

ANTIOXIDANT ACTIVITY OF THE METHANOL EXTRACT OF *DIOSPYROS PEREGRINA* FRUITS IN ALLOXAN INDUCED DIABETIC RATS

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Summary

Oxidative stress plays important role in diabetic complications. Thus the present investigation was undertaken to evaluate antioxidant effect of the methanol extract of *Diospyros peregriana* fruits on alloxan induced diabetic rats. Experimental diabetic rats were treated with methanol extract at the dose of 150 and 300 mg/kg body weight *p. o.* daily for two weeks. The diabetic rats showed lower activities of superoxide dismutase, catalase, and reduced glutathione content in hepatic and renal tissues as compared with normal rats. The activities of superoxide dismutase, catalase, and reduced glutathione were found to be increased in extract treated diabetic rats in selected tissues. The increased levels of lipid peroxidation in term of thiobarbituric acid reactive substances and hydroperoxides level in diabetic rats were also found to be reverted back to near normal status in extract treated groups. It was found that the extract is more effective at the dose of 300 mg/kg body weight and this effect is almost comparable to that of standard glibenclamide.

Key words: alloxan, antioxidant, diabetes, *Diospyros peregriana*, Ebenaceae.

Diabetes mellitus is a chronic disease characterized by high blood glucose levels due aberration of carbohydrate, fat and protein metabolism characterized by insulin hyposecretion or insensitivity. Diabetes mellitus is associated with augmented oxidative stress (1) which leads major chronic complications namely retinopathy, neuropathy, nephropathy, atherosclerotic coronary artery disease, and peripheral atherosclerotic vascular disease (2). Hyperglycemia increases the production of reactive oxygen species (ROS) inside the aortic endothelial cells. ROS-induced activation of protein kinase-C isoforms, increased formation of glucose-derived advanced glycation end products, increased glucose flux through aldose reductase pathways, and activation of cytokines are some of the known biochemical mechanisms of hyperglycemia-induced tissue and cell injury (3, 4). The mammalian cells are operational with both enzymatic and nonenzymatic antioxidant defenses which minimize ROS mediated cellular damage (5). Recent studies showed that the majority of plasma antioxidants are depleted in diabetes patients (6). Thus antioxidant therapy in diabetes may be helpful in relieving symptoms and complications observed in diabetes patients. As plants often contain a substantial amount of antioxidants, so herbal hypoglycemic coupled with antioxidant property may serve as a wonderful antidiabetic agent (7).

Diospyros peregrina Gurke. (Ebenaceae) is a small middle sized tree grows luxuriantly in the plains of costal West Bengal, India. Ripe fruits are edible with ethnomedicinal significance as tonic and aphrodisiac (8). Unripe fruits are used for the treatment of diarrhoea, dysentery, cholera, ulcer of mouth and in wounds (9). The fruits contain triterpenes, alkanes, flavonoids and tannins (10-14). The stem barks of the plant have been reported to possess hypoglycemic activity (15). The dried unripe fruits of *Diospyros peregrina* are successfully employed by the villagers of costal West Bengal for the management of diabetes. Since the fruits of this plant contain substantial quantity of flavonoids which are known for their important antioxidant activity (16). The present investigation was undertaken to evaluate the effectiveness the methanol extract of *Diospyros peregrina* fruits in augmented oxidative stress of experimental diabetic rats.

Material and Methods

Plant material

Matured unripe fruits of *Diospyros peregrina* were collected in the month of June 2006 from the villages of costal South 24 Parganas, West-Bengal, India. The plant was authenticated by H J Chowdhury, Joint Director, Botanical Survey of India, Shibpur, Howrah, India. A voucher specimen JU/PPRT/DP/PT/01/06 was deposited at our laboratory for future reference.

Preparation of methanol extract

Methanol extract of fruits was prepared in accordance to the method of National Institute of Health and Family Welfare, New Delhi, India. Matured unripe fruits of *Diospyros peregrina* were dried in an incubator for two days at 40°C, crushed in a mechanical grinder to fine powder of mesh 40. The powder (500 g) was then extracted with 2.5 l of 90% methanol in a Soxhlet apparatus at 65°C, until the powder became exhausted totally. Resulting extract was filtered, concentrated, and dried *in vacuo* (yield 8.75% w/w). The extract was stored in a desiccator for use in subsequent experiments.

Chemicals

Alloxan monohydrate, a most widely used chemical diabetogen was procured from Loba chemie, Mumbai, India. Chemically alloxan is 2, 4, 5, 6 tetra oxo hexahydro pyrimidine. Glibenclamide an oral hypoglycemic agent used in this experiment as standard drug purchased from Aventis Pharma. Ltd., Goa, India. 5, 5-dithio bis-2-nitro benzoic acid, and reduced glutathione were procured from SISCO Research Lab, Mumbai, India. Thiobarbituric acid, nitroblue tetrazolium and nicotinamide adenine dinucleotide were purchased from Loba Chemie, Mumbai, India. All chemicals and reagents used were of analytical grade.

Animals

Healthy adult Wister strain albino rats of both sex between 2-3 months of age and weighing 180-240 g were used for this study. Animals were allowed to be acclimatized for a period of 2 weeks in our laboratory environment prior to the study. Rats were housed in polypropylene cages (3 animals per cage), maintained under standard laboratory conditions (*i.e.* 12:12 h light and dark sequence; at an ambient temperature of 25 ± 2°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 (17) were followed and instructions given by our institutional animal ethical committee were followed throughout the experiment.

Induction of diabetes

Hyperglycemia was induced in overnight fasted adult Wister strain albino rats weighing 180-240 g by a single intraperitoneal injection of freshly prepared alloxan monohydrate in normal saline (150 mg/kg body weight) in a volume 1 ml/kg body weight (18). Hyperglycemia was confirmed by the elevated fasting glucose level in plasma, determined at 48 hours after injection (19). The rats found hyperglycemic were screened for the Antihyperglycemic study.

Experimental design

Animals were divided into five groups of six rats each.

Group I: Normal rats administered distilled water daily for 14 days. Group II: Diabetic control rats administered distilled water daily for 14 days. Group III: Diabetic rats administered methanol extract (150 mg/kg, orally) daily for 14 days. Group IV: Diabetic rats administered methanol extract (300 mg/kg, orally) daily for 14 days. Group V: Diabetic rats administered standard drug glibenclamide (1 mg/kg, orally) daily for 14 days.

All doses were started 48 h after alloxan injection.

After 14 days of treatment, all the rats were anaesthetized and sacrificed by cervical dislocation; livers and kidneys were excised and washed thoroughly to clear off blood. The tissues were immediately transferred to ice-cold saline and homogenized in 0.1N Tris-HCl buffer (pH 7.4). This tissue homogenates were used for the estimation of thiobarbituric acid reactive substances (20), hydroperoxides (20), reduced glutathione (21), superoxide dismutase (22), and catalase (23).

Statistical analysis

Data were statistically calculated by utilizing one way ANOVA and expressed as mean \pm SEM followed by Dunnett's *t*-test using computerized Graph Pad InStat version 3.05, Graph pad software, U.S.A.

Results

The antioxidant effect of the extract on tissue antioxidant markers was studied (Table 1). The diabetic rats showed a significant elevation ($p < 0.01$ when compared to the normal group) of thiobarbituric acid reactive substances in both hepatic and renal tissues of diabetic rats. Oral administration of extract significantly reduced ($p < 0.01$ when compared to the diabetic control group, at 300 mg/kg) these to near normal status as compared with non diabetic rats.

The hydroperoxides level in diabetic rats also significantly elevated in hepatic ($p < 0.05$) and renal tissues ($p < 0.01$) when compared with normal rats. Oral administration of extract to diabetic rats significantly reduce hydroperoxides level in liver ($p < 0.05$) and kidney ($p < 0.01$) tissues.

A significant reduction in reduced glutathione level ($p < 0.01$) was observed in hepatic and renal tissues of experimental diabetic rats. Extract administration to diabetic rats significantly increased liver ($p < 0.01$) and kidney ($p < 0.01$) reduced glutathione to near normal value.

The enzymatic antioxidants like superoxide dismutase and catalase were significantly lower ($p < 0.01$) in the selected tissues of diabetic rats as compared with normal animals. Administration of methanol extract significantly increased ($p < 0.01$, when compared with diabetic control group) the activities of all the enzymatic antioxidants.

Experimental results indicate that the methanol extract of *Diospyros peregrina* produce its antioxidant effect in a dose dependant manner in experimental diabetic rats. The extract was found to be more effective at the dose of 300 mg/kg body weight. The readings obtained from the extract treated group at 300 mg/kg were comparable to that of standard drug glibenclamide (1 mg/kg).

Table 1. Effect of methanol extract of *Diospyros peregrina* on tissue thiobarbituric acid reactive substances, hydroperoxide, reduced glutathione, superoxide dismutase and catalase in diabetic rats.

Parameters	Normal control (Distilled water)	Diabetic control (Distilled water)	Diabetic + DPME (150 mg/ kg)	Diabetic + DPME (300 mg/ kg)	Diabetic + Glibenclamide (1 mg/kg)
TBARS- Liver *	0.96 ± 0.07	1.61 ± 0.10 ^a	1.22 ± 0.13*	1.02 ± 0.12**	1.11 ± 0.08**
TBARS – Kidney *	1.48 ± 0.10	2.22 ± 0.16 ^a	1.76 ± 0.12*	1.58 ± 0.10**	1.54 ± 0.09**
HP – Liver *	62.65 ± 5.76	95.08 ± 5.60 ^b	75.80 ± 8.78	68.48 ± 7.91*	67.95 ± 6.55*
HP – Kidney *	52.76 ± 3.82	88.55 ± 7.31 ^a	69.08 ± 7.94	58.27 ± 3.86**	59.42 ± 5.26**
GSH – Liver **	41.78 ± 4.11	23.08 ± 2.43 ^a	34.63 ± 2.80	40.06 ± 3.89**	38.95 ± 3.63*
GSH – Kidney **	21.33 ± 1.83	6.68 ± 1.02 ^a	14.68 ± 1.60**	19.35 ± 1.13**	17.6 ± 1.04**
SOD – Liver #	6.77 ± 0.34	3.32 ± 0.35 ^a	5.53 ± 0.36**	6.33 ± 0.43**	6.47 ± 0.33**
SOD – Kidney #	15.07 ± 0.81	7.63 ± 0.56 ^a	11.87 ± 1.15*	13.50 ± 1.16**	14.17 ± 0.92**
CAT – Liver ###	83.77 ± 5.07	52.73 ± 3.31 ^a	66.87 ± 4.95	76.38 ± 4.17**	79.15 ± 4.85**
CAT – Kidney ###	32.32 ± 1.92	19.87 ± 1.65 ^a	27.22 ± 2.06*	30.35 ± 1.80**	29.77 ± 1.72**

DPME - Methanol extract of *Diospyros peregrina* fruits, TBARS - Thiobarbituric acid reactive substances, HP – hydroperoxide, GSH - reduced glutathione, SOD - superoxide dismutase, CAT – Catalase.

Units - * mmoles/100g wet tissue.

- ** mg/100g wet tissue.

- # U/mg of protein, one unit is defines as the enzyme concentration require to inhibit the OD at 560 nm of chromogen produced 50% in 1 min.

- ## μmoles of H₂O₂ consumed/min/mg protein.

Values are expressed as mean ± S.E.M. (n = 6)

^a $p < 0.01$ compared with normal control group.

* $p < 0.05$, ** $p < 0.01$ compared with diabetic control group.

Discussion

Alloxan, a beta cytotoxin, destroys β cell by liberating oxygen free radicals, which cause lipid peroxide mediated pancreatic injury (24). Thus lipid peroxidation is one of the characteristic features of chronic diabetes (25). An increase in hepatic and renal thiobarbituric acid reactive substances is an index of enhanced lipid peroxidation in diabetes (26).

Increased level of thiobarbituric acid reactive substances in diabetic rats suggests enhanced oxidative stress that could be due to amplified generation or decrease destruction of ROS (27). Oral administration of extract to diabetic rats significantly lower this enhanced thiobarbituric acid reactive substances level in both the tissues.

Hydroperoxide molecules participate in destruction of enzymatic protein and cell membrane (28). Increased hydroperoxides levels in liver and kidney were observed in diabetic rats. It may be due to decrease activity of antioxidant enzymes which results uncontrolled generation of ROS. Oral administration of methanol extract significantly lowered hepatic and renal hydroperoxide in diabetic rats and it indicates that the extract is capable to alleviate lipid peroxidation.

Increased lipid peroxidation in diabetes can be due to enhance oxidative stress in the cells as a result of depletion of antioxidant scavenger system. Reduced glutathione is a major endogenous antioxidant which counteracts free radical mediated damage. Depletion of liver and kidney reduced glutathione levels represents enhanced oxidative stress (29).

Superoxide dismutase is an antioxidant enzyme which reduces superoxide radicals to water and molecular oxygen (30) whilst catalase reduces hydrogen peroxide (31). Diminished activity of these antioxidant enzymes result elevation of ROS and ROS mediated cell destruction. Reduced activities of superoxide dismutase and catalase in liver and kidney were observed in diabetic rats and these were reverted to near normal status on extract treatment.

Present investigation showed that the methanol extract of unripe matured fruits of *Diospyros peregrina* possesses considerable antioxidant activity in alloxan induced diabetic rats. This antioxidant effect of the extract capable to control the diabetic complications mediated by enhanced oxidative stress associated with diabetes mellitus. Scientists claimed that “the treatment of diabetes with antioxidant therapy is like applying water to a burning house and is certainly helpful in limiting the conflagration” (32). Preliminary phytochemical investigation confirmed that the methanol extract of the fruits of *Diospyros peregrina* contains substantial quantity of flavonoids which are the known antioxidant from plant sources (16). Now our aim will be guided to isolate the lead flavonoid molecule from the said extract which can counteract with diabetes as well its pathogenesis through antioxidant defense mechanism.

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