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STUDY ON *IN VITRO* ASSESSMENT OF ANTIOXIDANT ACTIVITY OF *BURSERA PENICILLATA* ENGL (BURSERACEAE) STEM

Prabhakar Tirumani[a*], G. Sridhar[a], A. V. Rajashekhar[a]
[a]Department of Zoology, Osmania University, Hyderabad-07, India
Email: prabhu.venkat2010@gmail.com

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Abstract

Nowadays, the interest in discovering natural antioxidants, main resource select as plants, which have antioxidant compounds like poly phenols, flavonoids and carotenoids and their application to control onset of multiple diseases. Therefore, the present study was designed to evaluate phytochemical constituents and antioxidant activity of petroleum ether, benzene, ethyl acetate and methanol extracts of stem of *Bursera penicillata* have been tested using various antioxidant model systems viz., DPPH, ABTS, hydroxyl and reducing power. Total phenolic and flavonoid contents were determined by colorimetric methods using gallic acid and quercetin as standard. Ethyl acetate and methanol extracts showed the highest amount of total flavonoid and total phenolic content respectively. The methanol extract of stem of *B.penicillata* showed potent hydroxyl radical and DPPH scavenging assay and ethyl acetate extract exhibited potent reducing power and ABTS radical cation scavenging activity with low IC_{50} values. Based on our results, *B. penicillata* stem gains significance with regard to its antioxidant potential and which can be exploited for the treatment of various ailments.

Key Words: Antioxidant activity, *Bursera penicillata,* DPPH, Free radicals, Reducing power.

Introduction

Oxidative stress is induced by free radicals are also known as reactive oxygen species (ROS) and they can be generated in living organisms through cellular respiration, pollution, radiation and metabolism[1]. In moderate concentrations of free radicals are control many physiological functions of organisms by performing as secondary messengers[2]. The over production of these free radicals may attack the macromolecules of cell like lipids, DNA, RNA, proteins and carbohydrates leading to cause various degenerative diseases such as cancer, diabetic mellitus, inflammation, heart

diseases, rheumatoid arthritis, Parkinson's disease and ageing[3-8]. Organisms possess several defence mechanisms to control the level of ROS, but these defence mechanisms become unbalanced, antioxidant supplement can be used to reduce the oxidative damage[9].

Antioxidants are substances that can prevent (or) repair the body's cells especially through preventing the generation and scavenging of ROS, quenching of singlet oxygen[10]. Antioxidants are mainly consists of synthetic and natural and certain commonly used antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are synthetically available with restricted for their toxic effects[11-12]. Antioxidant compounds are natural sources of all parts of the plants also known as phytoconstituents such as phenols, vitamins, flavonoids, carotenoids and tannins etc. have good antioxidant activity[13].

Therefore, the continued search for natural antioxidants has gained importance during the last decade and the increasing awareness of herbal remedies for many chronic diseases. Scientific scrutiny and confirmation of the traditional therapeutic use of the plant medicines may produce the development of novel and effective drugs as has occurred in the past[14]. The genus *Bursera,* native from the tropical dry forests of Mexico and The family **Burseraceae** includes more than 600 species in 20 genera distributed in the neotropical and temperate regions of the world[15]. *Bursera penicillata* is an aromatic essential oil plant introduced into India from Mexico at the beginning of the 20th century for the exploitation of its essential oils[16]. These plants are locally named as Indian lavender (or) Indian linaloe since they release a characteristic aroma from their aerial parts. The primary rich constituents of Burseras' resin are terpenoid essential oils mainly linaloe and these resins are used topically in folk medicine[17-18].

In the traditional system, *B.penicillata* is used against toothache, burns, headache, gastric disorders, fever and stomachic and economically important due to diverse usage in cosmetic, perfumes and pharmaceuticals industries[19]. Since there are no reports with regard to *B. penicillata's* antioxidant activity and the present study has been undertaken to investigate the antioxidant potential, reducing power, total phenolic and flavonoid content of stems of plant extracts.

Material and Methods

Chemicals: DPPH (1,1-Diphenyl-2-picrylhydrazyl), ABTS (2,2-azinobis(3-ethyl benzothiazoline-6-sulphonic acid diammonium salt), rutin and quercetin obtained from Sigma-Aldrich (MO, USA) and sodium carbonate, sodium phosphate, potassium ferricyanide, ascorbic acid, gallic acid, potassium persulphate and Folin-Ciocalteu reagent were

purchased from Merck India Ltd. All other chemicals and solvents are analytical grade were obtained from indigenous companies in India.

Collection and extraction of plant material

Stems of *B. penicillata* were collected from the botanical garden of Osmania University, Hyderabad, India and the Plant material was identified and authenticated by Prof. P. Ramachandra Reddy, Taxonomist, Department of Botany, Osmania University, Hyderabad. A voucher specimen (Bot/OU/0119/HYD) was deposited in the herbarium of the department of botany for future reference. After collection, the stems of plants were shade dried for 10 days then grounded into coarse powder. About 100g of coarse powder of *B. penicillata* stem was extracted successfully with petroleum ether, benzene, ethyl acetate and methanol each 250 mL in a Soxhlet apparatus for 48 hrs. All the solvent extracts were filtrated through Whatman No.41 filter paper and then concentrated by using a rotary evaporator to yield a semisolid mass. The crude extracts were measured and stored in refrigerator until further use.

Determination of total phenolic content (TPC)

The total phenolic content was estimated using Folin-Ciocalteu reagent method[20] and gallic acid has used as a standard for plotting calibration curve. A volume of 1 mL of the plant extract (1mg/1mL) was mixed with 2 mL of Folin-Ciocalteu reagent (diluted 1:10 with distilled water) and was neutralized with 2 mL of 20% sodium carbonate solution. The reaction mixture was incubated at room temperature for 30 min with intermittent shaking for color development. The absorbance of the resulting blue color was measured at 765 nm using double beam UV-VIS spectrophotometer (Hitachi U-2910). The total phenolic contents of all the extracts were determined from the linear equation of a standard curveprepared with gallic acid and calculated as mean±SEM (n=3) and expressed as mg/Gallic acid equivalent (GAE)/g of dry extract.

Estimation of total flavonoid content (TFC)

Total flavonoid content of the different fractions was determined using the aluminum chloride colorimetric method as described, with some modifications[21]. A volume of 0.5 mL of the plant extract (1mg) was mixed with 0.1 mL of 10% aluminum chloride, 0.1 mL of potassium acetate (1M) and 4.3 mL of 80% methanol were mixed. The reaction mixture was incubated at room temperature for 30 min for color development. The absorbance was measured at 415 nm using UV-VIS spectrophotometer (Hitachi U-2910). The calculation of total flavonoids in the extracts were determined from

the linear equation of a standard curve prepared with quercetin and calculated as mean±SEM (*n*=3) and expressed as µg/mg quercetin equivalent (µg/mg QE/mg) of dry extract.

Determination of DPPH radical scavenging activity

The free radical scavenging activity of all the extracts of B. penicillata was evaluated by using DPPH radical scavenging method as described[22]. The assay mixture contained 2 mL of 0.004% DPPH solution prepared in methanol and 0.2 mL of standard (or) sample solution of various concentrations (5-300 µg/ml) in methanol. The mixture were shaken vigorously and allowed to stand at room temperature for 30 min and absorbance of the resulting solution was measured at 517 nm using a spectrophotometer (Hitachi U-2910) and ascorbic acid was used as a positive control. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The percentage inhibition of activity was calculated by using the following formula: DPPH scavenging activity (%) = $(A_0-A_1) / A_0 \times 100$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of samples and reference. All the tests were performed in triplicate and the results were mean±SEM.

Hydroxyl radical scavenging activity

The scavenging ability for hydroxyl radical was measured according to the modified method[23]. The assay was performed by adding 0.1 mL EDTA (1mM), 0.01 mL of $FeCl_3$ (10Mm), 0.1 mL H_2O_2 (10mM) 0.36 mL of deoxyribose (10mM), 1.0 mL of the extract of different concentrations (0-300 µg/mL) or ascorbic acid (0-100 µg/mL) dissolved in distilled water and add 0.33 mL of phosphate buffer (50mM, pH 7.9). The mixture was then incubated at 37 ^{0}C for 1 hour. 1.0 mL portion of the incubated mixture was mixed with 1.0 mL of 10%TCA and 1.0 mL of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to develop the pink color measured at 532 nm. The percentage inhibition of activity was calculated by using the following formula:

The hydroxyl radical scavenging activity (%) = $(A_0-A_1) / A_0 \times 100$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of samples and reference. All the tests were performed in triplicate and the results were mean±SEM.

Determination of ABTS radical scavenging activity

The total free radical scavenging activity of *B. pencillata* was measured by decoloration of $ABTS^+$ radical cation using the modified method[24]. ABTS radical cation ($ABTS^{+)}$ was generated by oxidation of ABTS (7mM) with potassium

persulfate (2.4mM) which was dissolved in 5 ml of distilled water. After incubation for 12-16 h at room temperature in dark condition, blue/green $ABTS^{+}$ chromophore was produced. The resulting solution was then diluted with ethanol by mixing 1 mL of freshly prepared ABTS solution to obtain an absorbance of (0.700±0.001) at 734 nm using spectrophotometer. Fresh ABTS solution was prepared for each assay. The assay mixture contained 2 mL of ABTS solution and 0.2 mL of standard (or) sample solution of various concentrations (0-300µg/mL) in ethanol. The absorbance of the resulting solution was measured at 734 nm and the extracts was compared with that of ascorbic acid and percentage inhibition was calculated by using this formula: ABTS radical scavenging activity = $(A_0-A_1) / A_1 \times 100$

Where A_0 is the absorbance of ABTS radical with methanol and A_1 is the absorbance of ABTS radical with sample extract/standard. All the tests were performed in triplicate and the results were mean±SEM.

Determination of reducing power

The reducing power of the extracts was quantified using the method described by Yildirim *et al*[25]. Briefly, 2 mL of reaction mixture containing various concentrations of (0-300 µg/mL) standard (or) plant extracts in sodium phosphate buffer (1.0 mL, 0.2 M, pH 6.6), incubated with 1% potassium ferricyanide (1.0 mL) at 50 ^{o}C for 20 min. The reaction was terminated by adding 10% TCA (2 mL) solution and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (2 mL) was mixed with 2 mL of distilled water and 0.5 mL of 1% ferric chloride solution (Freshly prepared) and absorbance was read at 700 nm and the experiments was performed trice and results were mean±SEM. Increased absorbance value indicates grater reducing power and the results were compared with that of the rutin as standard.

Statistical analysis

The determination of total phenolic content, total flavonoid content, ABTS, DPPH, Hydroxyl radical scavenging activity and reducing power were carried out for three replicates and mean values and standard error mean (SEM) were calculated and the results were processed by computer programs using Excel and Origin 6.1.

Results

Yield of extraction

The yield of petroleum ether, benzene, ethyl acetate and methanol extracts of stem of *B. penicillata* have been calculated and shown in Table 1 and among all fractions methanol extract has been found to be maximal (12.61 % w/w) followed by petroleum ether (8.52 % w/w).

Table 1: Quantitative analysis of yield and phyto-chemical constituents

Extracts	Total phenolic content (mgGAE/g)	Total flavonoid content (µgQE/mg)	% of yield (w/w)
Pet ether	62.91±2.97	3.37±0.16	8.52
Benzene	30.62±3.38	1.51±0.17	5.21
Ethyl acetate	116.47±6.75	28.83±0.5	3.17
Methanol	174.9±6.93	16.86±0.81	12.61

Total phenolic and flavonoid content of *Bursera penicillata* stem extracts.

Data are expressed as mean ± SEM.

Total phenolic content

Total phenolic content of all the extracts of *B. penicillata* stem have been calculated using the linear regression equation of the calibration curve ($y = 0.0009x - 0.0144$, $R^2 = 0.9983$) and expressed as gallic acid equivalent (GAE) shown in Table 1. The present study denote that total phenolic content of petroleum ether, benzene, ethyl acetate and methanol extract of stem of *B. penicillata* were 62.91±2.97 mg/GAE/g, 30.62±3.38 mg/GAE/g, 119.47±6.76 mg/GAE/g and 174.9±6.93 mg/GAE/g, respectively.

Total flavonoid content

Table 1. shows total flavonoid content of all the extracts of *B. penicillata* stem were determined as Quercetin equivalent (QE) and calculated by using the linear regression equation of the calibration curve ($y = 0.0099x - 0.0483$, $R^2 = 0.992$). Among the all extracts ethyl acetate extract was containing the highest amount (28.83±0.5 µg of QE/mg) followed by methanol extract (16.86±0.81 µg of QE/mg).

DPPH radical scavenging activity

The free radical scavenging activity of *B. penicillata* stem extracts were investigated with DPPH and it was compared with standard (L-ascorbic acid) and results have been expressed as % inhibition (Figure 1.C). The higher DPPH radical scavenging activity is associated with lower IC_{50} values. Among the solvent tested, methanol extract indicated highest DPPH radical scavenging ability with lowest IC_{50} value (131.75µg/mL) and followed by ethyl acetate (162.2 µg/mL) as shown in Table 2.

Table 2: Antioxidant activity of different solvent extract of *B. penicillata* stem (µg/mL)

Extracts	DPPH assay	ABTS assay	Hydroxyl assay
Pet ether	193.13*	195.65*	336.35*
Benzene	278.97*	164.08*	207.9*
Ethyl acetate	162.2*	114.68*	167.6*
Methanol	131.75*	149.34*	139.91*
Ascorbic acid	12.04	14.17	42.19

The IC50 values of different solvent extract of *B. penicillata* stem (µg/mL) and it was obtained by linear regression equation.

Superscript * denotes in column are statistically significant ($p \leq 0.05$) compared with standard.

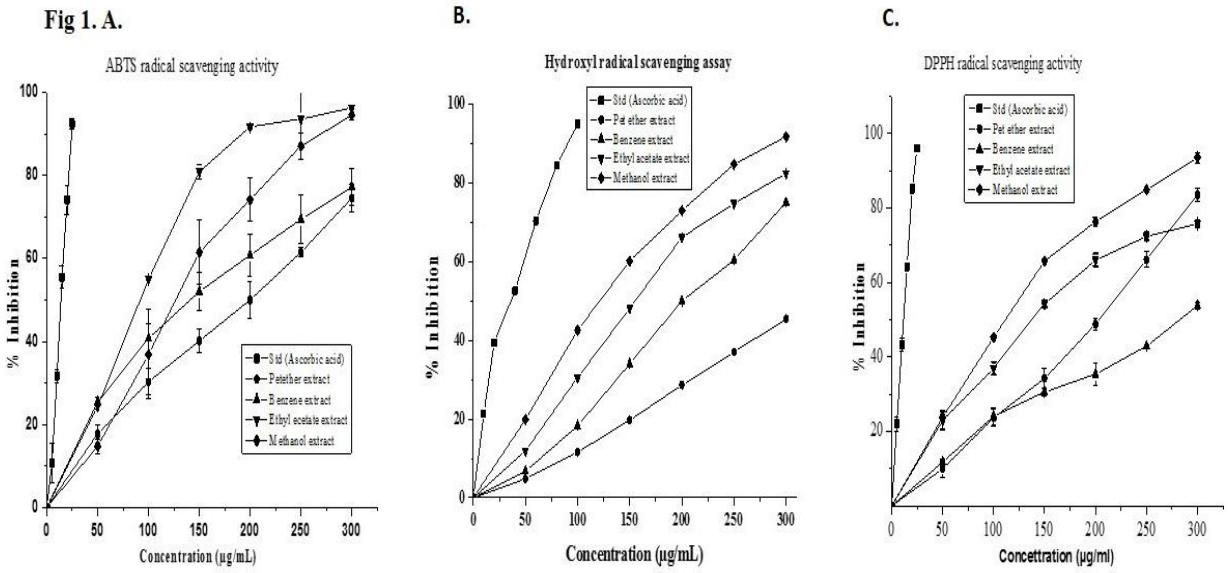

Figure 1: Antioxidant activities of different solvent extracts of *Bursera penicillata* stem.

Fig. 1. Antioxidant activities (µg/mL) of different solvent extracts of *B. penicillata* stem were determined by using DPPH, hydroxyl radical and ABTS assay and results are expressed as mean±SEM (*n*=3). Pet ether= petroleum ether.

Hydroxyl radical assay

Figure 2.B. depicts the hydroxyl radical scavenging ability of various solvent extracts of *B. penicillata* stem. In this assay, the highest antioxidant activity was exhibited by methanol extract then other solvent performed. The IC_{50} values are indirectly proportional to the antioxidant ability of various plant extracts and as shown in Table 2.

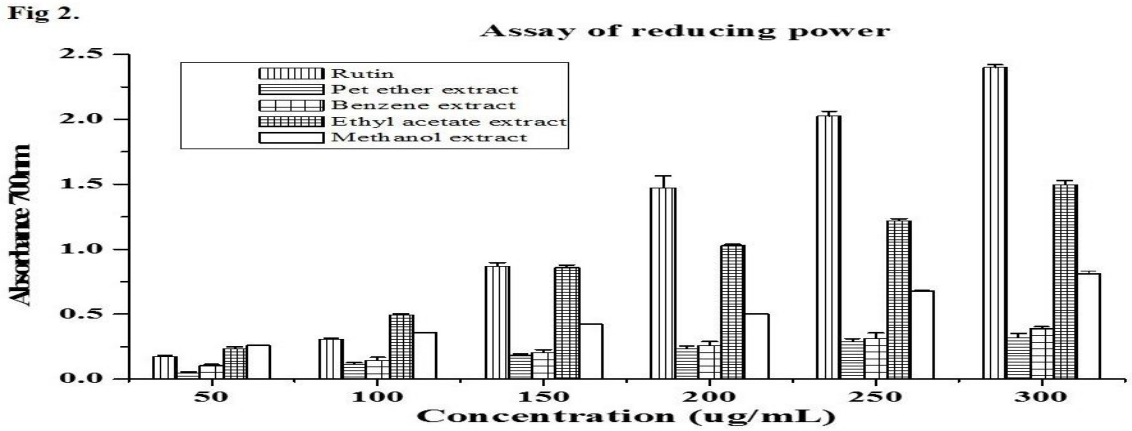

Figure 2: The assay of reducing power.

Fig. 2. The assay of reducing power of different extracts of *B. penicillata*. The results were the average of three determination ±SEM.

ABTS radical cation scavenging assay

In this study, different solvent extracts of *B. penicillata* stem were subjected to ABTS radical scavenging activity and the result have been shown in Figure 1.A. and this activity was compared to that of ascorbic acid. The ethyl acetate extract of stem registered better ABTS radical cation scavenging ability with low IC_{50} value (114.68 µg/mL) and followed by methanol extract (149.34 µg/mL) and result were presented in Table 2.

Reducing power assay

Reducing power activity was found to increase with the increase in concentration of the extract. In the present study, ethyl acetate extract has been found to be highest reducing power (1.50 at 700nm) when compared to other solvent performed (Figure 3). The reducing power ability of extracts have been shown in descending order (standard (rutin) > ethyl acetate > methanol > benzene > petroleum ether extracts.

Discussion

Since ancient times, human being are consume medicinal plants or natural products to treat their acute or chronic diseases. Some of these plants are have rich source with phytoconstituents such as polyphenols, flavonoids, tannins, vitamins, steroids, etc. possess different pharmacological actions like a natural antioxidants. Phenols and flavonoids have been recognised as free radical (or) active oxygen scavengers and can prevent damage caused by oxidative stress[26-27]. Many reports have suggested that the active compounds/molecules are effective in reducing risk & prevention of many acute and chronic diseases like cancer, cardiovascular, neurodegenerative and diabetic complications with their

antioxidant properties[28-29]. Phenolic compounds are vital plant constituents which are shown good antioxidant potential due to their ability of redox properties, hydrogen donors & singlet oxygen quenchers[30]. The antioxidant ability of flavonoids is due to their activity of prevention of radical formation and scavenge free radicals[31-32]. The antioxidant potential of this plant may be due to the existence of phenolic matters. The present study indicate that stems of *B. penicillata* are rich in phenols and flavonoids in polar solvent extracts such as methanol and ethyl acetate then in non-polar solvents like petroleum ether and benzene. The existence of high phenolic and flavonoid content in all solvent extracts of *B. penicillata* has donated directly to the antioxidant activity by counteracting the free radicals.

Yildirim *et al*. (2000) has described that the antioxidants were ability to protecting a biological system against the reactive oxygen species due to the variety mechanisms such as inhibition of chain initiation, free radical scavenging and reducing capacity[33]. Therefore, the antioxidant potential of medicinally important plant is find out by using the variety of antioxidant mechanisms were performed because no one method results may not reflect the accurate antioxidant ability[34-35]. DPPH assay is an easy and frequently used technique for testing *in vitro* antioxidant activity of regular compounds or plant extracts[36]. A number of methods available but these assay employing the stable 2, 2-diphenyl-1-picryl-hydrohyl radical (DPPH) at room temperature, purple in colour has expected the extreme attention owing to its ease of use and its conveniences. The reduction of colour capability to accept an electron or hydrogen radical from antioxidants is determined by measuring decrease in its absorbance value at 517 nm. DPPH assay postulated that the extracts of *B. penicillata* stem reduces the DPPH radical due to equivalent hydrazine when its counters with hydrogen donors in antioxidant principles and methanol extracts shown higher antioxidant potential then remaining of the plant extracts. Further evidence of the hydroxyl radical scavenging activity of extracts has been found in the deoxyribose system[37]. They were produced in this system by incubating ferric-EDTA with ascorbic acid and H_2O_2 at pH 7.4, and react with 2-deoxy-2-ribose to generate a melonoldehyde (MDA)-like product. This reaction mixture forms a pink chromogen upon heating with TBA at low pH. When the extracts of *B. penicillata* stem were added to the reaction combination, it detached the hydroxyl radicals from the sugar & prevented the reaction ultimately chromogen unicolor. Methanol extract shows a better hydroxyl radical scavenger then other extracts with their IC_{50} values shown in Table 2. The free radical scavenging ability of the stem extracts of *B. penicillata* have been tested through ABTS assay and results are presented in the (Figure 1.A). The summery of ABTS method is based on the inhibition of the absorbance of

radical cation, $ABTS^{+}$, which has a distinctive wave length at 734 nm, by antioxidants. The principle behind the procedure contains the reaction between ABTS and potassium per sulphate to produce the ABTS radical cation ($ABTS^{+}$) which is a blue green chromogen. In the attendance of antioxidant redactant, the coloured radical is converted back to colourless ABTS[38] and discolouration indicates the scavenging activity of the sample antioxidant such as phenolic compounds. In this study, the four extracts of *B. penicillata* stem were able to decolourise ABTS and free radical scavenging activity of the extracts have been found to be in the order of ethyl acetate extract > methanol > benzene > petroleum ether. The fact that the polyphenol compounds are good electron donors, they exhibit reducing power and have ability to reduce ferric ion to ferrous ion by donating electron may describe the importance of reducing power assay in determining the antioxidant property of plant extracts[39].In this study, the yellow colour of the test solution changes to various shades of green and blue, based on the reducing power of test sample. The existence of redactones, which have been exposed to be on significant antioxidant activity by breaking the free radical chain by donating hydrogen atom. The attendance of redactones (antioxidants) in the test sample might cause the redaction of Fe^{3+}/ferric cyanide complex to ferrous form which can be measured at 700 nm using spectrophotometer and the higher absorbance directs a greater reducing power[25]. The relative reducing power of different solvent extracts of *B. penicillata* stem from results are Rutin (standard) >ethyl acetate > benzene > petroleum ether and the reducing power of the extracts indicate that the plant having the biologically active compounds (or) rich source antioxidants.

Overall, the results concluded that among all tested sample, methanol extract contained the highest quantity of phenolic contents and flavonoids content found to be highest in ethyl acetate extract and these extracts exhibited significant antioxidant potential, as measured in DPPH, hydroxyl radical, ABTS and reducing power assays. In the present study clearly indicate that polar solvent extracts showed higher poly phenolic content and possess better antioxidant potential then non polar extracts like benzene and petroleum ether. So far we known this is the first report that examine the antioxidant activity of *B. penicillata* stem and this study clearly indicate this plant could be a good source of antioxidants. Further investigations are needed for exploit its antioxidant potential which could be of immense use in countering oxidative insult in biological systems.

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