EVALUATION OF ANTIOXIDANT ACTIVITY OF ETHANOL EXTRACT OF ROOTS OBTAINED FROM ASPARAGUS RACEMOSUS BY IN VITRO AND EX VIVO MODELS

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ABSTRACT

The objective of the study was to explore the antioxidant activity of ethanol extract of roots of Asparagus racemosus by in vitro and ex vivo models to substantiate the folklore claim of the traditional practitioners. The roots of the plant showed sufficient antioxidant activity by in vitro (determination of total antioxidant activity through estimation of the conjugated dienes and thiobarbituric acid reactive substances formed, total phenolic content, reducing power, free radical scavenging activity by DPPH method, nitric oxide scavenging activity, hydroxyl radical scavenging activity and super oxide scavenging activity by NBT and DMSO methods) and ex vivo (inhibition of haemolysis of RBC induced by phenyl hydrazine, inhibition of lipid peroxidation induced by ferrous sulphate and carbon tetrachloride) models. The experimental data were compared with that of standard antioxidant like ascorbic acid, alpha-tocopherol acetate, butylated hydroxyl anisol (BHA) and butylated hydroxyl toluene (BHT). All observed results indicated ethanol extract of roots of Asparagus racemosus to possess antioxidant activity in a concentration dependant manner and the activity of the extract was found to be very much comparable to that of the selected standard drugs.

Keywords: Asparagus racemosus, Antioxidant, Ethanol, Reactive oxygen species. Free Radical Scavenging.

INTRODUCTION

Reactive oxygen species (ROS) namely superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxy-nitrite radicals play a significant role in oxidative stress associated with a number of disease complications [1]. In healthy individuals, the production of free radicals is balanced with the antioxidative defense system. When equilibrium gets disbalanced, it results in the generation of free radicals beyond the limit and ultimately depletion of antioxidant levels. The oxidation of cellular lipids, nucleic acids, proteins, carbohydrates and other biomolecules by ROS is thought to be one of the major risk factors for cancer, atherosclerosis, diabetes mellitus, coronary heart disease and various other degenerative diseases [2, 3]. On the contrary, free-radical-scavenging antioxidants derived from dietary sources play an important role in preventing oxidative damages. The flavonoids existing abundantly in vegetables and fruits, are good radical scavengers. Many methods to determine the radical scavenging activity of plant extracts have been reported, all of which have different advantages and limitations [4-7].

Asparagus racemosus Willd. (Family: Liliaceae) commonly known as satawar, satavari or shatavari is a tall climber, undershrub, distributed in low jungles of tropical and subtropical parts of India, Ceylon, Africa, Java and Ausrtalia. The roots of the plant has been used as antidiarrhoeal, appetizer, stomachic, expectorant, laxative, tonic, inflammations biliousness, antidysenteric and diuretic. The plant contains a number of secondary metabolites namely as sterols and saponins [8, 9]. The present investigation is directed to the exploration of antioxidant activity of ethanol extract of roots of Asparagus racemosus by in vitro and ex vivo models keeping in mind that the plant contains sterol.

MATERIALS AND METHODS

Plant Materials

Roots of *Asparagus racemosus* were collected in the month of April and May from Purnia district, Bihar, India. The plant was authenticated by the Botanist of Govt. P. G. College, Uttarkashi, Uttarakhand. A voucher specimen of the herbarium was deposited in our laboratory for future reference.

Preparation of the Ethanol Extract

Ethanol extract of roots was prepared in accordance to the method of National Institute of Health and Family Welfare (NIHFW), New Delhi, India. Dried matured leaves were crushed in an electrical grinder to fine powder of mesh 40. The powder was then extracted with ethanol in a soxhlet apparatus until the powder became exhausted totally. Resulting extract was filtered with coarse sieve filter paper. The filtrate was dried under reduced pressure with the help of rotary vacuum evaporator. The extract was stored in a desiccator for use in subsequent experiments.

Chemicals

Ethylene di-amine tetra acetate (EDTA), phenyl hydrazine hydrochloride, dimethyl sulphoxide (DMSO), thiobarbituric acid (TBA), ferrous sulphate ($FeSO_4$), trichloroacetic acid (TCA) and acetic acid were procured from SD fine chemicals Ltd., India. Nitro blue tetrazolium chloride (NBT) was purchased from Hi-Media Ltd., India, while ascorbic acid and á-tocopherol acetate were procured from Cadila Pharmaceutical Ltd., India. All reagents used in the experiment were of analytical grade and other reagents were obtained from Sigma (Sigma-Alrich GmbH, Sternheim, Germany).

Determination of Total Antioxidant Activity

The antioxidant activity of the ethanol extract of the roots of *Asparagus racemosus* was determined according to the method using linoleic acid emulsion system [10]. Linoleic acid emulsions were prepared by mixing 0.285 g of linoleic acid, 0.289 g of Tween-20 as emulsifier and 50 ml of phosphate buffer (pH 7.2). The mixture was homogenized for 5 min and the antioxidant was added at the final concentrations of 25-800 ìg/ml of the extract. The mixture was incubated in an oven at 37°C for 24 hr and the course of oxidation was monitored by measuring the formation of conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS).

Estimation of CD Formation

Aliquots of 20 ìl were taken every hour from the emulsion during 24 hr of incubation. To each aliquot, 2 ml of methanol in deionized water (60 %) were added and the absorbance of the mixture was measured at 233 nm by UV-visible spectrophotometer.

Estimation of TBARS

Sample (100 ìl) was taken every hour from the emulsion and the following chemicals were sequentially to it, 100 ìl BHA (3.6 %) and 2 ml of TBA-TCA solution [20 mM TBA in 15% trichloacetic acid (TCA)]. The mixture was heated in a water bath at 90°C for 15 min and cooled at room temperature. 2 ml chloroform was added to it, the mixture was mixed and centrifuged at 2000 rpm for 15 min. The chloroform layer was separated and absorbance of the supernatant was measured at 532 nm agains a blankcontaining 0.1 ml of double distilled water and 2 ml of TBA-TCA solution. Lipid peroxidation was measured in terms of malondialdehyde (MDA) content.

The solutions without extract and containing equivalent volumes of solvent were used as blank samples. All data about total antioxidant activity were the averages of triplicate analyses. The inhibition of lipid peroxidation in percentage was calculated by the following equation:

% Inhibition = $(A_o - A_t)/A_o$ x 100,

where A_o = absorbance of the control reaction and A_t = absorbnce in the presence of the sample of the extracts [11, 12].

Determination of Total Phenolic Content

Total soluble phenolic compound in the ethanol extract of the roots of *Asparagus racemosus* was estimated with Folin-Ciocalteu reagent by the method [13]. Gallic acid was used as a standard phenolic compound. 1 ml of extract solution (containing 1mg extracts) was kept in a volumetric flask diluted with water (46 ml), 1ml of Folin-Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min, 3ml of sodium carbonate (2%) was added, then the mixture was allowed to stand for 2 hr with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer. The concentrations of the total phenolic compounds in the extracts were estimated as equivalent microgram of gallic acid by using an equation that was obtained from standard gallic acid graph:

Absorbance = 0.0053 × total phenols (gallic acid equivalent µg) – 0.0059

Reducing Power

The reducing power of ethanol extract of roots of *Asparagus racemosus* was determined according to reported method [14] with little modifications. The three different concentrations of extract (20ìg/ml, 40ìg/ml and 80ìg/ml) were mixed with 2.5 ml of 200mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potsssium ferricyanide. The mixture was mixed properly and then centrifuged at 650 rpm for 10 minutes. The upper layer 5 ml was mixed with 5 ml of distilled water and 1 ml of 1% ferric chloride and absorbance was measured at 700 nm in a spectrophotometer. Higher absorbance of reaction mixture indicated greater reducing power.

Free Radical Scavenging Activity by DPPH Method

This assay was based on the measurement of the scavenging ability of antioxidant test substances towards the stable radical. The free radical scavenging activity [15] of the ethanol extract was examined *in vitro* using DPPH radical. Different concentrations (25-800 ìg/ml) of the test extract was used in the study. The reaction mixture consisted of 1 ml of 0.1mM DPPH in ethanol, 0.95 ml of 0.05M Tris-HCl buffer (pH 7.4), 1 ml of ethanol and 0.05 ml og the herbal extract. The absorbance of the mixture was measured at 517 nm exactly 30 sec after adding the extract. The experiment was performed (in triplicate) and % scavenging activity was calculated using the formula

% Scavenging = $(A_o - A_t)/A_o$ x 100,

where A_o = absorbance of the control reaction and At = absorbance in the presence of the sample of the extracts. The activity was compared with ascorbic acid, which was used as a standard antioxidant.

Nitric Oxide Scavenging Activity

Sodium nitroprusside [16] (5 µM) in standard phosphate buffer solution was incubated with different concentration of the test extracts dissolved in standard phosphate buffer (0.025M, pH 7.4) and the tubes were incubated at 250°C for 5 hr. After 5 hr, 0.5 ml of incubation solution was removed and diluted with 0.5 ml Griess reagent (prepared by mixing equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in water). The absorbance of chromophore formed was read at 546 nm. The control experiment was also carried out in similar manner, using distilled water in the place of extracts. The experiment was performed (in triplicate) and % scavenging activity was calculated using the same formula, as mentioned above.

The activity was compared with ascorbic acid, which was used as a standard antioxidant.

Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extract for hydroxyl radicals generated from the ferric ions/ascorbate/EDTA/hydrogen peroxide system. The reaction mixture contained deoxy ribose (2-8mM), Fecl3 (0.1mM), EDTA (0.1mM), hydrogen peroxide (1mM), ascorbate (0.1mM), KH2P04-KOH buffer (20mM, pH 7.4) and various concentrations (25-800 ug/ml of extracts and 5-80 ug/ml of the standard drug) in the final volume of 1 ml. The reaction mixture was incubated for 1 hr at 37°C, deoxyribose degradation was measured at 532 nm [17].

Super Oxide Scavenging Activity

Super oxide scavenging activity by NBT model:

Super oxide scavenging activity was measured using reported method [18] with little modification. The reaction mixture consisted of 50mM sodium carbonate buffer (pH 10.2), 24ìM NBT, 0.1mM EDTA, 1mM hydroxylamine and 0.03% Trifonx-100 along with the compound to be tested in a total volume of 1 ml incubated for 20 minutes at 37°C and the absorbance was measured at 560 nm with the help of spectrophotometer. Ascorbic acid was used as standard antioxidant.

Super oxide scavenging activity by alkaline dimethyl sulphoxide (DMSO) method:

Superoxide scavenging activity was determined by using alkaline DMSO method [19] with minor modifications. Solid potassium sulphoxide was kept in contact with dry DMSO for at least 24 hours and the solution was filtered immediately before use. Filtrate (200 µl) was added to 2.8 ml of an aqueous solution containing NBT (56ìM), EDTA (10ìM) and potassium phosphate buffer (10mM). Ethanol extract of the leaves of 1 ml at different concentrations were taken and the absorbance was observed at 560 nm against a control in which pure DMSO was taken instead of alkaline DMSO. Ascorbic acid was used as standard antioxidant.

Inhibition of Haemolysis of RBC Induced by Phenyl Hydrazine

20% packed cell volume (PCV) of RBC suspension (from human blood) was prepared according to the procedure described by Hill and Thornalley. The assay was carried out according to the method [20] with certain modifications. The incubation mixture comprised of 1 ml of phenyl hydrazine hydrochloride (0.5mM), different concentrations of the sample extracts and 0.1 ml of 20% RBC suspension made to a total volume of 3.0 ml with phosphate buffered saline (PBS) solution. The mixture was incubated at 37°C for one hr and centrifuged at 100 rpm for 10 min. The extent of haemolysis was measured spectrophotometrically by recording the absorbance of the supernatant at 540 nm. Suitable controls were kept to nullify the effect of solvents and inherent haemolysis. á-tocopherol acetate was used as a positive control for the inhibition of phenyl hydrazine induced haemolysis of RBC.

Inhibition of Lipid Peroxidation

Inhibition of lipid peroxidation induced by $FeSO_4$

Animals: Male Wister strain albino rats (160-180 gm) were procured from M/s B N Ghosh Co. Kolkata, India. Rats were housed in standard polypropylene cages (two animals per cage), maintained under standard laboratory conditions (i.e. 10:14 hour light and dark order; at an ambient temperature of 26 ± 2 ºC; 40-55% of relative humidity); the animals were fed with standard rat pellet diet (Hindustan Liver Ltd. Mumbai, India) and water ad libitum.imalserellowedoequainted for a period of a week inur laboratory environment prior to the experiment.

Preparation of rat liver homogenate: Rat liver homogenate was prepared by the following method

[21]. Randomly selected 6 rats were fasted over night and were sacrificed by cervical dislocation, dissected and abdominal cavities were perfused with 0.9% normal saline. Livers of sacrificed rats were separated out and visible clots were removed. Then 10% liver homogenate was prepared in cold phosphate buffer saline (pH 7.4) using glass teflon homogeniser and filtered to get a clear homogenate.

Assay method: The degree of lipid peroxidation was assayed by estimating the thiobarbituric acid reactive substances (TBARS) by using standard method [22] with minor modifications [23]. Briefly, different concentrations of extract (50-300 ìg/ml) were added into 10% liver homogenate. Lipid peroxidation was initiated by adding 100 ìl of 15mM FeSO4 solution to 3 ml of liver homogenate (final concentration was 0.5mM). After 30 min 100 ìl of this reaction mixture was taken in a tube containing 1.5 ml of 10% TCA. After 10 min tubes were centrifuge and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. The mixture was heated in a hot water bath to complete the reaction. The intensity of pink coloured complex formed was measured at 535 nm in a spectrophotometer. The percentage inhibition of lipid peroxidation was calculated as per the following formula:

Inhibition (%) = 100 x (Control Test)/Control

Inhibition of lipid peroxidation induced by CCl^4

Inhibition of lipid peroxidation induced by CCl^4 was measured by the method [24]. Rat liver (30% w/v) homogenate in ice-cold 0.15M potassium chloride was prepared in a homogenizer. Aliquots of 0.5 ml of homogenates were taken in different small conical flasks. These were incubated at 37ºC in a constant shaker bath (150 cycles/min) for 45 min with 1.5 ml of potassium sulphate buffer (pH 7.4), 2 ml of 0.15M potassium chloride CGM at (25- 800) ìg/ml and ascorbic acid (5-100 µg/ml) in different flasks and finally 10 µl of CCl4 was added. The reaction was stopped by the addition of 4 ml of 10% w/v TCA and after incubation. The contents were centrifuged at 4000 rpm for 10 min and about 2 ml of clear supernatant was transferred to a graduated tube. 2 ml of 0.67% w/v of TBA was added and heated in a boiling water bath for 15 min the tubes were cooled bringing the mixture to pH 12-12.5 with potassium hydroxide stabilized the colour developed and the absorbance was measured at 543 nm. In case of control, only drug was excluded.

RESULTS AND DISCUSSION

Total antioxidant activity was estimated by calculating formation of CD and TBARS, as reported in Table1. Total phenolic content was 15.67 µg gallic acid equivalent of phenols as was determined in 1 mg of the ethanol extract of roots of Asparagus racemosus, reported in Table 2. Phenolic compounds are most vital constituents because of their scavenging ability due to their hydroxyl groups [25]. The phenolic compounds may contribute directly to the antioxidant effect. According to recent reports, a highly positive corelation exists between total phenols and antioxidant activity was found in many plant species [26, 27]. In addition, it was reported that phenolic compounds were associated with antioxidant activity and play a vital role in stabilizing lipid peroxidation [28].

Reducing power of the extract and standards followed the order (BHA>BHT >ascorbic acid> extract), as shown in Table 3. Reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [29]. The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging activity [30].

The ethanol extracts of the leaves of the plant showed prominent free radical scavenging effect of DPPH in a concentration dependant manner up to a concentration of 800 µg /ml. IC_{50} values of extract and ascorbic acid were found to be 180.0 µg/ml and 11.6 µg/ml, respectively, by this method (Table 4). The free radical scavenging activity of the extracts was determined on the basis of ability to scavenge the synthetic DPPH. This method is a widely used method to evaluate antioxidant activities in a relatively short time compared with other models due to prominent discolouration from purple to yellow in visible spectrum.

The ethanol extract showed significant scavenging activity against the nitric oxide radical when compared with the standard ascorbic acid in a dose dependant manner. IC50 values of the extract and ascorbic acid were found to be 265.0 µg/ml and 15.2 µg/ml, respectively, by this method (Table 4). Nitric oxide scavenging activity was determined by the formation of the chromophore during diazotization of the nitrite with sulphanilamide and subsequent conjugation with napthylene diamine. The ethanol extracts significantly scavenged the hydroxyl radicals when compared with the standard ascorbic acid in a dose dependant manner. IC_{50} values of the extract and ascorbic acid were found to be 235.0 µg/ml and 16.4 µg/ml, respectively, by this method (Table 4). For tissue injury hydroxyl radical is the one of the main factor. The extract scavenged the hydroxyl radical formed in the Fenton reaction and it was quantified using 2-deoxy-D-ribose degradation.

Super oxide scavenging activity was measured by *in vitro* assay methods employing both NBT and alkaline DMSO models. The ethanol extract of roots of *Asparagus racemosus* at a concentration range of 25-800 µg/ml significantly scavenged the superoxide radicals. The percentage scavenging of superoxide radicals by the extract was increased in a dose dependant manner in both NBT and DMSO models. IC_{50} values of extract and ascorbic acid were found to be 130.0 µg/ml and 18.2 µg/ml, respectively, as found in NBT model, whilst in DMSO method the IC_{50} values were found to be 285.0 µg/ml and14.1 µg/ml for extract and ascorbic acid, respectively.

The extract inhibited the haemolysis of RBC induced by phenyl hydrazine in a dose dependant manner. The IC_{50} values were found for the extract was 65.0 µg/ml, whilst standard drug, á-tocopherol acetate had shown IC_{50} of 11.6 µg/ml.

The extract had shown a concentration dependant inhibition of $FeSO_4$ induced lipid peroxidation in rat liver homogenates. The IC_{50} values were found to be 175.0 µg/ml and 17.5 µg/ml, respectively, for the extract and the standard antioxidant ascorbic acid. Inhibition of CCl_4 induced lipid peroxidation was also found to increase in a dose dependant manner. The IC_{50} value was found to be 152.5 µg/ml and 12.5 µg/ml, respectively, for the extract and standard antioxidant ascorbic acid. The results of superoxide scavenging activity with DMSO and NBT method, inhibition of haemolysis of RBC induced by phenyl hydrazine and inhibition of lipid peroxidation induced by $FeSO_4$ & CCl_4 model had been presented in Table 5.

Table 1: Determination of total antioxidant activity of ethanol extract of roots of *Asparagus racemosus* (EEAR)

Concentration of EEAR(µg/ml)	% Inhibition	
	Conjugated dienes	TBARS
25	17.43 ± 0.65	23.67 ± 0.54
50	26.84 ± 0.23	37.40 ± 0.36
100	33.76 ± 0.19	45.62 ± 0.74
200	44.22 ± 0.11	58.32 ± 0.65
400	53.44 ± 0.23	66.82 ± 0.25
800	59.44 ± 0.54	71.07 ± 0.43
IC50 (EEAR) (µg/ml)	215.0	132.5
IC50 (Standard) (µg/ml)	46.9	34.2

Values are mean ± S.E.M. of 3 replications

Table 2: Total phenolic content of EEAR

Parameter	Level (µg/g)
The extract (EEAR)	12.89

Table 3: Total reducing property of ethanol EEAR

Sample	Absorbance		
	20 ìg/ml	40 ìg/ml	80 ìg/ml
Ethanol extract (EEAR)	0.092±0.064	0.117±0.082	0.210.45±0.023
Ascorbic acid	0.190±0.016	0.365±0.015	0.543±0.013
Butylatedhydroxy lanisole(BHA)	1.040±0.025	1.756±0.017	2.810±0.028
Butylated hydroxyl toluene (BHT)	0.546±0.016	0.996±0.012	1.640±0.015

Values are mean ± S.E.M. of 3 replications

Table 4: *In vitro* antioxidant effect of EEAR through superoxide scavenging activity with DMSO and NBT method, free radical scavenging activity by DPPH method, nitric oxide and hydroxyl scavenging method

Concentration of EEAR (µg/ml)	% of Scavenging				
	SOD scavenging effect		Free Radical		
			DPPH method	Nitric oxide	Hydroxyl
	NBT model	DMSO model			
25	23.25 ± 0.12	27.45 ± 0.44	24.59 ±0.53	19.56 ±0.32	28.21 ±0.32
50	34.35 ± 0.54	31.76 ± 0.28	33.48 ±0.37	31.63 ±0.33	33.62 ±0.22
100	47.35 ± 0.38	38.95 ± 0.43	44.77 ±0.32	36.73 ±0.23	43.85 ±0.16
200	56.25 ± 0.39	45.63 ± 0.34	51.23 ±0.13	45.34 ±0.22	49.13 ±0.35
400	66.21 ± 0.63	54.65 ± 0.12	63.32 ±0.23	59.15 ±0.22	54.23 ±0.13
800	75.87 ± 0.23	73.54 ± 0.47	67.85 ±0.42	70..92 ±0.18	59.33 ±0.27
IC_{50} (EEAR) (µg/ml)	130.0	285.0	180.0	265.0	235.0
IC_{50} (Standard) (µg/ml)	18.2	14.1	11.6	15.2	16.4

Values are mean ± S.E.M. of 3 replications

Table 5: Ex vivo antioxidant effect of EEAR through inhibition of haemolysis of RBC induced by phenyl hydrazine and inhibition of lipid peroxidation induced by $FeSO_4$ andCCl_4 model

Concentration of EEAR (µg/ml)	% Inhibition		
	RBC membrane stabilization activity	Inhibition of lipid peroxidation	
		$FeSO_4$ model	CCl_4model
25	35.12 ± 0.16	27.54 ± 0.13	30.32 ± 0.14
50	44.98 ± 0.12	34.87 ± 0.34	37.01 ± 0.15
100	58.80 ± 0.32	42.62 ± 0.22	44.42 ± 0.32
200	67.52 ± 0.18	51.52 ± 0.32	55.37 ± 0.53
400	73.55 ± 0.23	55.32 ± 0.35	60.71 ± 0.28
-	76.75 ± 0.15	67.33 ± 0.43	68.32 ± 0.22
IC_{50} (EEAR) (µg/ml)	65.0	175.0	152.5
IC_{50} (Standard) (µg/ml)	11.6	17.5	12.5

Values are mean ± S.E.M. of 3 replications

CONCLUSION

The living cells producing ROS during several metabolic pathways lead to oxidative stress, which is associated with several pathological complications. Production of ROS depends upon rapid uptake of oxygen, activation of NADPH oxidase and the production of superoxide free radical. Oxidative stress can be prevented by different endogenous antioxidants like superoxide dismutase, reduced glutathion, catalase and glutathion peroxidase by escaping ROS and lipid peroxidation dependant injury. Antioxidants work as radical scavengers, peroxide decomposers, hydrogen donor, electron donor, enzyme inhibitors, singlet oxygen quenchers, synergist and metal chelating agents. The requirement of antioxidants of human beings is solely filled by dietary vegetables, which play an important role in preventing oxidative damages. Plants also need to protect themselves from free radical damage with the help of their own metabolites, so they develop a number of different classes of antioxidant. The pigments, flavonoids, coumarines, phytosterols, pro-anthocyanidines, tannins, essential oils, resins and gums are responsible phytochemicals for antioxidant activity. Thus it has fetched interest of researchers to isolate novel antioxidant from plant sources.

Preliminary phytochemical screenng indicated the presence of sterols and saponins in the extract. Sterols isolated from different sources are reported to have antioxidant activity. So the lead compound may be sterol. Now our intention is guided to isolate bioactive sterols from the extract and substantiate its antioxidant efficacy.

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