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Comparative Anti-Inflammatory and Anti-Oxidant Evaluation of *Jotropha gossypifolia* and *Croton bonplandianum*

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ABSTRACT:

The present investigation was undertaken to appraise the antioxidant and anti-inflammatory properties of *Jotropha gossypifolia* and *Croton bonplandianum* by using both *in-vitro* and *in-vivo* methods. The extracts of the leaves were prepared with the hydro-alcoholic solution (Methanol: Water = 70:30). Radical attenuating capacity of extracts was ascertained by 2, 2- diphenyl 1-picryl hydrazyl (DPPH) radical scavenging assay. The total phenolic contents of extracts were also determined by Folin–Ciocalteumethod. The *in-vivo*antioxidant potential of extracts was evaluated against CCl_4 induced toxicity in rats.The prevention of hypotonicity induced human red blood cells (HRBC) membrane lysis and protein inhibition was taken as a measure of the *In-vitro* anti-inflammatory activity where as in-*vivo*study was carried out by using carrageenan induced paw edema in rats.The *Croton bonplandianum* extract shows more anti-inflammatory activity which was also found to show more antioxidant activity.

KEYWORDS: Anti-inflammatory, Antioxidant, *Jotrophagossypifolia,Croton bonplandianum*

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INTRODUCTION:

The herbal medicine is gaining wide currency and acceptability and the documentation of valuable indigenous knowledge about medicinal plant species is assuming urgent priority due to the recent controversies of illegal Biopyracy[1]. Even though the development in the allopathic medicine also very rapid the side effects associated with these synthetic drug molecules changing the focus of the patient back to the herbal remedies. So, there is an urge in finding new molecules hidden in the herbs for development of safer medicines.

The presence of free transition metals in the biological system leads to excessive generation of free radicals [2].However when the natural antioxidant enzymes are not sufficient to scavenge these active free radicals, then their unusual longer persistence in the cell, causes peroxidation of cellular lipids and proteins, which results to damage of cell organelles. Further these oxidized macromolecules behave as foreign proteins and affect the immune system. They may activate the inflammatory cascade, resulting in initiation of various degenerative diseases and autoimmune disorders [3].Free radicals have been implicated in the causation of several problems like atherosclerosis, urolithiasis, ulcers, asthma, cancer, cardiovascular disease, cataract, diabetes, gastrointestinal inflammatory disease, liver disease, muscular degeneration and other inflammatory process[4].It was reported that some active oxygen species produced in the Fenton reaction or Harber- Weiss reaction, such as super oxide anion radicals and hydrogen peroxide, play important roles in the inflammatory process [5]. Age related chronic diseases are associated with generation of excessive free radicals and associated with inflammation. Therefore, search for potent antioxidants with anti-inflammatory potential has always been in demand [6].

The plant *Jatrophagossypifolia*(family: *Euphorbiaceae*) is a bushy gregarious shrub, grows wildly almost throughout India. It possesses significant anticancer, hepatoprotective and pesticidal activities [7, 8, 9]. Various preparations of *Jatrophagossypifolia*is used for bathing wounds, bleeding, itching of cuts and also in the management of leprosy, snakebite and in urinary complaints[10-14]. *Croton bonplandianum*, (family: *Euphorbiaceae*) is a perennialherb and can be found in waste lands and roadside areas. Flowering and fruiting time of this plant isSeptember to December. The part which hasmedicinal value is seed and seed oil. The seeds areused for the treatment of jaundice, acuteconstipation, abdominal dropsy and internalabscesses[15].Leaves are used for controlling hypertension, treatment of skin diseases, wounds and also as antiseptic and antidote [16].

Present study was aimed to evaluate the antioxidant and anti-inflammatory activities of *Jotrophagossypifolia*and *Croton bonplandianum*both by *in-vitro* and *in-vivo* methods.

MATERIALS AND METHODS:

Chemicals:

1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) and Carrageenanwas obtained from Sigma Aldrich Co., St. Louis, USA.All other chemicals used were of analytical grade obtained from E. Merck and HIMEDIA, Mumbai, India.

Animals:

Adult Wistar Albino rats of either sex (150-200g) were used for the present investigations. Animals were housed under standard environmental conditions at temperature (25±2^{0}C) and light and dark (12:12 h). They were acclimated to laboratory conditions for seven days before commencement of experiments, and were allowed free access to standard dry pellet diet and water ad libitum. The experimental protocol was approved by the IAEC, for using animals in present study (1633/PO/a/12/CPCSEA). Animals were fasted overnight with free access to water prior to each experiment.

Plant Materials and Extraction:

Leaves of both plants were collected from local areas of Kakinada, Andhra Pradesh, India. The plants are authenticated by Botanical Survey of India, Hyderabad (BSI/DRC/2013-14/Tech./522) and (BSI/DRC/2013-14/Tech./275). The plant leaves were shade dried at room temperature of (32±2) °C and the dried leaves were ground into fine powder using pulverizer. The powdered part was sieved and kept in deep freezer until the time of use. One hundred grams of dry powder was suspended in 500 mL of hydro-alcoholic (Methanol: Water = 70:30) solution for 72 h. The extract was filtered using a muslin cloth and concentrated at (40±5) °C.

Acute Toxicity Studies:

Acute oral toxicity was performed by following OECD-423 guidelines (acute toxic class method), albino rats (n=6) of either sex selected by random sampling were used for acute toxicity study. The animals were kept fasting for overnight and provided only with water, after which the extracts were administrated orally at 5mg/kg body weight by gastric incubations and observed for 14 days. Mortality was not observed.

***IN-VITRO* ANTI OXIDANT ACTIVITY:**

DPPH *Radical Scavenging Assay*[17]*:*

DPPH (2, 2-diphenyl picrylhydrazyl) is a commercially available stable free radical, which is purple in colour. The antioxidant molecules present in the herbal extracts, when incubated, react with DPPH and convert it into di-phenyl hydrazine, which is yellow in color. The degree of discoloration of purple to yellow was measured at 517 nm, which is a measure of scavenging potential of plant extracts. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extracts prepared in methanol containing 0.025 - 0.5 mg of the plant extracts and standard drugs separately (ButylatedHydroxyToluene). The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The ability of the plant extract to scavenge DPPH radical was calculated by the equation:

% Inhibition of DPPH radical = $Abs_{Control} - Abs_{Test} / Abs_{Control} \times 100$

Where; $Abs_{control}$ is the absorbance of DPPH radical + methanol; Abs_{Test} is the absorbance of DPPH radical + sample extract or standard.

Determination of Total Phenolics[18]*:*

Phenols react with phosphomolybdic acid in Folin-ciocalteau reagent in alkaline medium and produce a blue colored complex (molybdenum blue) that can be estimated colorimetrically at 650 nm. Weighed exactly 1.0 g of the plant sample and ground it with a pestle and mortar in 10ml of 80% ethanol. Centrifuge the homogenate at 10000 rpm for 20 minutes. Save the supernatant. Re-extracted the residue with five times the volume 80% ethanol, centrifuged and pool the supernatants. The supernatant was evaporated to dryness. Dissolve the residue in a known volume of distilled water. Different aliquots (0.2 to 2 ml) were pipetted out into test tubes. The volume in each tube was made up to 3.0 ml with water. 0.5 ml of Folin-Ciocalteau reagent was added. After 3 minutes, added 2.0 ml of 20% sodium carbonate solution to each tube. Thoroughly mixed and placed the tubes in a boiling water bath for exactly 1 minute, cooled and measured the absorbance at 650nm against reagent blank.

In-Vivo Anti Oxidant Activity[19]*:*

Group-I animals served as normal control, treated with vehicle (gum acacia 3% solution). Group-II animals served as toxic control, treated with CCl_4 in a single dose of 1.5 ml/kg, i.p., to produce acute toxicity. Group III served as a standard group, and was administered Liv-52 in a dose of

56 mg/kg, p.o. Group-IV, V animals were treated with *Jotrophagossypifolia*extract at a daily doses of 200 and 400mg/kg p.o respectively and Group-VI, VII animals were treated with *Croton bonplandianum*extract at a daily doses of 200 and 400mg/kg, p.o., respectivelyfor 7 days. The animals of Groups III-VII were given single dose of CCl_4, 1.5 ml/kg, i.p., 6 h after the last treatment. On day 8 the rats were sacrificed by carotid bleeding and liver was rapidly excised, rinsed in ice-cold saline, and a 10% w/v homogenate was prepared using 0.15M KCI, centrifuged at 800 rpm for 10 min at 4 °C. The supernatant obtained was used for the estimation of catalase, peroxidase, and other enzymes. Further, the homogenate was centrifuged at 1000 rpm for 20 min at 4 °C and the supernatant was used for biochemical estimation.

BIOCHEMICAL ESTIMATION:

Estimation of Glutathione:

Glutathione was estimated using Ellman's reagent (5,5- dithiobis-(2-nitrobenzoic acid) [DTNB]). The sulphydryl groups present in glutathione forms a colored complex with DTNB, which was measured by colorimeter at 412 nm. The amount of glutathione was determined using its molar extinction coefficient of 13600/m/cm and expressed in terms of µmol/mg of protein.

Estimation of Sod:

Estimation of SOD was done by auto oxidation of hydroxylamine at pH 10.2, which was accompanied by reduction of NBT, and the nitrite produced in the presence of EDTA was detected colorimetrically. One enzymatic unit of SOD is the amount in the form of proteins present in 100 ml of 10% liver homogenate required to inhibit the reduction of 24 mM NBT by 50% and is expressed as units per milligram of protein.

Estimation of Catalase:

Catalase activity was estimated by determining the decomposition of H_2O_2 at 240 nm in an assay mixture containing phosphate buffer. One international unit of catalase utilized is that amount that catalyzes the decomposition of 1 mM H_2O_2/min/mg of protein at 37°C. Catalase activity was calculated using the millimolar extinction coefficient of 0.07 and expressed in terms of micromole per minute per milligram of protein.

Estimation of Peroxidase:

Peroxidase estimation is based on periodide formation.Periodide can be spectrophotometrically determined at353 nm, and this is directly proportional to the peroxidaseconcentration in the reaction

mixture containingapproximate amounts of H_2O_2 and enzyme. One unitof peroxidase activity is defined as the change in absorbance per minute and expressed in terms of units per milligram of protein.

IN-VITRO ANTI INFLAMMATORY ACTIVITY[20]:

The human red blood cell membrane stabilization method (HRBC) has been used as a method to study the *invitro* anti-inflammatory activity. Blood was collected from healthy human volunteer who was not taken any anti-inflammatory drugs for two weeks prior to the experiment. The collected blood was mixed with equal volume of sterilisedAlsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% NaCl in water) and centrifuged at 3,000 rpm. The packed cells were washed with isosaline (0.85%, pH 7.2) and a 10% (v/v) suspension was made with isosaline. Various concentrations of extracts were prepared (100, 200and 400mg/ml) using distilled water and to each concentration 1 ml of phosphate buffer (0.15M, pH 7.4), 2 ml of hyposaline (0.36%) and 0.5 ml of HRBC suspension were added. It is incubated at 37°C for 30 min and centrifuged at 3,000 rpm for 20 min. The hemoglobin content in the supernatant solution was estimated spectrophotometrically at 560 nm.

Diclofenac sodium (5 mg/ml) was used as reference standard and a control (distilled water) was prepared omitting the extracts. The percentage hemolysis was calculated by assuming the hemolysis produced in presence of distilled water of as 100%. The percentage of HRBC membrane stabilization or hemolysis was calculated using the formula:

% Inhibition of haemolysis = 100 x [Absorbance of control – Absorbance of test/ Absorbance of control

IN-VIVO ANTI INFLAMMATORY ACTIVITY[21]:

The albino rats of either sex were divided into six groups of six animals each. Group- I received 5 ml/kg normal saline p.o.serves as a control group, Group- II, III received 100, 200 mg/kg body weight of extracts of *Jatrophagossypifolia*p.o., and Group- IV, V received 100, 200 mg/kg body weight of extracts of *Croton bonplandianum*p.o., Group- VI received 5 mg/kg of body weight of Diclofenac sodiumintraperitoneallytaken as a standard. After one hour of the administration of the drugs, acute inflammation was produced by the subplantar administration of 0.1 ml of 1 % (w/v) of freshly prepared suspension of λ-carrageenan in the right hind paw of each rat.

The paw volume of the rats were measured in the digital plethysmograph (Ugo basile, Italy), at the end of 60min., 120min., 180min. and 240min. The percentage increase in paw edema of the treated groups was compared with that of the control and the inhibitory effect of the drugs was studied. The

relative potency of the drugs under investigation was calculated based upon the percentage inhibition of the inflammation. Percentage inhibition was calculated using the formula,

Percentage of inhibition = 100 (1-Vt / Vc)

Where, Vc is Edema volume in control and Vt is Edema volume in test / standard compound.

STATISTICAL ANALYSIS:

The results obtained from all the above screening methods were analyzed by analysis of variance (ANOVA) and Student's t-test for significant difference between the grouped means at 95% confidence level ($p< 0.05$).

RESULTS:

Acute toxicity studies revealed the non-toxic nature of the hydroalcoholic plant extracts up to a dose level of 2000 mg/kg body weight in rats and there was no toxic reaction found.

The scavenging ability of both plant extracts on DPPH is shown in Figure 1 and compared with that of BHT. The scavenging effect of extracts and standard on the DPPH radical was expressed as percentage inhibition.It was observed that the *Jotrophagossypifolia*and *Croton bonplandianum*extracts has DPPH scavenging activity with IC_{50} values383.21µg/ml and 416.82µg/mlrespectively. *Croton bonplandianum* extract possessed higher phenolic content compared to *Jotrophagossypifolia*extract. This shows that there exists a good correlation between phenolic contents and the antioxidant activities in the plant.

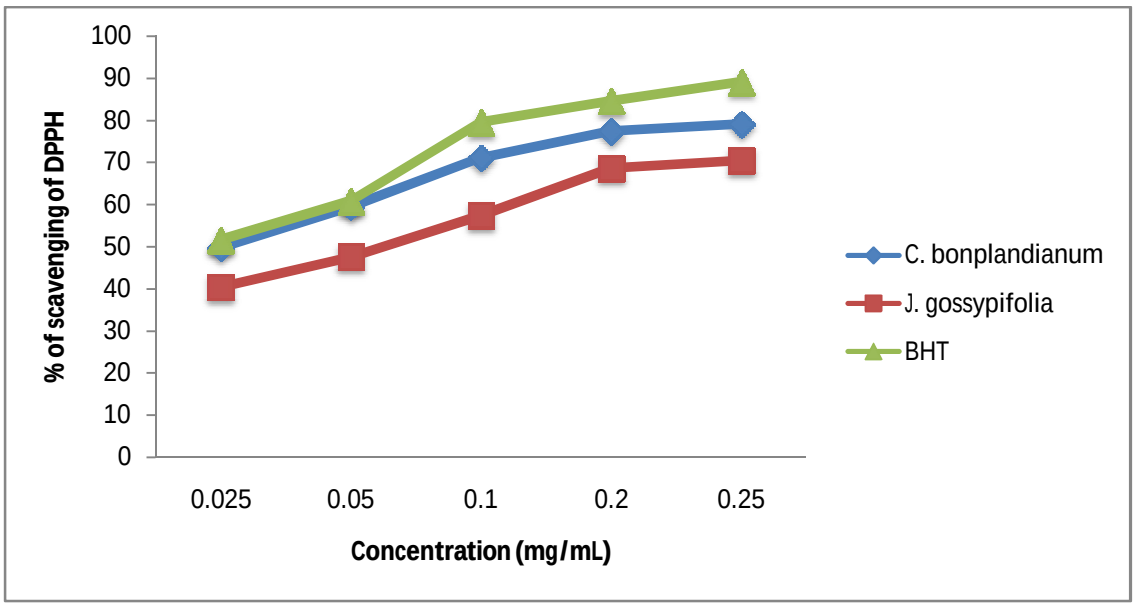

Figure 1: DPPH radical scavenging activity of plant extracts

The result showed that the activities of glutathione, SOD, catalase and peroxidase in group treated with CCl_4 declined significantly than that of normal group. Co-administration of plant extracts of at a dose of 200 and 400 mg/kg for 7 days markedly prevented these CCl_4 induced alteration and maintained enzymes level near to normal values (Table-1). Standard (Liv 52) treated group also significantly increased the level of glutathione, SOD, Catalase and peroxidase in CCL_4 induced toxic rats.

Table 1 Effect of plant extracts on biochemical parameters

Treatment	**Glutathione (µM/ mg of protein)**	**SOD (Units /mg of Protein)**	**Catalase (uM/min/mg of protein)**	**Peroxidase (Units /mg of Protein)**
Normal control (Vehicle treated)	20.4±0.09*	38±0.02*	13.2±0.02*	80±0.09*
Hepatotoxic Control (CCl_4 Treated)	10.3±0.76	25±0.06	1.8±0.16	11±0.02
Standard Liv 52	18.5±0.09*	37±0.08*	9.6±0.08*	69±0.03*
Croton bonplandianum (200 mg/kg)	13.6±0.08*	31±0.04	4.2±0.06	46±0.13
Croton bonplandianum (400 mg/kg)	15.0±0.06*	35±0.03*	8.5±0.10*	61±0.06*
Jotrophagossypifolia (200 mg/kg)	11.2±0.03*	28±0.05	2.4±0.08	37±0.05
Jotrophagossypifolia (400 mg/kg)	13.2±0.16*	32±0.01*	5.1±0.32*	54±0.02*

n= 6, Values are expressed as Mean ± SEM, *$P < 0.05$.

Plant extracts atdifferent concentrations (100, 200 and 400 mg/mL) showedsignificant stabilization towards HRBC membranes. Thepercentage protection of *Croton bonplandianum*extract at different concentrations shows higher activity than that of*Jotrophagossypifolia.* The results are tabulated in Table-2.

Table 2: In vitro anti-inflammatory activity of plant extracts

Extract/ Drug	Concentration (mg/ml)	Percentage protection
Croton bonplandianum	100	25.16
	200	33.41
	400	37.55
Jotrophagossypifolia	100	21.84
	200	30.07
	400	34.26
Diclofenac sodium	5	38.17

The characteristic swelling of the paw that occurs in the rat paw model of inflammation is due to edema formation.In the present study, both the plant extracts produced significant inhibition of carrageenan induced rat paw oedema after a period of 4 h (Table 3). This indicates the two test extracts were active in both the early and late phases of carrageenan induced acute hind paw inflammation in rats. The *Croton bonplandianum* extract was found to be more active than the *Jotrophagossypifolia* extract (Figure 2).

Table 3: Effect of plant extracts against carrageenan induced rat paw oedema.

Drug	Dose (mg/ kg)	Carrageenan induced oedema (Volume in mL)				Percent inhibition
		60 min	120 min	180 min	240 min	
Control		0.38±0.19	0.40±0.07	0.45±0.15	0.46±0.09	--
Diclofenac sodium	5	0.17±0.31*	0.15±0.06*	0.13±0.26*	0.10±0.64*	78.26
Croton bonplandianum	100	0.27±0.66*	0.24±0.71*	0.21±0.16*	0.17±0.33*	63.04
	200	0.18±0.45*	0.16±0.13*	0.14±0.21*	0.11±0.71*	76.09
Jotrophagossypifolia	100	0.28±0.10*	0.26±0.17*	0.24±0.33*	0.21±0.09*	54.35
	200	0.20±0.18*	0.19±0.33*	0.17±0.08*	0.16±0.05*	65.22

n= 6, Values are expressed as Mean ± SEM, *$P < 0.05$ When compared with control group

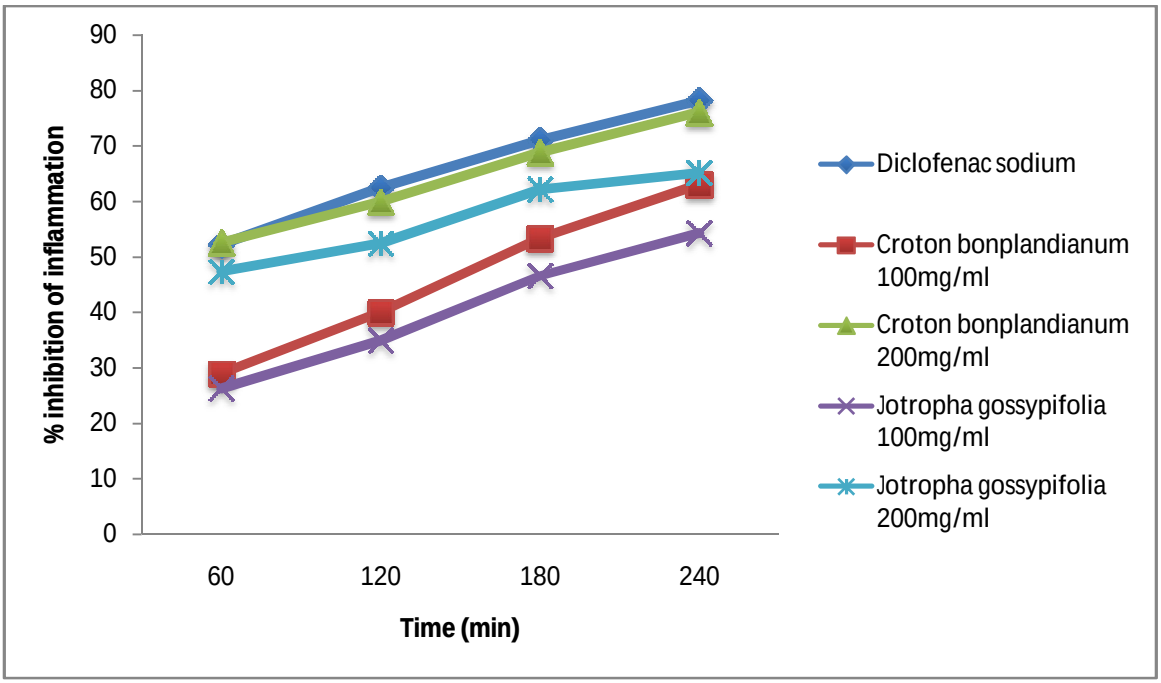

Figure 2: Comparative in vivo anti-inflammatory activity of plant extracts

DISCUSSION:

When we are screening a compound for particular activity, applying different pharmacological procedures and interpretation of those results will make us to ensure the activity and mode of action of a particular compound. Thus, both *invitro* and *invivo* methods are used in screening of antioxidant and anti-inflammatory properties of *Jotrophagossypifolia*and *Croton bonplandianum.* Reactive oxygen species (ROS) are reported to play a vital role in the inflammatory path ways. ROS propagate inflammation by stimulating the release of the cytokines such as IL-1, TNF-α, and IF-γ, which stimulate precipitation of extra neutrophil and macrophages. Both the plant extracts shows a significant antioxidant activity (Figure-1, Table-1). Then we extended our study to the preliminary anti-inflammatory screening by HRBC stabilization method. The lysosomal enzymes is said to be related to acute or chronic inflammation. The non-steroidal drugs (NSAIDs) act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membranes. But these drugs are associated with various and severe side effects [22]. Both the plant extracts exhibited membrane stabilization effect by inhibiting hypotonic induced lysis of erythrocyte membrane. The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may also well stabilize

lysosomal membrane [23]. The anti-inflammatory activity of the extracts is confirmed by Carrageenan induced hind paw edema which is the standard experimental model of acute inflammation. Carrageenan-induced acute inflammatory oedema is generally believed to be a biphasic response. The early phase (1-2 h) of the carrageenan model is mainly mediated by histamine and serotonin (5-HT). The late phase (2-4 h) is mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages [24]. In the present study, both the test extracts produced significant inhibition of carrageenan induced rat paw oedema after a period of 4 h (Table 3). This indicates the two test extracts were active in both the early and late phases of carrageenan induced acute hind paw inflammation in rats. All the results show the direct relation of antioxidant and anti-inflammatory properties. The *Croton bonplandianum* extract shows more anti-inflammatory activity which was also found to show more antioxidant activity.

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