosolic acid) was 0.012 µmol of TE /g wt extract.

Percentage inhibition on superoxide radical generation by the BLE was found increasing in a dose dependent manner, showing the IC_{50} value of 1.10 mg/mL, when compared to the IC_{50} value 3.35 µg/mL of ascorbic acid. With regard to scavenging of H_2O_2 and NO, the different concentrations of BLE showed decrease in percentage inhibition while increase in the concentrations with IC_{50} value of 14.95 mg/mL (IC_{50} of ascorbic acid-1.23µg/mL) and 0.23 g/mL (IC_{50} of BHT 0.15 µg/mL) respectively, and in case of the total reduction capability, IC_{50} value 2.49 mg/mL, exhibited an increase in a dose dependent manner (Fig. 1).

On post administration of banaba extract to diabetic models, it initially showed no effect up to 10 days exposure whereas the serum glucose levels decreased significantly ($P < 0.05$) on 15th day of extract administration and further remained constant in 30th, 45th, and 60th days of BLE exposure, indicating the hypoglycemic properties of the leaf extract of *L. speciosa,* as the substances with hypoglycemic properties would be effective in the management of diabetes (Table 1). Statistical analysis using two-way univariate analysis demonstrated that the serum glucose levels reduced at a dose of 150 mg/kg body weight suggesting the ameliorative role of BLE on the 15th day of exposure in extending protection to diabetic animals, compared to other doses. TBARS from liver and brain samples in BLE treated groups were significantly decreased, especially at 150 mg/kg body weight (Table 2). In both liver and brain samples, the levels of SOD and GST exhibited significant decrease and CAT activity and GSH levels significantly increased on banaba exposure, when compared to that of diabetic control mice. The dose response studies made on serum glucose as well as tissue antioxidant status showed that aqueous extract of banaba has significant effect after 15 days of exposure and the extent of increase was higher in group treated with 150 mg/kg body weight than other groups.

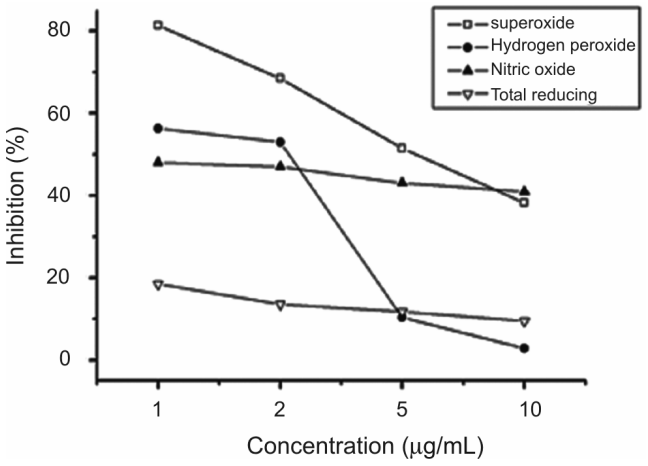

Fig. 1—Antioxidant potential of aqueous leaf extract of *L. speciosa* (banaba)

Table 1—Effect of banaba leaf extract (50-500 mg/kg bw/day) on blood glucose levels at 5th, 10th, 15th, 30th, 45th and 60th day in diabetic mice
[Values are mean ± SD of 6 observations in each group]

Groups	5th day	10th day	15th day	30th day	45th day	60th day
Control	55.69 ± 6.74†	54.68 ± 3.68†	58.02 ± 1.31†	56.82 ± 2.33†	56.41 ± 4.44†	58.11 ± 3.32†
STZ	118.58 ± 0.99*	122.75 ± 16.73*	119.75 ± 2.2*	121.58 ± 11.15*†	119.75 ± 5.56*†	119.75 ± 5.56*†
STZ + B 50	119.25 ± 7.75*	109.34 ± 6.37*	66.43 ± 0.80*†	69.69 ± 3.63*†	70.11 ± 6.12*†	8.71 ± 3.14*†
STZ + B 100	120.92 ± 3.57*	111.03 ± 4.86*	77.46 ± 2.07*†	85.16 ± 4.215*†	90.66 ± 1.37*†	91.53 ± 2.51*†
STZ + B 150	120.25 ± 4.08*	112.75 ± 4.02*	65.09 ± 2.81†	64.17 ± 1.17†	62.99 ± 0.79†	63.09 ± 0.82†
STZ + B 250	114.08 ± 4.36*	112.38 ± 5.83*	76.93 ± 0.59*†	79.29 ± 0.84*†	80.20 ± 0.80*†	79.68 ± 0.88*†
STZ + B 500	118.92 ± 5.46*	115.15 ± 4.83*	84.65 ± 0.47*†	85.65 ± 0.80*†	87.10 ± 1.72*†	91.99 ± 1.35*†

STZ = streptozotocin; B = banaba leaf extract
$P<0.05$ as compared to * control mice; † diabetic controls

Table 2—Effect of banaba leaf extract (50-500 mg/kg bw) on oxidative markers in liver (A) and brain (B) tissue of STZ diabetic mice [Values are mean ± SD from 6 observations in each group]

Groups		LPO (µM of MDA/g tissue)	SOD (IU/mg protein)	CAT (IU/mg protein)	GST (IU/mg protein)	GSH (mg of GSH/g tissue)
Control	A	0.34 ± 0.009	0.65 ± 0.01	0.80 ± 0.02	0.18 ± 0.004	4.29 ± 0.15
	B	0.89 ± 0.03	0.03 ± 0.002	0.19 ± 0.01	0.41 ± 0.01	1.08 ± 0.01
STZ	A	1.40 ± 0.02[a]	0.84 ± 0.01[a]	0.08 ± 0.01[a]	0.71 ± 0.01[a]	2.78 ± 0.08[a,d]
	B	1.69 ± 0.03[a]	0.07 ± 0.003[a]	0.07 ± 0.01[c]	1.32 ± 0.03[a]	0.85 ± 0.15[a]
STZ + BLE 50	A	0.55 ± 0.01[d]	0.32 ± 0.01[a,d]	0.15 ± 0.01[a]	0.57 ± 0.002[a,d]	6.84 ± 0.11[a,d]
	B	0.61 ± 0.02[d]	0.05 ± 0.004[a,d]	0.23 ± 0.01[d]	0.56 ± 0.02[b,d]	1.29 ± 0.04[d]
STZ + BLE 100	A	0.85 ± 0.08[a,d]	0.52 ± 0.02[a,d]	0.38 ± 0.02[c]	0.13 ± 0.008[c,d]	3.75 ± 0.06[d]
	B	1.11 ± 0.06[d]	0.03 ± 0.003[d]	0.14 ± 0.01	0.37 ± 0.04[d]	1.1 ± 0.03
STZ + BLE 150	A	0.39 ± 0.01[d]	0.47 ± 0.02[a,d]	0.52 ± 0.01[f]	0.21 ± 0.004 [d]	3.72 ± 0.07[f]
	B	0.73 ± 0.12[d]	0.02 ± 0.002[d]	0.47 ± 0.02[a,d]	0.34 ± 0.02[d]	1.04 ± 0.02[e]
STZ + BLE 250	A	0.88 ± 0.08[a,d]	0.47 ± 0.01 [a,d]	0.41 ± 0.22[a]	0.40 ± 0.004[a,d]	5.38 ± 0.32[a,d]
	B	1.16 ± 0.09[d]	0.05 ± 0.003[a]	0.07 ± 0.004[c]	0.34 ± 0.01[d]	1.15 ± 0.01[f]
STZ + BLE 500	A	1.06 ± 0.04[a,d]	0.73 ± 0.01[a,d]	0.70 ± 0.01[d]	0.58 ± 0.015[a,d]	4.45 ± 0.11[d]
	B	1.52 ± 0.02[a]	0.06 ± 0.003[a]	0.98 ± 0.05[a,d]	0.21 ± 0.01[a,d]	1.15 ± 0.04[f]
F - value	A	61.53	160.26	9.8	436.91	66.16
	B	37.72	53.34	235.44	219.78	4.83

P values: [a]< 0.001, [b]< 0.01, [c]< 0.05 compared to control group and [d]< 0.001, [e]< 0.01, [f]< 0.05 compared to STZ treated group

Discussion

There are numerous methods for evaluation of antioxidant activity; however, the total antioxidant activity of an extract or compound cannot be evaluated by using a single method, due to oxidative processes. Therefore, at least two methods should be employed in order to evaluate the total antioxidant activity[24]. In the total antioxidant activity, $ABTS^{+\bullet}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate and in the presence of the plant extract or trolox, preformed cation radical gets reduced. The calculated results with trolox standard curve (R^2 = 0.865) and the TEAC value explains the antioxidant potential of the banaba leaf extract.

The superoxide anion radicals are derived in PMS-NADH-NBT system, where the decrease in absorbance at 560 nm with BLE indicates the consumption of superoxide anion in the reaction mixture, thereby exhibiting a dose dependent increase in superoxide scavenging activity. H_2O_2 is highly important because of its ability to penetrate biological membranes and being non-reactive, where it acts as toxicant to cell by converting itself to hydroxyl radical. In this study, BLE in increasing concentrations (1-10µg) showed less effective in H_2O_2 scavenging activity. Similarly, nitric oxide (NO), being a potent pleiotropic mediator of many physiological processes and a diffusible free radical in the pathological conditions, reacts with superoxide anion and form potentially cytotoxic molecule 'peroxynitrite'. Comparatively, the BLE is found to be less scavenging on nitric oxide, when compared to ascorbic acid, the reference compound. The reducing capacity of any compound may serve as a significant indicator of its potential antioxidant activity. For measurements of the total reduction ability, the Fe^{3+} to Fe^{2+} transformation in the presence of BLE was investigated and an increasing absorbance was found in a dose dependent manner, with increasing concentration, and confirmed significant reducing ability.

Streptozotocin (STZ), a diabetogenic agent, is well known for its selective pancreatic islet beta cell cytotoxicity by damaging the beta cell membrane and breaks the DNA strand leading to the activation of poly (ADP-ribose) synthetase and NAD depletion[25]. The decomposition of STZ leads to formation of highly reactive carbonium ions which cause alkylation of DNA bases causing cell death. It's observed that in post STZ – injury, the surviving beta cells are able to maintain most of the metabolic functions but fail to maintain an adequate blood sugar levels. Monitoring the antihyperglycemic effect in the present study, the efficacy of plant extract on diabetic model was measured for two months. While exposure to BLE, initially up to 10 days showed no effect, after 15 days the blood glucose levels drastically reduced and further remained constant indicating the rejuvenation of beta cells which needs a period of 15 days of banaba therapy. As a result,

15-day exposure was considered significant and studies were continued to analyze the efficacy of plant extract on the biochemical variables. It is hypothesized that the chronic hyperglycemia in the STZ-induced mice causes abnormal glucose levels and banaba extract having 1% corosolic acid fraction influence the glucose and insulin release from destroyed pancreatic beta cells, either by regenerating the partially destroyed pancreatic beta cells or by the release of insulin stored in granules. Bioassay guided fractionation of banaba extract showed presence of a triterpenoid component known as corosolic acid (CA) which is responsible for the glucose transporter activity; translocation of glucose transporter 4 (GLUT4) from intracellular microsomal membrane to plasma membrane in genetically diabetic mice[26] and lowered the blood glucose level[27-29]. The presence of three active ellagitannins in banaba extract; lagerstroemin, flosin B and reginin A, in optimum concentrations are found to be activators of hexose uptake in rat adipocytes, similar to that employed by insulin[30] and could act intracellularly or bind and activate the insulin receptor (IR) extracellularly[31]. Studies on corosolic acid based banaba extract product glucosol; support the present result, which revealed dose dependent effect on glucose transport activation, which is likely to be the effect of corosolic acid[32]. In contrast, studies by Liu *et al*.[33] suggest that the glucose transport activity was caused by the tannin component of the extract, and not corosolic acid and while pure corosolic acid tested was found ineffective in stimulating glucose transport in cell model.

Growing evidence indicates that as a result of hyperglycemia, oxidative stress is increased in diabetes due to overproduction of reactive oxygen species (ROS) and decreased efficiency of antioxidant defences through enzymatic and non-enzymatic components[34-36]. Glucose auto-oxidize, in the presence of transition metal ions, to generate oxygen free radicals make the membrane vulnerable to oxidative damage[37]. Further, when exposed to free radicals, oxidation of lipids, proteins and other macromolecules such as DNA occurs during the development of diabetes and its complications[38]. In the present study, elevated LPO in the liver and brain samples of STZ diabetic mice indicated an increase in oxygen free radicals in diabetes primarily could be due to augmented blood glucose levels, which upon auto oxidation generate free radicals and secondarily, the effects of the diabetogenic agent, STZ. Studies suggest that the tissue content having relatively high concentration of easily peroxidizable fatty acids and increased activities of enzymes like fatty acyl coenzyme, coenzyme A oxidase due to hypoinsulinaemia initiate the beta-oxidation of fatty acids resulting in lipid peroxidation[39-41]. Increased LPO and its products (lipid radicals and lipid peroxide) are predominantly associated with atherosclerosis and brain damage[42]. Administration of BLE, at a dose 150 mg/kg body weight reduced the LPO markers to near normal in both liver and brain tissues showing a dose independent antiperoxidative effect of the BLE, supporting its *in vitro* antioxidant activity.

The altered balance of the antioxidant enzymes with an increase in the SOD and GST activities, in STZ diabetic condition, may be due to increased production of $O_2^{\bullet}$ and H_2O_2 by the auto-oxidation of the glucose and non-enzymatic glycation. Similarly, decrease in CAT level depicts the inactivation of the enzyme by $O_2^{\bullet}$ anions, as observed earlier[43]. These enzymes are suggested to play an important role in maintaining physiological levels of oxygen and H_2O_2 by hastening the dismutation of oxygen radicals and by eliminating the organic peroxides and hydroperoxides generated from inadvertent exposure to STZ[44]. GST is a family of enzymes involved in the binding, transport and detoxification[45] and cellular defence[46]. Increased free radicals in STZ diabetic liver and brain may have enforced GST detoxification thereby increasing its activity to a significant level[47]. The beneficial role of BLE, especially a dose of 150 mg/kg body weight, helps in ameliorating the oxygen free radicals, as seen in the *in vitro* studies, may have brought the enzyme levels nearly to normal and may help to control free radical generation during diabetes.

GSH plays an important role in the detoxification and metabolism as a cofactor or as a substrate for some enzymes and as an antioxidant agent protecting tissue from oxidative stress. In the present study, the decreased GSH levels in diabetic liver and brain tissues, may be due to the enhanced GST activity, supporting the earlier studies[48,49]. Elevated GSH content in liver and brain samples on BLE administration to diabetic animals may help in offering protection to cellular proteins against oxidation through glutathione redox cycle and further also helps in detoxifying reactive oxygen species generated during diabetic state.

In conclusion, *L. speciosa* has the potential to inhibit LPO and effectively intercept/neutralize ROS such as super oxide radical, H_2O_2 and NO based

free radicals. Thus the aqueous leaf extract of *L. speciosa* (banaba) especially at a dose of 150 mg/kg body weight duly reduced STZ generated reactive intermediates and radical species helping to keep normal levels of enzymatic and non-enzymatic antioxidants. It possesses optimal antioxidative, antiperoxidative and hypoglycemic property, which could exert an ameliorating action in the pathological alterations.

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