

Modulatory effect of polyphenolic extracts of *Ichnocarpus frutescens* on oxidative stress in rats with experimentally induced diabetes

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Background & objectives: The role of oxidative stress in the development of diabetes mellitus and its vascular complications are extensively studied. Hyperglycaemia causes oxidative damage by generation of reactive oxygen species and results in the development of complications. The present study was undertaken with the objective of exploring the anti-hyperglycaemic potential of polyphenolic enriched extract of *Ichnocarpus frutescens* in streptozotocin induced (n-STZ) neonatal diabetic rats (pups) for six weeks and to study oxidative stress and antioxidant status.

Methods: Two days old pups were rendered diabetic by single injection of streptozotocin (90 mg/kg body wt, ip). At the end of the treatment period, the level of blood glucose, serum biochemical markers, serum lipid levels and liver malondialdehyde, tissue antioxidant levels were measured.

Results: A marked rise was observed in the levels of fasting blood glucose (230.33 mg/dl), lipid profiles, lipid peroxidative products and a significant decrease in tissue antioxidants (superoxide dismutase, catalase and reduced glutathione) and serum high density lipoprotein cholesterol levels in STZ treated rats. Oral administration of polyphenolic extract (150 and 300 mg/kg body wt, po) decreased fasting blood glucose levels (187.66 and 170.50 mg/dl, respectively) of STZ-treated diabetic rats significantly ($P<0.01$), when compared with control rats. In addition, the polyphenolic extract showed favourable effect ($P<0.01$) on the reduced tissues antioxidants level, liver glycogen level, high density lipoprotein level, with significant ($P<0.01$) reduction of elevated lipid peroxidation products. Histopathological study of the pancreas showed the protective role of polyphenolic extract.

Interpretation & conclusions: Our study showed the antioxidant effect polyphenolic extract of *I. frutescens* in STZ induced experimental diabetes. The results also suggested that this polyphenolic rich extract could be potentially useful for hyperglycaemia treatment to correct the diabetic state.

Key words Antioxidants - diabetes mellitus - *Ichnocarpus frutescens* - neonatal rats - oxidative stress - polyphenolic extract - streptozotocin

Diabetes mellitus is the fastest growing metabolic disorder in the world and a major cause of morbidity in developing countries¹. Diabetes mellitus is shown

to be associated with increased oxidative stress, which could be a consequence of either increased production of free radicals or reduced antioxidant defenses².

Oxygen free radicals are formed disproportionately in diabetes mellitus by glucose oxidation, non-enzymatic glycation of proteins and the subsequent degradation of glycosylated proteins. Diabetic complications are also associated with overproduction of free radicals and accumulation of lipid peroxidation by-products. Enhanced oxidative stress has been well documented in both experimental and human diabetes mellitus³. Thus attempt has been made to reduce the oxidative stress in patient with diabetes by supplementation with naturally occurring antioxidants⁴. The major goals of antioxidant treatment have been to reduce oxidative stress by preventing or delaying the progression or reversing the complications of diabetes. Medicinal plants often contain substantial amounts of antioxidants such as polyphenols, flavonoids, anthocyanins and tannins.

Ichnocarpus frutescens L. Br. (common name: Sarsaparilla, local name: Paalvalli; Family: *Apocynaceae*) has been used as folk medicine and as an ingredient in Ayurvedic and Unani preparations for diseases of blood, skin, for headache, snake bite and inflammation. Leaves of *I. frutescens* are rich in polyphenols and flavonoids. Distribution of various phenolic acid compounds and flavonoids in the leaves of *I. frutescens* has been systematically studied and well documented^{5,6}. The decoction of leaves of *I. frutescens* is used in the treatment of jaundice and diabetes, and this plants is also used by the tribals of Karnataka and Utter Pradesh for treating diabetes and jaundice⁷. Our earlier studies showed hepatoprotective, anti-hyperlipidemic, *in vitro* anti-oxidant properties and attenuation of diabetic complications with *I. frutescens*^{8,9}. This study was carried out to evaluate the antioxidant potential of polyphenol enriched extract (PPE) of *I. frutescens* on streptozotocin induced diabetes in neonatal (n-STZ)-type II Wistar rats.

Material & Methods

Chemicals and instruments: Streptozotocin (STZ) was purchased from Sigma Chemical Co (MO, USA). Glibenclamide and pentobarbitone sodium have been purchased from Ranbaxy Laboratories, India. Anthrone reagent, bovine serum albumin (BSA), thiobarbituric acid, reduced glutathione (GSH), nitroblue tetrazolium, trichloroacetic acid, nicotinamide adenine dinucleotide (NADH), Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] were purchased from SISCO Research Laboratories Private Limited, Mumbai, India. Serum biochemical assay kits for aspartate aminotransferase (AST), alanine aminotransferase

(ALT), alkaline phosphatase (ALP), total bilirubin, total cholesterol (TC), triglycerides (TGs) and total protein were purchased from Span Diagnostics Limited, Mumbai, India. Fasting blood glucose (FBG) levels were estimated by glucose oxidase peroxidase reactive strips (Accu-chek, Roche Diagnostics, USA). All other chemicals and solvents were of analytical grade. The equipment used were ultraviolet spectrophotometer (Spekol 1200, Japan), homogenizer (Remi, India), cold centrifuge (Remi, India), pH meter (Systronics, India), rotary vacuum evaporator (Superfit, India), lyophilizer (Instrumentation India, India), and light microscope (Kyowa, Japan).

Plant materials: The fresh leaves of *I. frutescens* were collected from delta region of Cauvery River, Thiruchirappalli, India, in February 2005 and authenticated at Botanical Survey of India, Central National Herbarium Howrah, India (Ref No: CNH/I-1/87/2005-TECH/1326). An authentic voucher specimen was deposited in the Herbarium of Division of Pharmacognosy, Phytotherapy Research Laboratory, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

Extraction of polyphenol enriched extract (PPE): The leaves were air dried for one wk at room temperature without exposure to sunlight and coarsely powdered. The leaf powder (300 g) was macerated for 5 days at room temperature three times with 800 ml of hydro-alcoholic mixture (double distilled water: 99% absolute alcohol; 30:70% vol/vol). The three macerates were combined and concentrated using a rotary evaporator under reduced pressure at <35°C. The residue was first dissolved in water and aqueous layer was washed with petroleum ether several times until a clear upper layer of petroleum ether was obtained. The concentrated solution of lower layer was extracted four times with 200 ml of ethyl acetate containing glacial acetic acid (10 ml/l) each time. The four ethyl acetate extracts were combined, evaporated to remove ethylacetate and polyphenolic extract (PPE) of *I. frutescens* was obtained as a lyophilized powder and stored at -70°C.

Animals: Two day old Wistar rat (M/s Ghosh Enterprises, Kolkata, India) were housed with their respective mother in macrolon cages under standard laboratory conditions. The mother rats were fed with commercial diet from Hindustan Lever Ltd (Bangalore, India) and free access to water during the experiments. The study protocol was approved by the Institutional Animal Ethical Committee (IAEC) of the Jadavpur University, Kolkata, India.

Induction of experimental type II diabetes: Wistar rat pups (n=40) were injected (ip) with 90 mg/kg STZ in 0.9 per cent sodium chloride solution. Control pups received equivalent vol of 0.9 per cent sodium chloride solution alone. Twelve weeks after the injection of STZ, animals exhibiting FBG>150 mg/dl were considered as neonatal- STZ (n-STZ)-diabetic¹⁰. These animals were divided into five groups. The treated control (glibenclamide 600 µg/mg) and treated diabetic rats received polyphenolic extract (150 and 300 mg/kg, po) for six wk.

After the last treatment of polyphenolic extract (after 6 wk), rats were fasted overnight and sacrificed by cervical decapitation. Blood was collected and serum was used for the estimation of biochemical parameters. Liver, kidney, pancreas and heart tissues were excised immediately and stored in ice-cold containers. The tissues were homogenized with appropriate buffer, centrifuged and the supernatant was collected. Tissue antioxidant estimations were carried out in the homogenates on the same day of sacrifice.

Assay of biochemical parameters: FBG levels were estimated by glucose oxidase peroxidase reactive strips¹¹ (Accu-Chek, Roche Diagnostics, USA). The biochemical parameters evaluated were serum lipid profiles^{12,13} (TGs and TC), liver biomarkers such as, bilirubin¹⁴, AST¹⁵, ALT¹⁵ and ALP¹⁶, serum total protein¹⁷ using diagnostics kits. High density lipoprotein cholesterol¹⁸ (HDL-C) and low density lipoprotein cholesterol¹⁹ (LDL-C) were estimated by standard methods using spectrophotometric method.

Assay of tissue antioxidant enzymes: Liver glycogen was estimated by the standard method and expressed as mg/g of liver tissue²⁰. Thiobarbituric acid reactive

substances (TBARS) and GSH^{21,22} were estimated in all the tissues (liver, heart, pancreas, and kidney). Further, the activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) were assayed by standard methods^{23,24}.

Histopathological studies: For histopathological studies, rats from control and experimental groups were perfused with 10 per cent neutral formalin solution. Pancreas was removed immediately from the rat; paraffin sections of 5 µm thickness were made and stained by hematoxylin-eosin (H&E) stain. After staining, the sections were observed under light microscope.

Statistical analysis: Data were statistically evaluated by using one-way analysis of variance (ANOVA), followed by Dunnett 't-test using GraphadInstat Statistical software version 4.01(San Diego, CA, USA).

Results

Tables I and II show the changes in the level of FBG, TGs, TC, AST, ALT, ALP and total protein in normal and experimental groups of rats. There was a significant elevation in FBG, AST, ALT, ALP, TGs, TC, LDL-C and while the levels of HDL-C, protein decreased during diabetes when compared to control group. Administration of polyphenolic extract brought back the levels to near normal values as that of standard drug glibenclamide. Streptozotocin induced diabetic rats showed a significant decrease in body weight compared to normal rats. Oral administration of polyphenol extract showed a significant increase ($P<0.01$) in body weight when compared to untreated diabetic rats.

Table I. Effect of polyphenolic extracts of *I. frutescens* on fasting blood glucose and body wt on streptozotocin induced neonatal diabetic rats

Treatment groups	Basal	Body wt (g)		Basal	Fasting blood glucose (mg/dl)	
		After diabetes induction			After diabetes induction	
		Initial	Final		Initial	Final
Normal control	211.33 ± 1.76	219.34 ± 3.092*	218.66 ± 3.08*	89.16 ± 2.62	90.16 ± 2.40*	90.00 ± 2.73*
Diabetic control	212.66 ± 2.40	161.56 ± 3.27	193.44 ± 4.38	85.66 ± 1.66	220.67 ± 5.05	230.33 ± 3.91
PPE (150 mg/kg)	209.67 ± 4.11	168.67 ± 3.63	188.68 ± 3.81*	87.5 ± 2.12	221.5 ± 4.97	187.66 ± 6.00*
PPE (300 mg/kg)	214.33 ± 3.63	157.04 ± 4.31	172.70 ± 5.52*	88.83 ± 2.22	224.00 ± 4.56	170.50 ± 5.42*
Glibenclamide (600 µg/kg)	212.81 ± 2.81	174.79 ± 7.49	191.21 ± 5.16*	87.33 ± 1.60	235.5 ± 2.52	128.00 ± 4.00*

Values are expressed as mean ± SEM; n = 6, * $P<0.01$ compared to STZ diabetic control rats
PPE, polyphenolic extract

Table II. Effect of polyphenolic extracts of *I. frutescens* on serum biochemical parameters and liver glycogen levels on streptozotocin induced neonatal diabetic rats

Parameters	Normal control	Diabetic control	PPE (150 mg/kg)	PPE (300 mg/kg)	Glibenclamide (600 µg/kg)
Cholesterol (mg/dl)	70.2 ± 3.72**	185.51 ± 2.81	117.55 ± 3.23**	99.56 ± 3.03**	101.88 ± 3.32**
Triglyceride (mg/dl)	92.52 ± 3.0**	167.18 ± 3.03	132.68 ± 3.36**	111.36 ± 3.32**	113.10 ± 3.57**
HDL (mg/dl)	56.63 ± 1.64**	39.9 ± 2.14	46.76 ± 1.66*	50.51 ± 0.844**	51.17 ± 1.28**
LDL (mg/dl)	128.08 ± 3.05**	152.61 ± 2.74	143.2 ± 2.16	133.35 ± 2.87**	138.15 ± 2.92**
AST (U/l)	37.91 ± 2.30**	120.47 ± 6.91	96.45 ± 3.85**	62.83 ± 2.19**	52.34 ± 2.39**
ALT (U/l)	44.73 ± 2.65**	82.20 ± 2.62	68.09 ± 2.42*	55.48 ± 3.32**	46.95 ± 3.83**
ALP (U/l)	183.60 ± 4.21**	273.47 ± 4.04	224.46 ± 4.92**	197.08 ± 3.33**	212.13 ± 2.34**
Total bilirubin (mg/dl)	0.82 ± 0.047**	1.60 ± 0.145	1.15 ± 0.069**	1.07 ± 0.060**	1.11 ± 0.049**
Total protein (g/dl)	7.36 ± 0.138**	6.11 ± 0.357	6.91 ± 0.079*	7.13 ± 0.122**	7.05 ± 0.13**
Liver glycogen (mg/g tissue)	4.38 ± 0.232**	0.956 ± 0.097	1.056 ± 0.075	1.84 ± 0.278*	2.85 ± 0.262**

Values are expressed as mean ± SEM; n = 6, *P<0.05 and **<0.01 compared to STZ diabetic control rats.

LDL, low-density-lipoprotein; AST, aspartate aminotransferase; ALT, alanine transaminase; ALP, alkaline phosphatase; n-STZ, neonatal-streptozotocin; SEM, standard error mean; PPE, polyphenolic extract

Table III. Effect of polyphenolic extracts of *I. frutescens* on tissues antioxidant enzymes and lipid peroxidation levels in streptozotocin induced neonatal diabetic rats

Parameters	Normal control	Diabetic control	PPE (150 mg/kg)	PPE (300 mg/kg)	Glibenclamide (600 µg/kg)
SOD (mg/protein)					
Liver	27.42 ± 1.08**	11.82 ± 1.31	15.34 ± 0.860	20.04 ± 1.404**	22.05 ± 1.90**
Kidney	15.43 ± 1.52**	5.50 ± 0.443	7.05 ± 0.375	12.35 ± 1.104**	13.18 ± 1.11**
Pancreas	11.72 ± 0.741**	4.3 ± 0.337	7.15 ± 0.466*	11.00 ± 0.819**	11.86 ± 1.06**
Heart	11.02 ± 0.420**	4.26 ± 0.446	6.10 ± 0.491*	6.67 ± 0.429**	6.50 ± 0.356**
CAT (mg/protein)					
Liver	184.33 ± 9.21**	3.13 ± 1.51	70.68 ± 4.89**	164.69 ± 11.71**	109.30 ± 21.77**
Kidney	18.5 ± 1.08**	3.01 ± 0.834	18.03 ± 1.994**	37.05 ± 3.43**	31.65 ± 0.533**
Pancreas	10.10 ± 0.562**	0.83 ± 0.070	8.62 ± 1.006**	4.30 ± 0.430**	10.90 ± 0.640**
Heart	6.42 ± 0.874**	0.71 ± 0.252	3.63 ± 0.572**	4.41 ± 0.318**	5.09 ± 0.437**
GSH (mg/100 g tissue)					
Liver	31.80 ± 2.58**	7.21 ± 0.864	12.64 ± 1.445	31.04 ± 1.515**	49.38 ± 2.00**
Kidney	31.46 ± 1.99**	8.11 ± 0.784	15.65 ± 1.11**	30.85 ± 3.06**	31.18 ± 2.96**
Pancreas	29.18 ± 2.01**	7.56 ± 0.773	25.82 ± 1.742**	39.66 ± 2.634**	30.81 ± 1.326**
Heart	27.46 ± 2.24**	12.71 ± 0.920	28.81 ± 2.22**	39.36 ± 2.09**	28.62 ± 1.91**
LPO (nm MDA/protein)					
Liver	4.57 ± 0.171**	15.77 ± 0.358	7.58 ± 0.406**	5.31 ± 0.140**	5.06 ± 0.252**
Kidney	3.85 ± 0.099**	16.68 ± 0.583	6.51 ± 0.109**	10.70 ± 0.390**	7.23 ± 0.139**
Pancreas	2.65 ± 0.140**	0.182 ± 0.182	3.87 ± 0.196**	7.57 ± 0.224**	3.70 ± 0.076**
Heart	3.18 ± 0.051**	10.41 ± 0.337	7.08 ± 0.336**	3.95 ± 0.100**	3.79 ± 0.096**

Values are expressed as mean ± SEM; n = 6, *P<0.05, **<0.01 compared to STZ diabetic control rats

n-STZ, neonatal-streptozotocin; SEM, standard error mean; PPE, polyphenolic extract; LPO, lipid peroxidation; MDA, malondialdehyde; GSH, reduced glutathione; CAT, catalase; SOD, superoxide dismutase

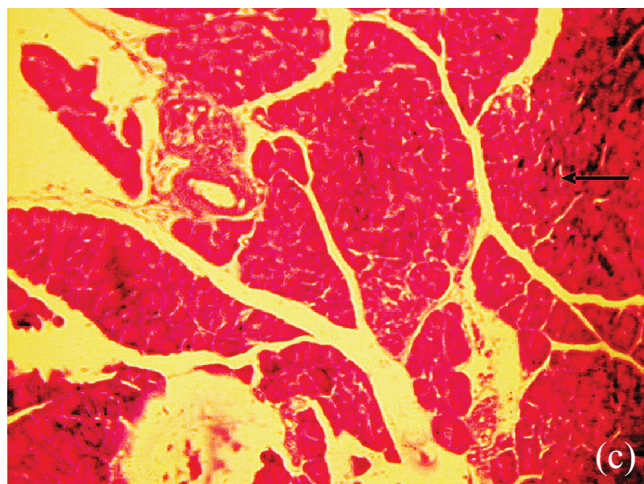
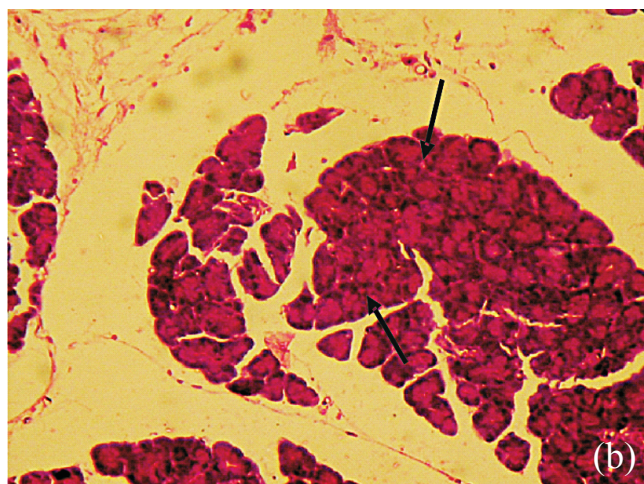
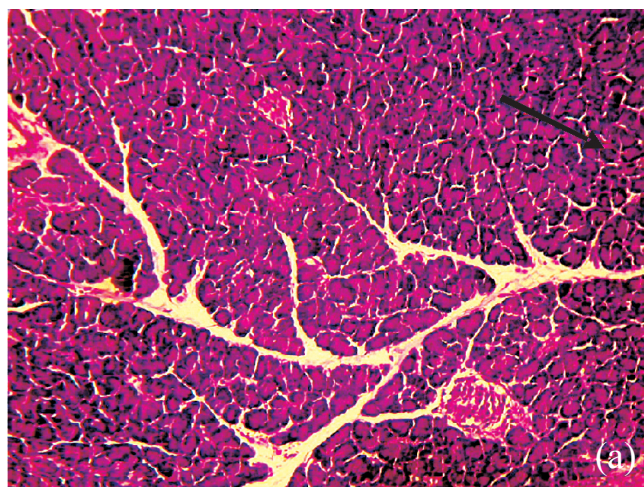


Fig. (a). Section from pancreas of non-diabetic rat showing normal islets (→). **(b).** Section from pancreas of diabetic control rat showing minute islets (→) with disturbed arrangement. **(c).** Section from pancreas of PPE treated diabetic rat showing recovery of islets and its arrangement (→) that are comparable to that of non-diabetic rat (a-c: H & E stain, 100 X).

The antioxidant enzymes SOD, CAT and non-enzymatic GSH levels were determined in the liver, pancreas, heart and kidney of diabetic and diabetic rats treated with polyphenolic extract and glibenclamide (Table III). In the negative control rats, the highest antioxidant levels were found in the liver, followed by kidney, heart and pancreas. In diabetic rat liver, pancreas, heart and kidney the levels of SOD, CAT and GSH were decreased significantly compared to age matched control rats. Treatment with polyphenolic extract normalized the altered antioxidant levels of all tissues occurring due to diseases.

The lipid peroxide levels were increased significantly in liver, heart, kidney and pancreas in STZ-treated rats. The polyphenolic extract was able to reverse the altered peroxidative damage to near normal values (Table III).

Section from the non-diabetic vehicle treated rats showed normal architecture of pancreas (Fig. a) and no histopathological alterations were observed in these animals. Pancreatic section from control diabetic rats (Fig. b) showed minute and reduced number of islets. Section from polyphenolic extract treated diabetic rats showed regenerating tiny islets (Fig. c) which could be comparable to that of non-diabetic control rats. These histological observations showed the protective role of polyphenolic extract on pancreas in STZ induced diabetic rats.

Discussion

The aim of the present study was to demonstrate the efficacy of polyphenolic extract in the reduction of FBG level as well as to determine the recovery in altered biochemical variables indicative oxidative stress and various organ damages in rats with STZ induced diabetes. The high blood FBG levels were observed in STZ treated rats indicating the establishment of oxidative stress mediated diabetic state²⁵. Administration of graded dose of polyphenolic extract significantly decreased FBG concentration when compared to the diabetic control. Oral administration of polyphenolic extract resulted in a significant decrease in serum TC and TGs. The TGs, TC and VLDL-C contents in plasma registered a significant hike in diabetic control group, which was retrieved to near normalcy in polyphenolic extract treated groups. This observation indicates the lipid lowering potential of *I. frutescens*.

Increase in the plasma ALT, AST and ALP are observed in the condition in which pancreas, liver, kidney and heart are destroyed by STZ. Moreover, the

activities of these enzymes have been used as indicators of tissue toxicity in experimental diabetes. Increased levels of AST, ALT and ALP were seen in STZ induced diabetic rats, over a six weeks period. Treatment with polyphenolic extract showed potential hepatoprotective activity as reported in our earlier study¹⁰.

Glycogen synthesis in the rat liver and skeletal muscles is impaired during diabetes²⁶. The regulation of glycogen metabolism occurs by the multifunctional enzyme glycogen synthase and glycogen phosphorylase that play a major role in the glycogen metabolism²⁷. The reduced glycogen store in rats with experimentally induced diabetes has been attributed to reduced activity of glycogen synthase and increased activity of glycogen phosphorylase. In the present study the experimental diabetic rats treated with polyphenolic extract and glibenclamide treated groups restored the level of hepatic glycogen by means of increasing the activity of glycogen synthase enzyme.

Measurement of tissue TBARS help to assess the extent of tissues damage and elevated TBARS observed in the various tissues of diabetic rat can be related to overproduction of lipid peroxidation by-products and diffusion from damaged tissues²⁸. Enhanced TBARS and declined antioxidants observed in the liver, pancreas, heart and kidney of diabetic rats can be attributed to increased biomembrane lipid peroxidation process and thereby contributing to alteration in antioxidant status²⁹. The decrease in thiobarbituric acid reactive substance (TBARS) of various tissues clearly showed the antioxidant property of polyphenolic extract. These findings suggest that the polyphenolic extract may exert antioxidant activity and protect the tissue from lipid peroxidation.

In the current study, the SOD, CAT and GSH activities of diabetic rat liver, pancreas, heart and kidney were significantly reduced. This may be due to the production of reactive oxygen free radicals that can themselves reduce the activity of these enzymes. The lowered glutathione level in diabetes has been considered an important indicator of increased oxidative stress³⁰.

Flavonoids have been shown to be potential antioxidants in the treatment of STZ induced oxidative stress in diabetic rats³¹. It is possible that the delay in STZ induced oxidative stress in various tissues of polyphenolic extract treated rats is predominantly due to its antioxidant activity. Polyphenolic extract of *I. frutescens* may also act by either directly scavenging reactive oxygen metabolites due to the presence of

various antioxidant compounds or by increasing the level of endogenous antioxidant molecules or enzymes. Further, pharmacological and chemical studies are required to explore the mechanism of action of active ingredient(s) responsible for the antioxidant activity observed.

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