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IN VITRO ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF *ALTERNANTHERA SESSILIS*

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ABSTRACT

The present study was aimed to evaluate the antioxidant activity of *A. sessilis* plant extracts in different solvents. The antioxidant activity was studied by phosphomolybdate method and DPPH method. In phosphomolybdate method the highest activity was shown by methanolic extract (12.044 mM of ascorbic acid eqvt/gm of sample). The highest radical scavenging activity by DPPH method was found in methanol extracts (IC₅₀ 587.093µg/ml). Ferrous chelating activity, superoxide radical scavenging activity, nitric oxide radical scavenging activity was found high in acetone, acetone and methanol extracts respectively. Total Flavonoids and crude phenolics were found to be 0.370 mg/gm dry wt. and 1.529 mg/gm dry wt. respectively. The antioxidant activity increases with increase in the concentration. This study indicates that *A. sessilis* is a potential source of natural antioxidant.

Keywords:

Antioxidant,
Phosphomolybdate,
DPPH,
Ferrous chelating,
Superoxide,
Nitric oxide

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INTRODUCTION: Production of free radicals in the biological system results the imbalance in the level of pro-oxidant and antioxidant known as oxidative stress. These ROS are able to oxidize cellular bio-molecules like nucleic acids, proteins, lipids and carbohydrates ^{1, 2, 3, 4}. Fruits and vegetables are rich source of phytochemicals especially polyphenols ^{5, 6}. It is evident from the study that there is an inverse relationship between the dietary intake of antioxidant rich foods and the incidence of a number of human diseases ⁷. Leafy vegetables apart from being a good source of minerals also contain antioxidant vitamins and pigments. They are also known for their therapeutic value ⁸.

Alternanthera sessilis is a prostrate or perennial herb. The branches are raised from the root and are up to 50 cm long. This plant is found in damp places, wet headlands, roadsides and sometimes as weed of plantations. In South East Asia this plant is taken as

vegetables. In India it is used for treatment of gastrointestinal problems.

In the present study an attempt has been made to evaluate the antioxidant and radical scavenging activity of *A. sessilis* by different *in vitro* models.

MATERIALS AND METHODS:

Plant material: Fresh mature leaves and stem of *Alternanthera sessilis* were collected during the month of March and April from Dibrugarh, Assam, India.

Preparation of plant extract: The leaves and stem of *Alternanthera sessilis* was dried at room temperature, powdered and used for extraction. 1 gm of powder extracted with 10 ml of 90% methanol, 70% acetone and 80% ethanol for 12 hours with occasional shaking. The supernatant was collected and concentrated under reduced pressure in a rotary evaporator. All extracts were kept in a refrigerator until use.

Determination of phenolic compounds:

Preparation of crude phenolic extracts: The soluble crude polyphenols were isolated by standard method⁹. The air dried grounded sample of *A. sessilis* (1 gm) was extracted six times with a 20 ml mixture of acetone/methanol/water (7:7:6 by vol.) at room temperature. After each centrifugation, the supernatant were collected, combined and evaporated to near dryness. This residue was dissolved in 25 ml methanol. The methanolic extract was referred to as Extract A.

Isolation of phenolic acids: 10 ml of extract A was evaporated to dryness and the residue was suspended in double distilled water and treated with 30 ml of 4 M NaOH. The resulting hydrolyzed solution was acidified to pH 2 with 6 M HCl and extracted six times with diethyl ether (1:1, vol/vol). The diethyl ether extracts were combined and evaporate to dryness. The residue containing phenolic acids, both liberated form esters was dissolved in methanol. The methanolic extract was referred to as Extract B.

Quantification of phenolics: Total content of phenolics in extract A and extract B was estimated by Folin-Ciocalteu's method¹⁰. 0.5 ml of sample extract was taken and final volume was adjusted to 3 ml by addition of distilled water. 0.5 ml of Folin-Ciocalteu's (50% v/v) was added to the reaction mixture. After 5 min incubation at room temperature 2 ml of 20% (w/v) was added. After 3 min incubation absorbance at 760 nm was taken along with blank. Results were expressed as gallic acid equivalent per gram of dried sample.

Determination of Flavonoids: The amount of flavonoids was determined spectrophotometrically¹¹. 1 ml of plant extract was mixed with 1 ml of 2% aluminum trichloride in ethanol. The mixture was diluted with ethanol to 25 ml and allowed to stand for 40 min at 20°C and the absorbance was measured at 415nm against the sample blank. The results were expressed as rutin equivalent per gram of dried sample.

Total antioxidant activity by phosphomolybdenum method: The antioxidant activity is based on the

reduction of Mo (VI) to Mo (V) by the test sample and the subsequent formation of green phosphate/Mo (V) complex at acidic pH¹². An aliquot of 0.1 ml sample was mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were incubated at 95°C for 90 min and cooled at room temperature. The absorbance was measured at 695 nm against blank. The antioxidant activities of samples were expressed as mM of ascorbic acid eqvt./g of sample.

DPPH radical scavenging assay: The radical scavenging activity of Alternanthera extracts and TROLOX was measured by DPPH method¹³. 1 ml of 0.135mM DPPH solution in methanol was mixed with 1 ml of extract. The reaction mixture was vortexed and left in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. A reaction mixture without test sample was served as control. The ability to scavenge DPPH radical was calculated by the following equation:

$$\% \text{ scavenging activity} = \frac{\text{Control}_{\text{Abs}} - \text{Test}_{\text{Abs}}}{\text{Control}_{\text{Abs}}} \times 100$$

Ferrous ion chelating activity: Ferrous ion chelating activity assay was based on the principle of the Fe²⁺ chelating ability of the test samples by measuring the ferrous iron-ferrozine complex formed at 562 nm¹⁴. To different concentration of sample extracts were added 0.1 ml of 2mM ferrous chloride, 0.2 ml of 5mM ferrozine and 3.7 ml of methanol. The solution was allowed to react for 10 min. The absorbance at 562 nm was measured against blank. The percentage of ferrous ion chelating activity was calculated as follows:

$$\% \text{ ferrous ion chelating activity} = \frac{\text{Control}_{\text{Abs}} - \text{Test}_{\text{Abs}}}{\text{Control}_{\text{Abs}}} \times 100$$

Superoxide scavenging activity: Superoxide scavenging activity was based on the inhibitory action of superoxide dismutase on the rate of base catalyzed auto-oxidation of pyrogallol¹⁵. The assay medium contained 1 ml of different concentration of test sample, 2ml of water, 3ml of 0.05M Tris buffer, P^H 8.2, and the reaction was started by addition of 0.02 ml pyrogallol (60mM) and recorded during 1 min at 420

nm. The percentage of superoxide scavenging activity was calculated as follows:

$$\% \text{ scavenging activity} = \frac{\text{Control}_{\text{Abs}} - \text{Test}_{\text{Abs}}}{\text{Control}_{\text{Abs}}} \times 100$$

Nitric oxide radical scavenging activity: Nitric oxide radical scavenging activity was measured by spectrophotometric method¹⁶. Test samples at different concentration (1000 µg -9000 µg) were dissolved in DMSO. To the 1 ml of test solution 1 ml of sodium nitroprusside (5mM) in phosphate buffer saline was mixed and incubated for 30 min at 25°C. Assay medium without test solution served as control. After 30 min 1 ml of incubated solution was taken out and equal amount of Griess reagent was added. The absorbance of the chromophore formed during the diazotization of the nitrile with sulphanilamide and the subsequent coupling with naphthylethylene diamine dihydrochloride was measured at 546 nm. The percentage scavenging activity was calculated as follows:

$$\% \text{ scavenging activity} = \frac{\text{Control}_{\text{Abs}} - \text{Test}_{\text{Abs}}}{\text{Control}_{\text{Abs}}} \times 100$$

Statistical analysis: Analyses were performed in triplicate. The results obtained were presented as mean ± S.D. Statistical analysis was performed using minitab 15. Data were analyzed by ANOVA (p>0.05) followed by Tukey's test. The IC₅₀ values were calculated by linear regression and were compared by t-test, p < 0.05 was considered significant.

RESULTS AND DISCUSSION: The total amount of phenolic compounds and Flavonoids are presented in **Table 1**. Phenolic compounds may contribute directly to antioxidative action because their hydroxyl groups confer scavenging ability¹⁷. The total crude phenolic content was found to be 1.529 mg gallic acid eqvt/ gm of dried sample and the total phenolic acid was 1.404 mg gallic acid eqvt/ gm of dried sample. Total flavonoid content was 0.370 mg rutin eqvt/ gm of dried sample. The Flavonoids confer antioxidative property through scavenging or chelation⁷.

TABLE 1: AMOUNT OF TOTAL PHENOLIC AND FLAVONOID CONTENT IN A.SESSILIS

Sample	Total crude phenolic (mg/g dry wt.)	Total phenolic acid (mg/g dry wt.)	Flavonoids (mg/g dry wt.)
<i>A.sessilis</i>	1.529 ± 0.083	1.404 ± 0.069	0.370 ± 0.011

Values are mean ± S.D

Table 2 shows the total antioxidant activity of different extract of *A.sessilis* by phosphomolybdenum method. The data was subjected to statistical analysis using ANOVA and Tukey's test. Tukey's test revealed that the differences in the total antioxidant activity among methanol and ethanol; ethanol and acetone was significant. There was no significant difference among methanol and acetone extract.

TABLE 2: ANTIOXIDANT ACTIVITY OF DIFFERENT SOLVENT EXTRACTS OF A. SESSILIS BY PHOSPHOMOLYBDATE METHOD

Solvent extracts	Methanol	Ethanol	Acetone
Total antioxidant activity (mM of ascorbic acid/g of sample)	6.025 ± 0.683	12.044 ± 1.437	7.612 ± 0.255

Values are mean ± S.D

Free radical scavenging activity of *A.sessilis* was measured using DPPH. The results are shown in **Table 3**.

TABLE 3: FREE RADICALS SCAVENGING ACTIVITY AND IRON CHELATING ACTIVITY (IC₅₀ VALUES) OF DIFFERENT SOLVENT EXTRACT OF A.SESSILIS AND REFERENCE COMPOUNDS

Activity	Extract/Reference	IC ₅₀ (µg/ml)
DPPH scavenging activity	Methanol	587.093 ± 18.683*
	Acetone	690.81 ± 34.958*
	Ethanol	641.25 ± 18.20*
	Trolox	2.42 ± 0.005
Superoxide scavenging activity	Methanol	6284.28 ± 389.24*
	Acetone	5048.59 ± 199.54*
	Ethanol	5602.01 ± 106.67*
	Rutin	510.85 ± 22.47
Nitric oxide scavenging activity	Methanol	1758.35 ± 49.36*
	Acetone	7001.007 ± 190.39*
	Ethanol	5941.67 ± 239.15*
Iron Chelating activity	Rutin	3592.31 ± 319.65
	Methanol	2684.68 ± 61.86*
	Acetone	850.28 ± 45.19*
	Ethanol	2807.34 ± 19.96*
	Rutin	25275.95 ± 175.61

Each value represents mean ± S.D. * p<0.05

The highest DPPH radical scavenging activity was detected in methanol extract (IC₅₀ 587.093µg/ml)

followed by ethanol and acetone extract (IC₅₀ 641.25 and 690.81 respectively). The t-test analysis showed that there is significant difference in the DPPH radical scavenging activity among the different extract of test sample and standard trolox.

Iron is capable of generating free radicals from peroxides by Fenton reactions, and minimization of the Fe²⁺ concentration in the Fenton reaction affords protection against oxidative damage¹⁸. The addition of different plant extracts interferes with the ferrous ferrozine complex and the formation of the red colored complex decreased with the increasing concentration. Among the extract tested acetone extract showed the highest ferrous chelating activity (IC₅₀ 850.28 µg/ml). The abilities shown by methanol and ethanol were 2684.68 and 2807.34 respectively. The activities were significantly higher than the standard rutin (25275.95µg/ml) (Table 3).

The superoxide radical scavenging activities of different solvent extracts of *A. sessilis* were determined using a base catalyzed pyrogallol auto-oxidation. As shown in Table 3 the highest superoxide radical scavenging activity was found in acetone extract (IC₅₀ 5048.59µg/ml) which is followed by ethanol and methanol. These activities are less than that of standard rutin (IC₅₀ 510.85µg/ml).

In vitro inhibition of nitric oxide radical is a measure of antioxidant activity of plant drugs¹⁹. Table 3 shows that acetone extract exhibited highest nitric oxide scavenging activity (IC₅₀ 5941.67µg/ml). All the extract showed significant difference in the nitric oxide scavenging activity with standard rutin except the methanol extract.

Nitric oxide plays an important role in various inflammatory processes but the overproduction of nitric oxide contributes to various diseases. The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxy nitrite anion²⁰. The plant extract compete with oxygen to react with NO thereby inhibiting the formation of nitrite.

On the basis of results obtained it may be concluded that *Alternanthera sessilis* showed the potent radicals scavenging activity and metal ion chelating activity.

The difference in the antioxidant activity in different extract may be due to the different phytochemical constituents present at different percentage. Further investigation is needed to evaluate the active principle, its isolation and characterization.

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