# ANTIOXIDANT ACTIVITY OF AQUEOUS EXTRACT OF AERIAL PARTS OF *MOLLUGO PENTAPHYLLA* LINN- AN INVITRO STUDY

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## Summary

*Mollugo pentaphylla* Linn. commonly known as carpet weed in English, is a perennial herb found throughout India and other parts of the world. The plant is use for many disorders as per the ethno medical claim. The present study was aim to establish *in-vitro* antioxidant activity of aqueous extract of aerial parts of *Mollugo pentaphylla* (Family: Molluginaceae) in different *in-vitro* experimental methods like 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical, peroxide radical, superoxide radical and nitric oxide radical scavenging activity with reference to standard antioxidant ascorbic acid. The total antioxidant potential and reducing power were also determined. The plant extract shown the total antioxidant activity of 98.66 mg ascorbic acid equivalent/g as compared to 117.83 mg of the reference standard ascorbic acid. The reducing power of the extract was found to significant and in a concentration dependent manner.

The extract shown marked antioxidant activity with an $IC_{50}$ value of 96.5 µg/mL for DPPH radical, 381.4 µg/mL for superoxide radical, 432.7 µg/mL for $H_2O_2$ radical and 247.5 µg/mL for nitric oxide radical. Hence basing on the above results it was concluded that the aqueous extract of leaves of *M.pentaphylla* showed significant antioxidant activity.

**Keywords:** *Mollugo pentaphylla,* free-radical, antioxidant, DPPH.

## Introduction

The potentially reactive oxygen species (ROS) such as $O_2$•−, $H_2O_2$ and •OH, are continuously generated inside the human body as a consequences of exposure to a plethora of exogenous chemicals in our ambient environment and/or a number of endogenous metabolic processes involving redox enzymes and bioenergetic electron transfer. Free radicals, especially the oxygen radical, superoxide, when formed could lead to the formation of other radicals. In fact, the toxicity of $O_2$•− in living organisms is due to its conversion into •OH and reactive radical .metal complexes. Superoxide and hydrogen peroxide are converted into •OH and other reactive radical complexes through the iron catalyzed Haber-Weiss reaction or the superoxide driven Fenton reaction (1-3). Superoxide anion radical is a strong oxidant and may initiate the oxidation of molecules like ascorbic acid or epinephrine following hydrogen abstraction due to its basicity. Hydrogen peroxide is a stable molecule, can act as both oxidizing and reducing agent and can generate hydroxyl radicals by an interaction with transition metal ions or a reaction with highly reactive oxidizing agents like NO and $NO_2$ (4).

Observable significant re-visit of ancient approach to prophylaxis and anaphylaxis (herbal therapy), though with modern dimensions of study envelopes our world of research today. In the light of recent scientific developments, the medicinal properties of plants have been investigated through out the world due to their potent pharmacological activities and economic viability.

Plants are a rich source of natural antioxidants such as phenolics and flavonoids that may occur in all parts such as fruits, vegetables, nuts, seeds, leaves, roots and barks (5). Therefore, much attention has been focused on the use of natural antioxidants to inhibit Reactive oxygen species (ROS) production and protect from damage due to ROS. ROS such as superoxides, peroxides and hydroxyl radicals have been identified as major contributors to all cell and tissue damage in many disease conditions. These free radicals induce damage to biomembranes, proteins and DNA. The ROS readily attack and induce oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA (6). This oxidative damage is a crucial etiological factor implicated in several chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, neurodegenerative diseases and also in the ageing process (7-10). The antioxidants mediate their effect by directly reacting with ROS, quenching them and/or chelating the catalytic metal ions (11). Several synthetic antioxidants, *e.g.*, BHA and butylated hydroxytoluene are commercially available but are quite unsafe and their toxicity like liver damage and mutagenesis is a problem of concern (12-13). Natural antioxidants, especially phenolics and flavonoids are safe and also bioactive. Therefore, in recent years, considerable focus has been directed towards identification of plants with antioxidant ability that may be used for human consumption.

*Mollugo pentaphylla* Linn. commonly known as carpet weed (English), Pitta saga (Oriya) is a perennial herb found throughout India, Ceylon, Malacca, China, Japan, Fiji etc. The urban people used this plant medicinally in paste form orally and externally for treatment of skin allergic condition, antimicrobials etc.(14,15,16) Highly esteemed by Hindus as a bitter vegetable which they eat occasionally on account of its stomachic, aperient and antiseptic properties (17). Ethnomedical Information on *Mollugo pentaphylla* cites the folkloric use of the plant as an emmenagogue on female human adult in India (18) and Indonesia (19). Hot $H_2O$ extract of dried entire plant in India used for whooping cough and in cases of atrophy in human (20) and Decoction of dried entire plant used to treat hepatitis in Taiwan (21).

*M. pentaphylla* is a component in an important folk medicine named "Peh-Hue-Juwa-Chi-Cao" in Taiwan, which is used as an anticancer, antitoxic and diuretic agent (22). Eaten as a pot-herb; it is also used medically for mouth infections. The original scientific studies on the plant reported to possess active antifungal activity (23, 24), antibacterial activity (25), spermicidal and spermiostatic effect (26), anti-inflammatory and hepatoprotective activity (27) and antioxidant activity (22). The plant is reported to contain Flavones such as Apigenin and Mollupentin (28), Mollugogenol A, an antifungal triterpenoid, Mollugogenol B, Mollugogenol D, Oleanolic acid and a steroid – Sitosterol Beta (23,26,29,30).

## Material and Methods

### Chemicals

All the solvents used in the study were of highest commercially available analytical grade purity and were procured from S.D. Fine Chemicals Limited, Mumbai, India. 1, 1-diphenyl-2-picrylhydrazyl (DPPH), sodium nitropruside (SNP), nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), ascorbic acid, phenazine methosulphate, Griess reagent, trichloroacetic acid, ammonium molybdate, potassium ferricyanide, naphthyl ethylene diamine dihydrochloride and other chemicals were obtained from Sigma Chemical Company, Mumbai, India.

### Plant Materials

Fresh and mature plant of *Mollugo pentaphylla linn*. was collected from Odisha, India and the plant was and authenticated by taxonomist, Dr. A. K. Pradhan, Professor, Department of Botany, PPD Mahavidyalaya, Tigiria, Cuttack, Orissa, India. A voucher specimen (Regdn. No. SPS/SOAU/2008/005) has been preserved in the institution herbarium of School of Pharmaceutical Sciences, Siksha 'O' Anusandhan University for future reference. After due authentication, fresh aerial parts were collected in bulk, cleaned thoroughly with distilled water, and subsequently dried under shade at $25^0$C. The shade dried leaves were powdered in an electrical grinder and stored in nylon bags in a deep freezer for further use.

**Preparation of the extract**

Powdered plant material (750 g) was refluxed with 2000 ml of distilled water for 48 h, after defatting with petroleum ether (60-80 $^0$C). Following filtration and concentration in a rotary evaporator, a dark brown viscous residue was obtained (yield: 29.72% (w/w) with respect to dried plant material).

**Antioxidant Studies**

**Evaluation of Total antioxidant activity**

The assay was done according to Prieto *et al,*1999 (31). The antioxidant activity of aqueous extract of aerial parts of *Mollugo pentaphylla* (AAMP) was evaluated through the principle of the formation phosphomolybdenum complex. In this method, an aliquot of 0.4 ml of sample solution (100 ppm in methanol) was mixed in a vial with 4 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The blank was prepared by replacing the sample with 0.4 ml of methanol. The vials were capped and incubated in a water bath at 95 $^0$C for 90 mins. After cooling the samples at room temperature, the absorbances were measured at 695 nm against the blank. The antioxidant activity was expressed relative to that of ascorbic acid.

**Assay of Reducing Power**

This Ferric Reducing Antioxidant Power (FRAP Assay) of AAMP was performed based on the method Yildrim et al 2000; Lu and Foo (32). The assay mixture i.e. 1 ml of plant extract solution (final concentration 100- 500 mg/l) was mixed with 2.5 ml phosphate buffer( 0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [ K3Fe(CN6)] (10g/l), then mixture was incubated at 50 degree C for 20 minutes. Two and one-half, 2.5 ml of trichloroacetic acid (100g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml Fecl3 (1g/l) and absorbance measured at 700nm in V-600 UV-Visible Spectrophotometer (JASCO Ltd, JAPAN). Ascorbic acid was used as standard and phosphate buffer used as blank solution. The absorbances of the final reaction mixture were expressed as mean ± standard error mean. Increased absorbance of the reaction mixture indicates stronger reducing power.

**Free Radical Scavenging Activity**

**DPPH Scavenging Activity** (33-35):

The free radical scavenging capacity of the aqueous extracts of *Mollugo pentaphylla* was determined using DPPH (1, 1-diphenyl-2-picryl-hydrazyl). DPPH solution (0.004% w/v) was prepared in 95% methanol. The extract was mixed with 95% methanol to prepare the stock solution (100 mg/100mL). The concentration of this extract solution was 100 mg /100 ml or 1000µg/ml. From stock solution 1ml, 2ml, 3ml, 4ml & 5ml of this solution were taken in five test tubes & by serial dilution with same solvent were made the final volume of each test tube up to 10 ml whose concentration was then 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml & 500µg/ml respectively. Freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes containing extract (100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml & 500µg/ml) and after 10 min, the absorbance was taken at 517 nm using V-600 UV-Visible Spectrophotometer (JASCO Ltd, JAPAN). Ascorbic acid was used as a reference standard and dissolved in distilled water to make the solution with the concentration 100µg/ml. Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as blank. % scavenging of the DPPH free radical was measured using the following equation:

% DPPH radical-scavenging = [(Absorbance of control - Absorbance of test Sample) / (Absorbance of control)] x 100

**Superoxide free-radical scavenging activity:**

Measurement of superoxide anion ($O^{2-}$) scavenging activity of the test extract was based on the slight modified method described elsewhere (36, 37). $O^{2-}$ radicals are generated non-enzymatically in Phenazine methosulphate–Nicotinamide adenine dinucleotide phosphate (PMS–NADH) systems by the oxidation of NADH and assayed by the reduction of NBT. In this experiment, the superoxide radicals were generated in 1 mL of Tris-HCl buffer (16 mM, pH 8.0) containing NBT (50 µM) solution and NADH (78 µM) solution. The reaction was started by adding PMS solution (10 µM) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm in a spectrophotometer was measured against blank samples. Decreased absorbance of the reaction mixture indicated

increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$(\%)\ I = (A0 - A1) / (A0) \times 100$$

Where A0 was the absorbance of the control and A1 was the absorbance of extract and the standard compound.

**$H_2O_2$ scavenging activity**

Scavenging of $H_2O_2$ by AAMP was determined by the method of Ruch et al, 1989 (38). One millilitre of the test extract solution [prepared in phosphate buffered saline (PBS)] was incubated with 0.6 ml of 4mM $H_2O_2$ solution (prepared in PBS) for 10 min. The absorbance of the solution was measured at 230 nm against a blank solution containing the extract without $H_2O_2$. The concentration of $H_2O_2$ was spectrophotometrically determined from absorption at 230 nm using the molar absorptivity of 81 $M^{-1}$ $cm^{-1}$.

**NO scavenging**

Nitric oxide (NO) was generated from sodium nitropruside (SNP) and measured by Greiss reaction (39). SNP in aqueous solution at physiological pH instinctively generates NO, which intermingles with oxygen to generate nitrite ions that can be anticipated by Greiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). Scavengers of NO with oxygen leading to reduced production of NO. SNP (5 mM) in phosphate buffer saline (PBS) was mixed with different concentration of (100-500 µg/mL) drug dissolved in suitable solvent and incubated at 25$^{o}$C for 150 min. The above samples were reacted with Greiss reagent. The absorbance of chromophore created during diazotization of nitrite with sulphanilamide and following coupling with naphthyl ethylene diamine was read at 546 nm and referred to the absorbance of standard solution of potassium nitrite treated in the same way with Greiss reagent.

**Statistical analysis**

All the experimental results were expressed as mean ± SEM of three parallel measurements. One way ANOVA was performed followed by Dunnet's t-test, $p<0.05$ was considered as significant.

## Results and Discussion

### Total antioxidant activity and assay of Reducing Power

The aqueous extract of aerial parts of *M. pentaphylla* (AAMP*)* was made by hot extraction process using distilled water as solvent and found that the yield value of the extract is 29.72 % (w/w). The said plant extract is reported to possess the phenolic content (75.16µg of pyrocatechol equivalent/500mg) and flavonoid content (9.58mg equivalent of quercetin /gm) (40). It is well known that the presence of polyphenols and flavonoids in plants, mainly responsible for their **dynamic antioxidant activity**, the obtained amount of total phenolics & flavonoids in the test extract indicates to possess a high antioxidant activity. The total antioxidant activity and the ferric reducing power of AAMP and standard drug ascorbic acid were investigated by using, different in vitro methods and are presented in Fig. 1 and 2 respectively. Fig.1 shows that the plant extract have total antioxidant activity of 98.66mg ascorbic acid equivalent/gm in comparison to that of the reference standard ascorbic acid which registered 117.83mg ascorbic acid equivalent/gm. Similarly, Fig.2 represented the reductive capabilities of the test extract compared to that of ascorbic acid and found that AAMP potentiate in reducing the $Fe^{3+}$ / ferricyanide complex to the ferrous form ($Fe^{2+}$) which was monitored by measuring the formation of Perl's Prussian blue at 700nm. The reducing power of the extract was found to be significant and in a concentration dependent manner.

**Fig. 1.**

**Determination of Total Antioxidant activity of *AAMP***

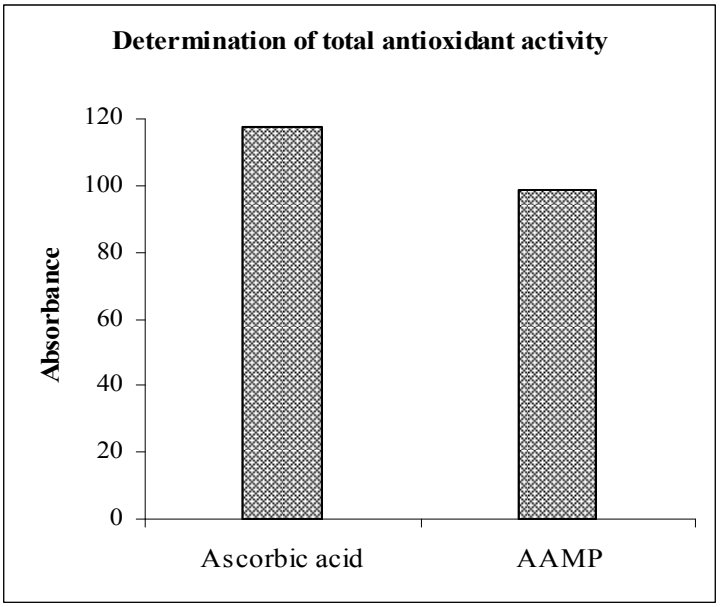
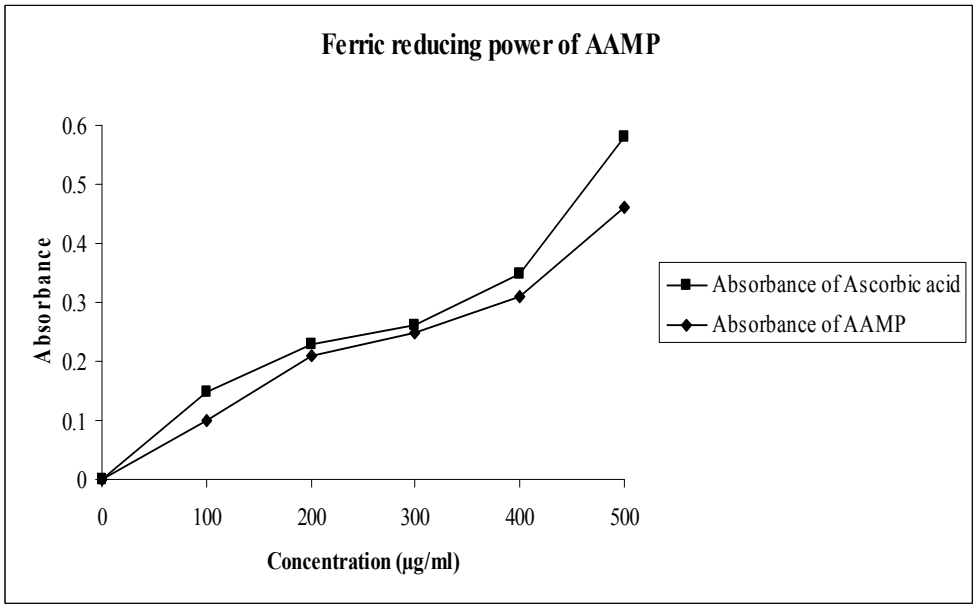



**Fig. 2.**

**Determination of Ferric reducing power of *AAMP***



**Free Radical Scavenging Activity**

The capacity of *M. pentaphylla* extract to scavenge DPPH, $O^{2}\bullet-$, •OH and NO were measured and the results are shown in Table-1. AAMP scavenges DPPH radical in a concentration dependent manner. The antioxidants react with DPPH, a purple colored stable free radical and convert it into a colorless α-α-diphenyl-β-picryl hydrazine. The amount of DPPH reduced could be quantified by measuring a decrease in absorbance at 517 nm. The test extract significantly and concentration dependently reduces DPPH radicals. However at a concentration of 500µg/ml, the extract significantly ($p< 0.001$) scavenged 98.0 % of DPPH radicals and had an $IC_{50}$ value of 96.5µg/ml. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. It has characteristic absorbance maxima at 517 nm, which is widely used to evaluate the free radical scavenging effect of natural antioxidants (41). Many researchers have reported positive correlation between free radical scavenging activity and total phenolic compound. DPPH radical scavenging activity increased with the increase of phenolic compound content (42-44). The DPPH scavenging ability of the extract may be attributed to its hydrogen donating ability. The primary free radical in most biological systems is Superoxide ($O^{2}\bullet-$). Although $O^{2}\bullet-$ itself is quite uncreative compared to the other radicals, but it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals (45). From the investigations, it was found that the *M. pentaphylla* extract scavenged $O^{2}\bullet-$ significantly and in a concentration dependent manner. The $O^{2}\bullet-$ scavenging activity was determined by Phenazine methosulphate/NADH-NBT system wherein $O^{2}\bullet-$ derived from dissolved oxygen by Phenazine methosulphate/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anions in the reaction mixture. The test extract exhibited a maximum of 68.8% superoxide scavenging activity with a significant extent ($p<0.001$) at a concentration of 500 µg/ml and the $IC_{50}$ value is 381.4 µg/ml. The spontaneous or catalytic dismutation of $O^{2}\bullet-$ leads to the formation of $H_2O_2$, which in the presence of a transition metal ion like $Fe^{3+}$, decomposes into •OH radicals, a highly damaging species in free radical pathology (46).

The extract is found to scavenge 66.8 % of $H_2O_2$ and in a significant extent ($p<0.001$) at 500 µg/ml with a calculated $IC_{50}$ value of 432.7 µg/ml. However, as compared to DPPH; $O^2\bullet-$, $H_2O_2$ and NO were weakly scavenged by the extract. *M. pentaphylla* extract at a concentration of 500 µg/ml also quenched 68.3% NO released by a NO donor, SNP in a significant manner ($p<0.001$) showing the $IC_{50}$ value is 247.5 µg/ml. Incubation of SNP solution in PBS at 25 $^0$C for 150 min resulted in the release of NO. The extract effectively and dose dependently decreased the release of NO (Table-1). Control experiments showed that, even at high concentrations, the extract did not interfere with the reaction between nitrite and Griess reagent. ROS like $O^2\bullet-$ may react with NO and give rise to various other reactive nitrogen species (RNS) such as $NO_2$, $N_2O_4$, peroxynitrite. Both ROS and RNS together attack and damage various cellular molecules. Virtually all cellular components including lipids, proteins, nucleic acids, carbohydrates are susceptible to oxidative damage (47). *M. pentaphylla* extract, owing to its radical scavenging ability may provide protection against oxidative damage induced to the biomolecules: proteins and lipids.

## Conclusions

It could be concluded that *M. pentaphylla* bear a potent antioxidant activity due to their phyto-constituents which in turn scavenge free radicals. The preliminary studies showed the presence of a number of polyphenols and flavonoids, which may be responsible for its antioxidant activities.

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**TABLE –1**

**Determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide ($O_2$•−), hydrogen peroxide ($H_2O_2$) and nitric oxide (NO) scavenging activity of *AAMP***

| Groups & Treatment | DPPH | $O_2$•− | $H_2O_2$ | NO |
|---|---|---|---|---|
| | % of control | % of control | % of control | % of control |
| **Control** | 100.0 ± 5.4 (00) | 100.0 ± 4.2 (00) | 100.0 ± 3.7 (00) | 100.0 ±2.7 (00) |
| **Ascorbic acid** | | | | |
| 100 µg/ml | 29.6 ± 2.1[c] (70.4) | 43.3 ± 3.7[b] (56.7) | 61.4 ± 4.8 (38.6) | 77.4 ± 3.6 (22.6) |
| 200 µg/ml | 13.2 ± 1.5[c] (86.8) | 37.1 ± 4.8[b] (62.9) | 45.7 ± 3.5 (54.3) | 68.3 ± 3.9[a] (31.7) |
| 300 µg/ml | 3.4 ± 0.41[c] (96.6) | 29.4 ± 2.6[c] (70.6) | 38.6 ± 2.4[b] (61.4) | 59.1 ± 2.4[b] (40.9) |
| 400 µg/ml | 0.00 (**100**) | 23.9 ± 3.1[c] (76.1) | 27.2 ± 1.7[c] (72.8) | 56.7 ± 3.8[b] (43.3) |
| 500 µg/ml | 0.00 (100) | 18.7 ± 2.3[c] (**81.3**) | 21.5 ± 1.8[c] (**78.5**) | 33.5 ± 4.9[c] (**66.5**) |
| **AAMP** | | | | |
| 100 µg/ml | 48.3 ± 5.3[a] (51.7) | 87.4 ± 5.3 (12.6) | 97.6 ± 4.9 (2.4) | 59.3 ± 2.8[b] (40.7) |
| 200 µg/ml | 33.5 ± 4.7[a] (66.5) | 63.8 ± 4.8 (36.2) | 89.8 ± 4.3 (10.2) | 57.6 ± 3.1[b] (42.4) |
| 300 µg/ml | 13.3 ± 2.7[b] (86.7) | 56.9 ± 3.9[a] (43.1) | 74.7 ± 3.8 (25.3) | 41.8 ± 4.5[c] (58.2) |
| 400 µg/ml | 4.1 ±1.2[c] (95.9) | 48.7 ± 2.9[c] (51.3) | 58.7 ± 2.3[b] (41.3) | 35.6 ± 2.3[c] (64.4) |
| 500 µg/ml | 2.0 ± 0.3[c] (**98.0**) | 31.2 ± 2.2[c] (**68.8**) | 33.2 ± 2.0[c] (**66.8**) | 31.7 ± 1.6[c] (**68.3**) |
| **$IC_{50}$ Value** | **96.5** | **381.4** | **432.7** | **247.5** |

Values are expressed in MEAN ± SEM (n =3). Values expressed in the parenthesis indicate % scavenging activity. (t-value denotes statistical significance at [a]p<0.05, [b]p<0.01 and [c]p<0.001 respectively, in comparison to control group)

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