

Radical Scavenging and Antioxidant Activity of Ethanolic Extract of *Mollugo nudicaulis* by *Invitro* Assays

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ABSTRACT

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In the present study, the ability of scavenging free radicals of the ethanolic extract of *Mollugo nudicaulis* was determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power, ferric reducing antioxidant power (FRAP), hydroxyl radical scavenging assay, superoxide radical scavenging (SOD), hydrogen peroxide radical scavenging assay, nitric oxide scavenging assay (NO) and total antioxidant capacity assay. The results showed that the ethanolic extract of *Mollugo nudicaulis* has a significant antioxidant activity. The amount of flavonoids, tannin, phenol, carotene and lycopene were also determined. The extract has the high amount of phenolic content. Thus, the study suggests that *Mollugo nudicaulis* has a better source of natural antioxidants, which might be helpful in preventing the progress of oxidative stress.

Keywords: Free radical scavenging, antioxidant, *Mollugo nudicaulis*, oxidative stress.

INTRODUCTION

Reactive oxygen species (ROS) generated in cells, are fundamental in modulating various physiological functions and represent an essential part of aerobic life and metabolism. Excessive generation of these radicals disrupts the antioxidant defense system of the body which may lead to oxidative stress¹. Oxidative stress plays a major part in the development of chronic and degenerative ailments such as cancer, autoimmune disorders, rheumatoid arthritis, aging, cardiovascular and neurodegenerative diseases².

Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection against infections and degenerative diseases. They can either directly scavenge or prevent generation of ROS³. The two most commonly used synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have begun to be restricted because of their toxicity and DNA damage induction⁴. The plant species have been investigated in the search for novel antioxidants, but generally there is still a demand to find more information concerning the antioxidant potential of plant species as they are safe and also bioactive. Therefore in recent years, considerable attention has been directed towards the identification of plants with antioxidant activity⁵.

Mollugo nudicaulis Lam (Molluginaceae) used in Indian phytotherapy for the treatment of inflammation, jaundice, urinary and kidney disorders⁶. *Mollugo nudicaulis* were also used along with other medicinal plants to treat wounds, cold, cough, fever and body pain⁷. Hence, the present study was aimed at evaluating the free radical scavenging activity of the ethanolic extract of *Mollugo nudicaulis*.

MATERIALS AND METHODS

Plant material and extraction:

Fresh plant materials were collected in the month of January 2009 from the rural areas of Coimbatore, Tamilnadu, India. The plant was authenticated by Dr. G.V.S. Murthy, Botanical Survey of India, Tamilnadu Agricultural University Campus, Coimbatore with the voucher number BSI/SRC/5/23/10-11/Tech 420. The plant materials were washed under running water, cut into pieces, air dried and pulverized into fine powder in a grinding machine. 100 g of the powder was then extracted with 500 ml of ethanol, filtered, squeezed off and evaporated under reduced pressure in a rotary evaporator to obtain crude extract.

Free radical scavenging activity:

DPPH radical scavenging assay:

The free-radical scavenging activities of these compounds were tested by their ability to bleach the stable radical DPPH. The antioxidant activity using the DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay was assessed by the method of Blois⁸. The reaction mixture contained 100 µM DPPH in methanol

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and different concentrations (0.5-2.5 µg/ml) of compounds. Absorbance at 517 nm was determined after 30 min at room temperature and the scavenging activity were calculated as a percentage of the radical reduction. Each experiment was performed in triplicate. BHT was used as a reference compound.

Reducing power assay:

The reducing power of extract was determined by the method of Yen and Duh⁹. Different concentrations of extracts (0.5-2.5 µg/ml) were mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1 % potassium ferricyanide. The mixtures were incubated at 50°C for 20 min. After incubation, 2.5 ml of 10% trichloroacetic acid were added to the mixtures, followed by centrifugation for 10 min. The upper layer (5 ml) was mixed with 5 ml of distilled water and 1 ml of 0.1 % ferric chloride and the absorbance of the resultant solution were measured at 700 nm.

Ferric Reducing Antioxidant Power (FRAP) assay:

The FRAP procedure described by Benzie and Strain¹⁰ was followed. Briefly, the FRAP reagent contained 5 ml of a (10 mmol/L) TPTZ (2, 4, 6- tripyridyl- S- triazine) solution in 40 mmol/L HCL plus 5 ml of FeCl₃ (20 mmol/L) and 50 ml of acetate buffer, (0.3 mol/L, pH=3.6) and was prepared freshly and warmed at 37°C. The sample extracts (0.5-2.5 µg/ml) were mixed with 3 ml FRAP reagent and the absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37°C for 10 min.

Hydroxyl radical scavenging assay:

The reaction mixture (3 ml) containing 1 ml FeSO₄ (1.5 mM), 0.7 ml hydrogen peroxide (6 mM), 0.3 ml sodium salicylate (20 mM) and varying concentrations of the extracts (0.5-2.5 µg/ml) were taken. After incubation for 1h at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm¹¹. Ascorbic acid was used as the standard.

Superoxide radical scavenging assay:

The measurement of superoxide scavenging activity is based on the method as described by Liu¹² and is assayed by the reduction of nitroblue tetrazolium (NBT). Tris HCl buffer (3 ml, 16 mM, pH 8.0) containing 1 ml NBT (50 µM) solution, 1 ml NADH (78 µM) solution and a sample solution of extract (0.5-2.5 µg/ml) in water were mixed. The reaction was started when 1 ml of phenazine methosulfate (PMS) solution (10 µM) was added to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance was read at 560 nm against the corresponding blank samples. Quercetin was used as a reference drug. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. Ascorbic acid was used as the standard.

Hydrogen peroxide radical scavenging assay:

The ability of the extracts to scavenge hydrogen peroxide (H₂O₂) was determined according to the method of Nabavi¹⁴. A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer, pH 7.4. The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Extracts (0.5-2.5 µg/ml) in distilled water were added to a hydrogen peroxide solution at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as the standard.

Nitric oxide scavenging assay:

The interaction of ethanol extract of *Mollugo nudicaulis* with nitric oxide (NO) was assessed by the nitrite detection method¹⁵. The chemical source of NO was sodium nitroprusside (10 mm) in 0.5 m phosphate buffer, pH 7.4, which spontaneously produced NO in an aqueous solution. NO interacted with oxygen to produce stable products, leading to the production of nitrites. After incubation for 60 min at 37°C, Griess reagent (a-naphthyl-ethylenediamine 0.1% in water and sulphanilic acid 1% in H₃ PO₄ 5%) was added. The same reaction mixture without the extract of sample but with equivalent amount of distilled water served as control. Ascorbic acid was used as positive control.

Total antioxidant capacity assay:

The total antioxidant capacity assay was determined as described by Prieto et al¹⁶

Different concentrations of the extract (0.5-2.5 µg/ml) were taken and added 1.0 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid or gallic acid¹⁶.

Bioactive compounds

Flavonoid content:

This was assayed as described by Jia et al¹⁷. 0.5 ml of the sample is added into a test tube containing 1.25 ml of distilled water. Then added 0.075 ml of 5 % sodium nitrite solution and allowed to stand for 5 min. Added 0.15 ml of 10 % aluminium chloride, after 6 min 0.5 ml of 1.0 M sodium hydroxide were added and the mixture were diluted with another 0.275 ml of distilled water. The absorbance of the mixture at 510 nm was measured immediately. The flavonoid content was expressed as mg catechin equivalents/g sample¹⁷.

Total phenolic content:

Total phenolic content was determined by the method described by Singleton and Rossi¹⁸. 1.0 ml of sample was mixed with 1.0 ml of Folin and Ciocalteu's phenol reagent. After 3 min, 1.0 ml of saturated Na_2CO_3 (~35 %) was added to the mixture and made up to 10 ml by adding distilled water. The reaction was kept in the dark for 90 min, after which its absorbance were read at 725 nm. A calibration curve was constructed with different concentrations of catechol (0.01-0.1 mM) as standard. The results were expressed as mg of catechol equivalents/g of extract.

Tannin content:

The quantitative tannin content in samples was estimated by the method of Price and Butler¹⁹ with some modifications. 0.1 g of dry plant sample was transferred to 100 ml flask, 50 ml water was added and boiled for 30 min. After filtration with cotton filter the solution was further transferred to a 500 ml flask and water was added to 500 ml mark. 0.5 ml aliquots were finally transferred to vials, 1 ml 1% $\text{K}_3\text{Fe}(\text{CN})_6$ and 1 ml 1% FeCl_3 were added, and was made up to 10 ml with distilled water. After 5 min, the solutions were measured spectrophotometrically at 720 nm.

β -Carotene and Lycopene content:

β -Carotene and lycopene were determined by the method of Nagata and Yamashita²⁰. The dried extract (100 mg) was vigorously shaken with 10 ml of acetone-hexane mixture (4:6) for 1 min and filtered through Whatmann No.4 filter paper. The absorbance of the filtrate was measured at 453, 505, 645 and 663 nm and calculated. The assays were carried out in triplicates, the results were mean \pm SD and expressed as mg of carotenoid / g of extract.

Statistical analysis:

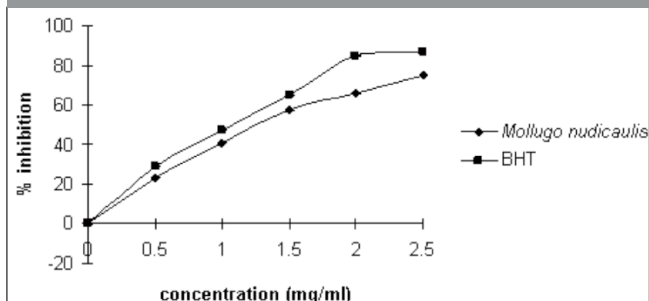
Results were expressed as mean \pm SD. The statistical comparison among the groups were performed with one way ANOVA test using a statistical package program (SPSS 10.0) at $p < 0.05$ significant level.

RESULTS

DPPH radical scavenging activity of ethanolic extract of *Mollugo nudicaulis* was compared with BHT. It was observed that the plant extract had higher scavenging activity. At a concentration of 2.5 mg/ml, the scavenging activity of ethanolic extract of *Mollugo nudicaulis* reached 74.96 %, while at the same concentration; the standard was 87 % (Fig.1.)

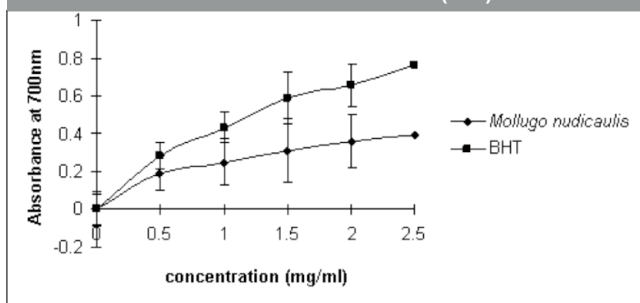
The reductive capabilities of the *Mollugo nudicaulis* were compared to BHT. The reducing power of the plant extract

Fig. 1: DPPH Scavenging of ethanolic extract of *Mollugo nudicaulis* compared to that of Butylated hydroxytoluene (BHT). Each value is expressed as mean \pm standard deviation (n=3).



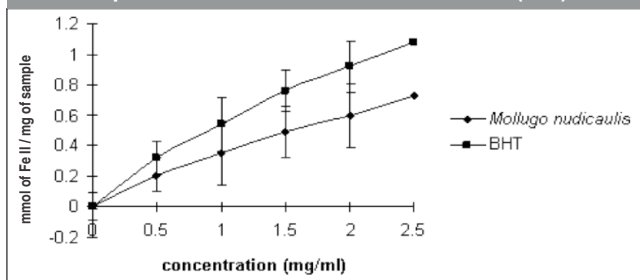
was observed to be 45 % at a concentration of 2.5 mg/ml, whereas the reducing power of the standard BHT was found to be 80 % (Fig.2).

Fig 2: Reducing power of ethanolic extract of *Mollugo nudicaulis* compared to that of Butylated hydroxytoluene (BHT). Each value is expressed as mean \pm standard deviation (n=3).



The ethanolic extract of *Mollugo nudicaulis* exhibited a lower FRAP radical scavenging activity than BHT and the scavenging potency is found to be 40 % and 55 % respectively (Fig.3).

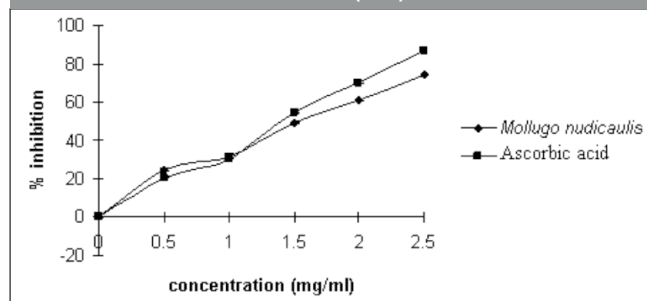
Fig 3: Ferric reducing antioxidant power of ethanolic extract of *Mollugo nudicaulis* compared to that of Butylated hydroxytoluene (BHT). Each value is expressed as mean \pm standard deviation (n=3)



The ethanolic extract of *Mollugo nudicaulis* showed a significant dose-dependent hydroxyl radical scavenging activity and it reached up to 73.84 % at the concentration of

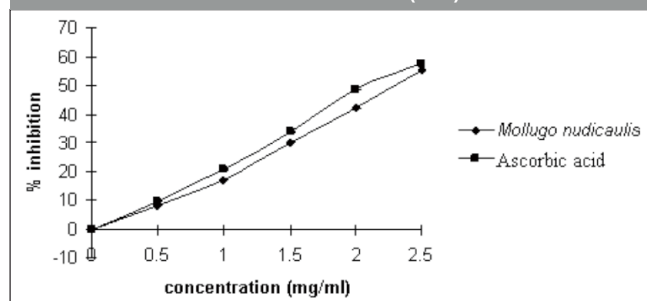
2.5 mg/ml. However, vitamin C which was used as a positive control showed better radical scavenging effect (90 % at the concentration of 2.5 mg/ml) (Fig.4).

Fig 4: Hydroxyl radical scavenging of ethanolic extract of *Mollugo nudicaulis* compared to that of Ascorbic acid (Vit C). Each value is expressed as mean ± standard deviation (n=3).



The ethanolic extract of *Mollugo nudicaulis* scavenges the superoxide radicals up to 58 % at 2.5 mg/ml concentration, whereas standard ascorbic acid at the same concentration scavenged 60 %. The abilities of the plant extract and ascorbic acid to quench superoxide radicals from reaction mixture is reflected in the decrease of the absorbance (Fig.5).

Fig 5: Super oxide anion scavenging of ethanolic extract of *Mollugo nudicaulis* compared to that of Ascorbic acid (Vit C). Each value is expressed as mean ± standard deviation (n=3).



The free radical scavenging activity of *Mollugo nudicaulis* was evaluated by hydrogen peroxide (H₂O₂) scavenging method. From the results, *Mollugo nudicaulis* showed concentration dependent activity and the H₂O₂ scavenging effect was 55.69 % at a concentration of 2.5 mg/ml. This was comparable to the scavenging effect of ascorbic acid (60%) (Fig.6).

A 76% of nitric oxide free radical scavenging activity is due to the scavenging ability of *Mollugo nudicaulis* at 2.5 mg/ml was evident, whereas the standard ascorbic acid exhibited 80% inhibition of activity at the same concentration. (Fig.7).

Fig 6: Hydrogen peroxide radical scavenging of ethanolic extract of *Mollugo nudicaulis* compared to that of Ascorbic acid (Vit C). Each value is expressed as mean ± standard deviation (n=3).

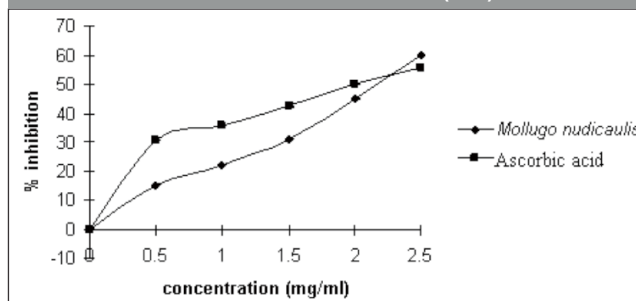


Fig 7: Nitric oxide scavenging of ethanolic extract of *Mollugo nudicaulis* compared to that of Ascorbic acid (Vit C). Each value is expressed as mean ± standard deviation (n=3).

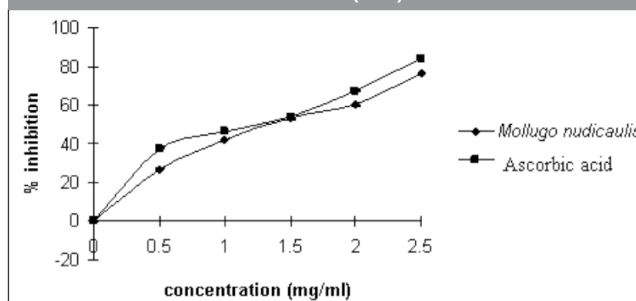


Table 1: IC₅₀ values of the free radical scavenging activities of the ethanol extract of *Mollugo nudicaulis*

| Parameters | IC ₅₀ Value (mg/ml) | | |
|--|--------------------------------|-----|-------|
| | <i>Mollugo nudicaulis</i> | BHT | Vit C |
| DPPH scavenging assay | 1.35 | 1.1 | – |
| Hydroxyl radical scavenging assay | 1.6 | – | 1.3 |
| Superoxide radical scavenging assay | 2.15 | – | 2.1 |
| Hydrogen peroxide radical scavenging assay | 2.25 | – | 2.0 |
| Nitric oxide radical scavenging assay | 1.35 | – | 1.4 |

Total antioxidant capacity of the extract is expressed as the number of equivalents of ascorbic acid. The total antioxidant capacity of *Mollugo nudicaulis* was found to be 194.87 ± 0.221 (Table 2).

Table 2: Total antioxidant capacity of ethanolic extract of *Mollugo nudicaulis*

| Solvent | <i>Mollugo nudicaulis</i> (nM/g Ascorbic acid) |
|---------|--|
| Ethanol | 194.87 ± 0.221 |

Values are expressed as mean ± SD (n=3).

Total flavonoid, total phenols, tannins, total carotenoids and lycopene contents of *Mollugo nudicaulis* were demonstrated (Table 3). Total flavonoid content of *Mollugo nudicaulis* was found to be 10.43 ± 0.151 . The contents of total phenols, tannins, total carotenoids, lycopenes were found to be 66.07 ± 0.014 , 1.51 ± 0.245 , 12.2 ± 0.147 and 16.32 ± 0.078 respectively.

Table 3: Bioactive compounds of ethanolic extract of *Mollugo nudicaulis*

| Parameters | Solvent |
|--|-------------------------------|
| | Ethanol |
| Total Flavonoid (mg/g) ^a | 10.43 ± 0.151 |
| Total Phenols (mg/g) ^b | 66.07 ± 0.014 |
| Tannins (mg/g) ^a | 1.51 ± 0.245 |
| Total Carotenoids (mg/g) | 12.2 ± 0.147 |
| Lycopene (mg/100g) | 16.32 ± 0.078 |
| Values are expressed as mean \pm SD (n = 3). | |
| ^a mg of catechin/g | ^b mg of catechol/g |

DISCUSSION

The present study investigated the antioxidant activity of the ethanolic extract of *Mollugo nudicaulis* in different *in vitro* model of screening. ROS has received considerable attention in the recent past, because of its role in several pathological conditions. ROS produced *in vivo* include superoxide radical O_2^- , hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl)²¹.

DPPH radical is considered to be a model of lipophilic radical. In this mode, scavenging activity is attributed to hydrogen donating ability of antioxidants²². Although ethanolic extract of *Mollugo nudicaulis* possess good DPPH scavenging activity, it was evident that the extract could serve as free radical inhibitors or scavengers.

A reducing power is an indicative of reducing agent having the availability of atoms which can donate electron and react with free radicals and then convert them into more stable metabolites and terminate the radical chain reaction²³. Accordingly, *Mollugo nudicaulis* might contain a sizable amount of reductants which may react with the free radicals to stabilize and terminate from free radical chain reaction.

FRAP assay, non-enzymatic antioxidants react with pro-oxidants and inactive them. In a redox reaction, antioxidants act as 'reductants'. In this context, the antioxidant power can be referred to as 'reducing ability'. In this FRAP assay, an easily reducible oxidant, Fe III is used in excess. Thus there is a reduction of Fe III-TPTZ complex by antioxidant²⁴. The decrease in the concentration of FRAP is a measure of the antioxidant activity of *Mollugo nudicaulis*.

Hydroxyl radical are the major active oxygen causing lipid peroxidation in enormous biological damage. The highly reactive hydroxyl radical can cause oxidative damage to DNA, lipid and protein²⁵. In this study, *Mollugo nudicaulis* was found to scavenge O_2^- significantly and in dose dependent manner and may protect the DNA, protein and lipid from damage.

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated after oxygen is taken into living cells. Superoxide anion changes to other harmful ROS and free radicals such as hydrogen peroxide and hydroxyl radical, which induce oxidative damage²⁶.

Hydrogen peroxide is a weak oxidizing agent and it is not very reactive, can cross biological membranes. Because of the possible involvement of hydrogen peroxide in the generation of hydroxyl radicals, this property places hydrogen peroxide in a more prominent role to initiate cytotoxicity than its chemical reactivity. Thus removing H_2O_2 is very important for the protection of living systems²⁷. *Mollugo nudicaulis* scavenged hydrogen peroxide which may be attributed the presence of phenolic groups that could donate electrons to hydrogen peroxidase, thereby neutralizing it into water.

Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological process including neurotransmission, vascular homeostasis, antimicrobial and antitumor activities. However, excess production of NO is associated with several diseases. It would be interesting to develop potent and selective inhibitors of NO for potential therapeutic use²⁸. In the present study, *Mollugo nudicaulis* exhibited potent nitric oxide radical scavenging activity, which competes with oxygen to react with nitric oxide and thus inhibits the generation of nitrite.

Antioxidant activity of plant is the most efficient way of combating tissue injuries undesired transformations and preventing health risks²⁹. Total antioxidant capacity of ethanolic extract of *Mollugo nudicaulis* may serve as a significant indicator of its potential antioxidant activity.

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides³⁰. Flavonoids and tannins seem to be a most promising polyphenolic compounds³¹. Carotenoids like β -carotene and lycopene present in plants exert antioxidant functions such as quenching of singlet oxygen and other electronically excited molecules and progression of many degenerative diseases³². The results suggest that the ethanolic extract of *Mollugo*

nudicaulis showed higher antioxidant activity due to the presence of phenols, flavonoids, tannins, carotenoid and lycopene.

CONCLUSION

This study suggests that the ethanolic extract of *Mollugo nudicaulis* possess high free radical scavenging activity and phytochemical constituent which might be useful for further studies to unravel novel treatment strategies for diseases associated with free radical induced tissue damage.

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