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Research Article **Zoology**

Free Radical Scavenging Activity of *Mollugo Cerviana* extract - An *In vitro* study

N. Markkas and Madhuramozhi Govindharajalu

ABSTRACT

Antioxidant activity of methanolic extract of *Mollugo cerviana* whole plant were carried out for proving its utility in free radical mediated diseases including diabetic, cardiovascular, cancer etc. The methanolic extract was screened for in vitro antioxidant activity by nitric oxide radical scavenging, oxygen radical scavenging such as DPPH scavenging, superoxide anion radical scavenging, total antioxidant, metal chelation and iron reducing power activity at different concentrations. Throughout the studies extract showed marked antioxidant activity. The antioxidant activity of the extract may be due to the phytochemicals present in it. The antioxidant activity was found to be concentration dependent and may be attributed to the presence of bioflavonoids content in the of *Mollugo cerviana*. Overall, the plant extract is a source of natural antioxidants which might be helpful in preventing the progress of various oxidative stress mediated diseases including aging.

Keywords: Antioxidant activity, Mollugo cerviana, Radical scavenging, Reactive oxygen speceis.

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INTRODUCTION

The adverse effects of oxidative stress on human health have become a serious issue. Under stress, our bodies produce more reactive oxygen species (ROS) (e.g., superoxide anion radicals, hydroxyl radicals and hydrogen peroxide) than enzymatic antioxidants (e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase) and non-enzymatic antioxidants (e.g., ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, carotenoids, and flavonoids). This imbalance leads to cell damage (Bhatia et al., 2003; Peuchant et al., 2004) and health problems (Steer et al., 2002). A lack of antioxidants, which can quench the reactive free radicals, facilitates the development of degenerative diseases, including cardiovascular diseases, cancers, neurodegenerative diseases, Alzheimer's disease and inflammatory diseases (Velavan, 2011; Alma et al, 2003). Natural and synthetic antioxidants are beneficial to free radical mediated diseases. Synthetic antioxidants, such as butylated

hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), have been widely used as antioxidants in the food industry and may be responsible for liver damage and carcinogenesis (Grice, 1988) For this reason, interest in the use of natural antioxidants has increased.

The World Health Organization (WHO) has estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts and their active components (Winston, 1999). Plant and its products are rich sources of a phytochemicals and have been found to possess a variety of biological activities including antioxidant potential (Velavan *et al*, 2007; Velavan, 2015). The majority of the active antioxidant constituents are flavonoids, isoflavones, flavones, anthrocyanins, coumarins, lignans, catechins, and isocatechins. In addition to the above compounds found in natural foods, vitamins C and E, beta-carotene, and tocopherol are known to possess antioxidant potential (Prior, 2003). With this background and abundant source of unique active components harbored in plants. The chosen medicinal plant namely as *Mollugo cerviana* (Tamil: Parpatakam). *Mollugo cerviana* is considered as stomachic, aperients and antiseptic. Flow and tender shoots are diaphoretic and given in fevers. Hence, the free radical scavenging activity of *Mollugo cerviana* was not evaluated. Therefore, the present study were to investigate the free radical scavenging activity of *Mollugo cerviana* through the free radical scavenging such as DPPH scavenging, superoxide anion radical scavenging, total antioxidant, metal chelation and iron reducing power activity.

MATERIALS AND METHODS

Chemicals

Nitro blue tetrazolium (NBT), ethylene diamine tetra acetic acid (EDTA), sodium nitroprusside (SNP), trichloro acetic acid (TCA), thio barbituric acid (TBA), potassium hexa cyano ferrate [$K_3Fe(CN)_6$], and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd., India. All other chemicals and solvents used were of analytical grade available commercially.

Plant materials:

The whole plant of *Mollugo cerviana* was collected in January 2015 from Tamil University, Thanjavur District, Tamil Nadu, India from a single herb. The whole plant was identified and authenticated by Dr. S. John Britto, The Director, the Rabiant Herbarium and centre for molecular systematics, St. Joseph's college Trichy-Tamil Nadu. India. A Voucher specimen has been deposited at the Rabinat Herbarium, St. Josephs College, Thiruchirappalli, Tamil nadu, India.

Preparation of alcoholic extract

The whole plant of *Mollugo cerviana* was first washed well and dust was removed from the plant. Whole plant was washed several times with distilled water to remove the traces of impurities from the plant. The whole plant was dried at room temperature and coarsely powdered. The powder was extracted with 70% methanol for 24 hours. A semi solid extract was obtained after complete elimination of alcohol under reduced pressure. The extract was stored in refrigerator until used.

DPPH Assay

The scavenging ability of the natural antioxidants of the plant extract towards the stable free radical DPPH was measured by the method of Shimada et al., (1992). Briefly, a 2 ml aliquot of DPPH methanol solution (25µg/ml) was added to 0.5 ml sample solution at different concentrations. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. Then the absorbance was measured at 517nm in a spectrophotometer. L-Ascorbic acid was used as the standard.

Radical scavenging activity (%)

$$= 100 - \frac{A_C - A_S}{A_C} \times 100$$

Where A_C = control is the absorbance of the control and A_S = sample is the absorbance of reaction mixture (in the presence of sample). All tests were run in triplicates (n = 3), and the average values were calculated.

Determination of total antioxidant capacity

The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method according to the procedure of Prieto, Pineda, and Aguilar (1999). The assay is based on the reduction of Mo(VI)–Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. 0.3 ml extract was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Superoxide anion scavenging activity assay

The scavenging activity of the *Mollugo cerviana* towards superoxide anion radicals was measured by the method of Liu et al. (1997).

Superoxide anions were generated in a non-enzymatidc phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 ml of Tris-HCl buffer (100mM, pH 7.4) containing 0.75 ml of NBT (300 μM) solution, 0.75 ml of NADH (936 μM) solution and 0.3 ml of different concentrations of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 μM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation:

% Inhibition = $[(A_0-A_1) / A_0 \times 100]$,

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Fe^{2+} chelating activity assay

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis et al. (1994). To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2 mM) was added. After 30 s, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as

Chelating rate (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Reducing power assay

The Fe^{3+} reducing power of the extract was determined by the method of Oyaizu (1986) with slight modifications. The extract (0.75 ml) at various concentrations was mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate $[K_3Fe(CN)_6]$ (1%, w/v), followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Statistical analysis: Tests were carried out in triplicate for 3–5 separate experiments. The amount of extract needed to inhibit free radicals concentration by 50%, IC_{50}, was graphically estimated using a nonlinear regression algorithm.

RESULTS AND DISCUSSION

Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans. Plant produces these chemicals to protect itself but recent research demonstrates that many phytochemicals can protect humans against diseases. There are many phytochemicals fruits and herbs and each works differently. The phytochemical screening of *Mollugo cerviana* reported that the presence of flavonoids, terpenoids, steroids, tannin, saponins, glycosides, phlopatannins, carbohydrate, triterpenoids, protein, alkaloids and anthroquinones (Markkas and Madhuramozhi Govindharajalu, 2016).

DPPH Assay

DPPH radical scavenging activity of *Mollugo cerviana* extract and standard as ascorbic acid are presented in Fig 1. The DPPH radical was widely used to evaluate the free-radical scavenging capacity of antioxidants (Nuutila *et al.*, 2003). Recently, the use of the DPPH$^{\bullet}$ reaction has been widely diffused among food technologists and researchers, for the evaluation of free radical scavenging activity on extracts from plant, food material or on single compounds. In the DPPH assay, the antioxidant was able to reduce the stable radical DPPH to the yellow colored 1, 1-diphenyl-1, 2-picryl hydrazine. The molecule of 2, 2-diphenyl-1-picryl hydrazine is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole. The proton transfer reaction of the DPPH$^{\bullet}$ free radical by a scavenger (A-H) causes a decrease in absorbance at 517 nm, which can be followed by a common spectrophotometer set in the visible region. The effect of antioxidants on DPPH$^{\bullet}$ is thought to be due to their hydrogen donating ability (Sindhu and Abraham, 2006). The half inhibition concentration (IC_{50}) of plant extract and ascorbic acid were 46.65μg ml^{-1} and 34.91 μg ml^{-1} respectively**.** The plant extract exhibited a significant dose dependent inhibition of DPPH activity. The potential of L-ascorbic acid to scavenge DPPH radical is directly proportional to the concentration. The DPPH assay activity is near to standard as ascorbic acid.

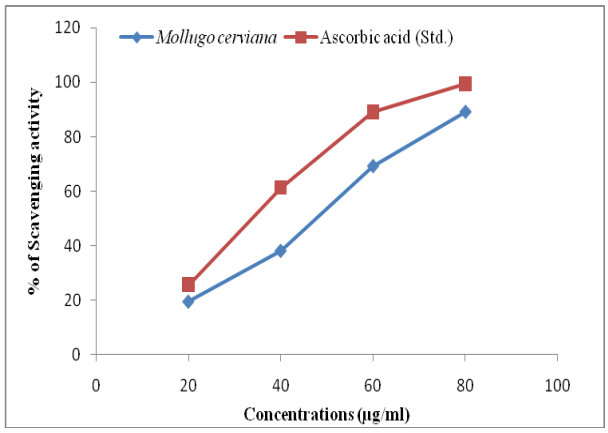

Fig. 1. DPPH radical scavenging activity of *Mollugo cerviana*

Total antioxidant activity

The yield of the methanol extract of the plant extract and its total antioxidant capacity are given in Fig. 2. Total antioxidant capacity of *Mollugo cerviana* extract is expressed as the number of equivalents of ascorbic acid. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/ Mo (V) complex with a maximal absorption at 695 nm. The assay is successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extract (Prieto et al., 1999). Moreover, it is a quantitative one, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The study reveals that the antioxidant activity of the extract is in the increasing trend with the increasing concentration of the plant extract. The half inhibition concentration (IC_{50}) of plant extract and ascorbic acid were 50.95µg ml^{-1} and 42.41 µg ml^{-1} respectively**.**

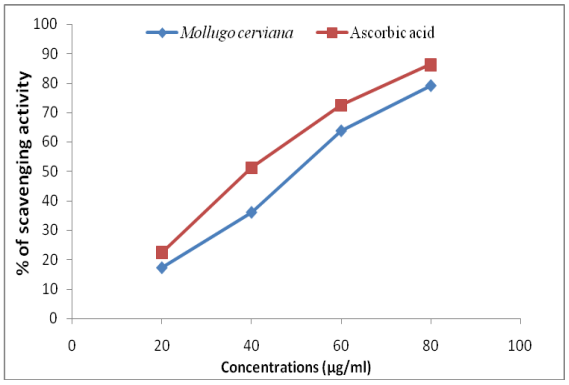

Fig. 2.Total antioxidant assay of *Mollugo cerviana*

Superoxide anion radical scavenging activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system (Korycka-Dahl & Richardson, 1978). The superoxide anion radical scavenging activity of the extract from Mollugo cerviana assayed by the PMS-NADH system was shown in Fig 3. The superoxide scavenging activity of *Mollugo cerviana* was increased markedly with the increase of concentrations. The half inhibition concentration (IC_{50}) of *Mollugo cerviana* was 53.59µg ml^{-1} and ascorbic acid were 31.62µg ml^{-1} respectively**.** These results suggested that *Mollugo cerviana* had notably superior superoxide radical scavenging effects.

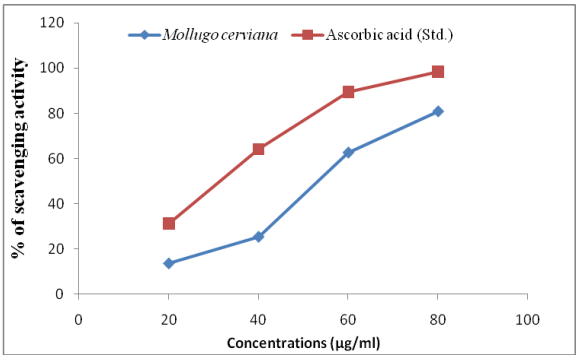

Fig. 3.Super oxide scavenging activity of *Mollugo cerviana*

The ferrous ion chelating activity

Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction. The formation of the ferrozine– Fe^{2+} complex is interrupted in the presence of aqueous extract of *Mollugo cerviana* , indicating that have chelating activity with an IC_{50} of 48.25µg ml^{-1} and ascorbic acid was 30.96µg ml^{-1} respectively (Fig. 4). Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Halliwell, 1991; Fridovich, 1995). Metal chelating activity can contribute in reducing the concentration of the catalyzing transition metal in lipid peroxidation. Furthermore, chelating agents that form s bonds with a metal are effective as secondary antioxidants because

they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion (Gordon, 1990). Thus, *Mollugo cerviana* demonstrate a marked capacity for iron binding, suggesting their ability as a peroxidation protector that relates to the iron binding capacity

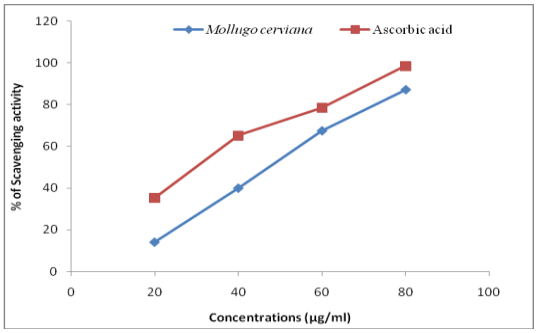

Fig. 4. Ferrous iron chelating activity of *Mollugo cerviana*

Reducing power activity

For the measurements of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of *Mollugo cerviana* . The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, and prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997; Yildirim *et al*, 2000; Thirumurugan *et al*., 2015). Fig. 5 depicts the reductive effect of *Mollugo cerviana* . Similar to the antioxidant activity, the reducing power of *Mollugo cerviana* increased with increasing dosage. All the doses showed significantly higher activities than the control exhibited greater reducing power, indicating that *Mollugo cerviana* consist of hydrophilic polyphenolic compounds that cause the greater reducing power.

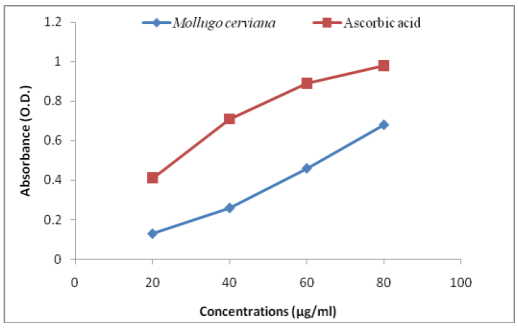

Fig.5 .Reducing power assay of *Mollugo cerviana*

CONCLUSION

The results of the present study showed that the extract of *Mollugo cerviana* extract which contains of flavonoids and polyohenols. These phytochemicals are exhibited the greatest antioxidant activity DPPH scavenging, superoxide anion radical scavenging, total antioxidant, metal chelation and iron reducing power activity which participate in various pathophysiology of diseases including cancer, diabetic, ageing etc. This work has gathered experimental evidence on the *Mollugo cerviana* extract as natural antioxidant for its capacity to scavenge reactive oxygen and nitrogen species and protect cells/organism from oxidative damage and thus could be an effective against oxidative stress. In addition, the *Mollugo cerviana* extract found to contain a noticeable amount of total phenols which plays a major role in controlling antioxidants. Thus, it can be concluded that *Mollugo cerviana* extract can be used as an accessible source of natural antioxidants with consequent health benefits.

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