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RESEARCH ARTICLE

Evaluation of *In-vitro* radical scavenging activity and polyphenolic content in the leaves of indigenous plant *Alternanthera sessilis* (L.)R.Br.Ex DC

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ABSTRACT

Plants are considered to be the elixir of life, as it has the power to cure all ills of life. The objective of the present study is to investigate the free radical scavenging activity of ethyl acetate, ethanol and aqueous extracts of *Alternanthera sessilis* leaves. The extracts were subjected to quantitative analysis of phenols, flavonoids and tannins. The antioxidant capacity of the plant extracts were studied by the methods such as 1,1- diphenyl- 2- picryl-hydrazil (DPPH) free radical scavenging ,Hydrogen peroxide scavenging , Nitric oxide radical inhibition assay , Ferric reducing antioxidant power assay, Superoxide anion radical scavenging, and Hydroxyl radical scavenging assay. Among the three extracts, ethanolic extract of the leaves of *Alternanthera sessilis* contains high amount of polyphenolic content and exhibited significant antioxidant activity. The free radical scavenging activity was compared with the positive control ascorbic acid. The results of the study indicates that the ethanolic extract of the indigenous plant *Alternanthera sessilis* was found to be a potent scavenger of DPPH, NO, H_2O_2, Hydroxyl, Super oxide anion and FRAP activity.

Keywords: Alternanthera, Polyphenols, DPPH, FRAP, Ascorbic acid.

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CONTENTS

1. Introduction

Plants are known to be Mother Nature's gift as it houses an enormous source of bioactive components, most of them are being evolved as chemical defense against predation or infection. The medicinal value and efficacy of plants have

been studied extensively, which covers a percentage of 2,65,000 flowering plants on earth[1]. Nowadays, medicinal plants have been considered as a valuable source of potential drugs and this has created awareness among the people. The choice of plants for therapeutic usage is highlighted, because of their ease in availability, low cost, safe and with fewer side effects [2]. Plant foods contain all the significant nutrients which promote a healthier and wealthier form of nutrition to human beings [3]. As stated by The World Health Organization (WHO), 80% of the population of developing countries depend on plant drugs. Therefore, the natural medicines from herbs are widely accepted by the majority of population [4]. Alkaloids, tannins, flavonoids and phenolic compounds are the significant phytochemicals present in the plants. The desirable feature of the plant to be used in indigenous treatment is the correlation between the phytoconstituents and the bioactivity of plant. These criteria's are necessary to synthesize compounds with specific activities to treat various health ailments and chronic diseases [5].

The important source of antioxidants are the phytochemicals, as they are the major line of defense for cell survival and also terminate the chain reaction of free radicals [6].Free radicals are highly reactive molecules results due to oxidation reaction. Free radicals are produced through oxidation reaction by losing electron and reacts with other molecules by replacing it. Free radicals could damage cells and may lead to disorders like Alzheimer's disease, arthritis, hemorrhoids, Parkinsonism, rheumatism, AIDS, cataract, Stroke, cancer and so on [7]. The principle components present in the whole plant parts (flowers, leaves, fruits, roots, barks) namely phytochemicals are a group of secondary metabolites that were found to be associated to impart protective and preventive role against many degenerative diseases and pathological processes such as in ageing [8, 11], coronary heart disease, Alzheimer's disease [9, 11] neurodegenerative disorders, atherosclerosis, cataracts, and inflammation [10, 11]. The therapeutic value of Leafy vegetables is attributed to the minerals, antioxidant vitamins and pigments present in them [12].

An annual herb *Alternanthera sessilis* linn which belongs to Amaranthaceae family possess many branches, simple leaves and small white flowers seen in hotter regions of India at an altitude of 1200m [13, 14]. In Tamilnadu and other Southern parts of India, the featured herb, *Alternanthera sessilis* is being widely used by people in cooking. Stigmasterol, campesterol, ß-sitosterol, α-, ß-spinasterol etc., are the numerous phytochemicals present in the vegetable [15].The leaves of *Alternanthera sessilis* is used in the treatment of various ailments such as skin and eye diseases and as an antidote for snake bite [16, 17]. The whole plant is reported to be antipyretic in nature and possess properties like wound healing. This plant is claimed to be a soft laxative and useful in purifying and nourishing blood [19]. The herb is also reported as febrifuge, galactagogue, abortificient, and used in the treatment of indigestion [17, 18]. The aim of the present study is to investigate the total phenol, total flavonoids, total tannins and antioxidant properties of the indigenous plant.

2. Materials and Methods

Collection of plant materials:

The dried leaves of *Alternanthera sessilis* were used as a source of plant material for the present investigation. This material was purchased from the local market of Koyambedu, Chennai. The plant materials were taxonomically identified and authenticated by Dr. P.T.Devarajan, Associate Professor, Department of Plant Biology & Biotechnology, Presidency College, Chennai.

Fresh leaves of *Alternanthera sessilis* were separated washed and shade dried for about 10 days. These dried leaves were ground to coarse powder using mechanical grinder.

Preparation of The Extract:

The dried leaves were subjected to extraction using ethanol, ethyl acetate andwater by Soxhlet extraction method. The collected extracts were stored and then taken up for further investigations. The resulted filtrates were used in invitro antioxidant studies.

Total Phenolic content (TPC):

Total phenolic content of extract was assessed according to the Folin–Ciocalteau method (Slinkard & Singleton) [20], with some modifications. In a test tube, 0.1 ml of extracts (200, 600 and 1000µg/ml), 1.9 ml distilled water and 1 ml of Folin–Ciocalteau's reagent, followed by 1 ml of 100 g/l sodium carbonate were added. The reaction mixture was incubated at 25 °C for 2 hours and the absorbance of the mixture was read at 765 nm. The sample was tested in triplicate and a calibration curve with six data points for catechol was obtained. The results were compared to a catechol calibration curve and the total phenolic content of sample was expressed as mg of catechol equivalents (CAE) per gram of extract.

Total flavonoids Content (TFC):

The total flavonoid content in the sample was estimated by the method of Chang et al [21]. A volume of 0.25 ml of the sample was diluted to 1.25 ml with distilled water. A volume of 75 µl of 5% sodium nitrite was added and after six minutes, 0.15 ml of aluminium chloride solution was added. A volume of 0.5 ml of 0.1M sodium hydroxide was added after 5 min and made up to 2.5 ml with distilled water. The solution was mixed well and the absorbance was measured at 510 nm in comparison with standard quercetin at 5-25 µg concentration. The results are expressed as mg of flavonoids as quercetin equivalent (QUE) per gm of dried sample.

Total tannins content (TTC):

Tannins – phenolics were determined by the method of Peri and Pompi [22]. 1ml of the sample extracts of concentration 1mg/ml was taken in a test tube. The volume was made up to 1ml with distilled water and 1ml of water served as the blank. To this 0.5 ml of Folin's phenol reagent (1: 2) followed by 5ml of 35% sodium carbonate was added and kept at room temperature for 5 minutes. Blue colour was formed and the colour intensity was read at 640nm. A standard graph (gallic acid – 1mg/ml) was plotted, from

which the tannin content of the extract was determined. The total tannin content was expressed in mg/g of extract.

Invitro antioxidant assays

DPPH free radical scavenging assay :

The ability of the fractions to annihilate the DPPH radical (1,1-diphenyl-2-picrylhydrazyl) was investigated by the method described by Blois, 1958 [23]. An equal volume of different concentration of the major fraction (200, 600 and 1000 µg) was added, to methanolic solution of DPPH (0.1mM). The tubes were incubated at room temperature for 30 minutes and the absorbance was recorded at 517 nm. The experiment was repeated for three times. Ascorbic acid was used as standard controls. The annihilation activity of free radicals was calculated in % inhibition.

% of Inhibition = (A of control – A of Test)/A of control x 100.

Nitric oxide radical inhibition assay:

GriessIllosvoy reaction Garratt, 1964 [24], explains the spontaneous interaction between sodium nitroprusside and oxygen in an aqueous solution to produce nitrite ions at physiological pH. The reaction medium generates nitric oxide. In the present investigation, Griess Illosvoyre agent was modified using naphthyl ethylenediamine dihydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). A total volume of 3 ml consists of 2 ml of 10 Mm sodium nitroprusside , 0.5 ml of phosphate buffer saline and different concentration of extracts (200– 1000 µg/ml) and 0.5 ml of standard solution were incubated at 25 °C for 150 minutes. After incubation, diazotization was completed on incubation with 0.5 ml of the reaction mixture containing nitrite and mixed with 1 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 minutes. Then 1 ml of naphthylethylenediamine dihydrochloride (1%) was added, mixed and allowed to stand for 30 minutes. The intensity of the pink colour formed was measured at 540 nm against the corresponding blank. Vitamin C was used as positive control.

% of Inhibition = (A of control – A of Test)/A of control x 100.

Hydrogen peroxide scavenging activity:

The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch *et al* [25]. A solution of H2O2(43 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). The extract at different concentrations in 3.4 ml phosphate buffer was added to 0.6 ml of H_2O_2 solution (43Mm). The absorbance value of the reaction mixture was recorded at 230 nm.

H_2O_2 scavenging activity (%) = (A0 – A1) /A0 ×100.

Where, A0 is the absorbance of the control, and A1 is the absorbance of the sample.

Hydroxyl radical scavenging activity:

The efficacy of various fractions of sample was studied by Hydroxyl radical scavenging activity assay to scavenge the hydroxyl radicals generated by the Fe^{3+} - ascorbate-EDTA-H_2O_2 system (Fenton reaction) Halliwell et al., 1987 [26]. 1.0 ml of final volume reaction mixture contained 100 µl of 2-deoxy2- ribose (28 mM in 20 mM Potassium dihydrogen phosphate buffer, pH 7.4), 500 µl of the sample at various concentrations (200, 600, 1000 µg/ml) in buffer, 200 µl of 1.04 mM EDTA and 200 µM $FeCl_3$ (1:1v/v), 100 µl of 1.0 mM hydrogen peroxide (H_2O_2) and 100 µl of 1.0 mM ascorbic acid. Test samples were kept at 37℃ for 1 hour. The free radical damage imposed on the substrate and deoxyribose was measured using the thiobarbituric acid test. One ml of 1% thiobarbituric acid (TBA) and 1.0 ml 2.8% trichloroacetic acid (TCA) were added to the test tubes and were incubated at 100°C for 20 min to develop the pink chromogen. After cooling, the absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Ascorbic acid (200, 600, 1000 µg/ml) was used as a positive control.

Superoxide scavenging activity:

Superoxide scavenging activities of the extracts were determined by monitoring the competition of those with NBT for the superoxide anion generated by the PMS–NADH system , Liu, Ooi, & Chang, 1997 [27]. The reaction mixture containing 1 ml of 20 mM Tris–HCl buffer (pH 8.0), 0.05 mM nitrobluetetrazolium (NBT), 0.01 mM phenazinemethosulphate (PMS) and different concentration of extracts (200–1000 µg/ml) were added and the tubes were pre incubated for 2 minutes. The reaction was initiated by the addition of 0.078 mM NADH. NBT reduction resulted in the formation of blue chromogen and it was measured at 560 nm. Results were depicted as percentage of inhibition of superoxide radicals. Ascorbic acid was used as a standard. The free radical scavenging activity was calculated.

Ferric Reducing Antioxidant Potential Assay (FRAP Assay):

The antioxidant potential was determined by the method given by Benzie and Strain [28], with slight modifications. A freshly prepared FRAP reagent comprising of 10 mM of 2,4,6-Tris(2-pyridyl)-1,3,5-triazine (TPTZ) (dissolved with 40 mM of HCl), 20 mM of Ferric chloride in water and 300 mM of acetate buffer (pH 3.6) in ratio of 1:1:10 was utilized for the assay. A blank containing sample and solvents only was used for colour correction. The plates were incubated at 37˚C for 90 minutes and absorbance was recorded at 593 nm. (L-ascorbic acid), was used as antioxidant standards and positive controls. A standard calibration curve of ferrous sulphate was compared with the test sample and the concentration of extract or chemical which gives the same absorbance as 1 mmol ferrous ion and results were expressed in terms of Ferrous Equivalent (FE).

[A (Sample Final) - A (Sample Initial) /A (Std. Final) - A (Std. initial)] × 2.

3. Results and Discussion

Quantitative Assessment of Phytoconstituents:

The data in Table 1 shows the amount of various phytoconstituents present in different extracts of *Alternanthera sessilis*. The present study revealed that the total phenolic content was found to be high in ethanolic extract as 501.63 ± 1.41 followed by aqueous extract as 208.83 ± 1.34 and ethyl acetate as 107.8 ± 1.46. The values for flavonoid content is shown as 24.65 ± 1.62 for ethyl acetate, 41.04 ± 1.07 for ethanol and 32.81 ± 1.33 for aqueous extracts. The amount of tannins present in the three extracts, ethyl acetate, ethanolic and aqueous was 106.06 ±

1.05, 378.08 ± 1.03 and 218.90 ± 1.19 respectively. The amount of phenols, flavonoids and tannins were found to be comparatively higher in the ethanolic extract of the leaves of the green leafy vegetable, *Alternanthera sessilis*. The presence of various polyphenols signifies the nature and bioactivity of the plant.

Assessment of Antioxidant activity

DPPH Radical Scavenging Assay:

DPPH assay is the widely used antioxidant method for the evaluation of free radical scavenging activity. The antioxidant potential of the plant is evident from the presence of phenolic compounds in the leaf extracts. IC_{50} value of ethyl acetate, ethanol and aqueous extracts were found to be 550 ± 4.24, 202.5 ± 3.53 and 326 ± 6.36 respectively. The antioxidant efficacy of ascorbic acid was 249.5 ± 7.77. Among the three extracts, ethanolic extract exhibits significant antioxidant activity. (Table 1 and Fig 1).

Nitric oxide radical inhibition assay:

The ethanolic extract of Alternanthera sessilis showed high radical inhibition activity when compared to the standard ascorbic acid. It has effectively inhibited the formation of nitric oxide. The maximum activity for the ethanolic extract was 229 ± 7.07 when compared with ascorbic acid (337 ± 2.12). The other two extracts ethyl acetate and aqueous showed 537 ± 2.82 and 283.5 ± 4.94. (Table1 and Figure 2)

Hydrogen peroxide scavenging activity:

Scavenging activity of *Alternanthera sessilis* leaf extracts showed a significant decomposition activity in a concentration dependent manner. The ethyl acetate, ethanolic and aqueous extracts exhibited potent scavenging effect as that of the standard ascorbic acid with IC_{50} values as 642.1 ± 6.36, 343.8 ± 5.65, 460 ± 3.53 and 512.6 ± 9.19 respectively. (Table 2 and Figure 3)

Hydroxyl radical scavenging activity:

The scavenging ability of the leaf extracts expressed as IC_{50} was presented in the Table 1. The most active extract was the ethanolic extract with IC_{50} value in µg/mL as 240.8 ± 3.53. The least active extracts were ethyl acetate and aqueous with IC_{50} of 438.8 ± 4.24 and 336.6 ± 2.82 and the standard ascorbic acid showed IC_{50} value as 280.5 ± 4.94. (Figure 4).

Superoxide radical scavenging activity:

In the present study, the leaf extracts of A. sessilis was examined for its scavenging activity. The superoxide scavenging effect of different extracts was compared with the standard ascorbic acid. The study revealed that the ethanolic extract behave as a powerful superoxide anion scavenger with the IC_{50} value as (249.5 ± 2.12) whereas the extracts like ethyl acetate (799.6 ± 1.41) and aqueous (539.6 ± 0.707) exhibited low activity than the standard ascorbic acid (424 ± 2.82). (Table1 and Figure 5)

Ferric Reducing Antioxidant Potential Assay (FRAP Assay): The FRAP assay is a robust and potentially useful test over a wide concentration range. The reducing ability of leaf extracts were found in the decreasing order as ethanol (11.08 ± 1.69) > aqueous (8.88 ± 1.90) > ethyl acetate (7.35 ± 1.41) ascorbic acid (7.03 ± 1.69) . (Table 1 and Figure 6)

Discussion: Based on the chemistry, source and similar properties, plants comprise of an array of molecules categorized as primitive and auxiliary metabolites. In the current study phytochemical content and radical scavenging activities of the plant were performed in the leaf portion of the edible vegetable. Analysis of the plant revealed the presence of various phytochemicals like alkaloids, tannins, saponins, terpenoids, phenol, glycosides, carbohydrates and steroids. Primary metabolites contribute to the growth and development whereas the secondary metabolites act as a biocatalyst. [29, 30]

The presence of bioactive constituents indicates that the plant *Alternanthera sessilis* could be used in folk medicine for various ailments. Among the plant metabolites, phenols are the largest and most ubiquitous group [31, 32]. Generally, phenolic compounds are found to act against apoptosis, aging, inflammation, atherosclerosis and so on. Phenolic compounds are said to be good antioxidants. They are also involved in cardiovascular protection and improvement of endothelial function, Inhibition of angiogenesis and cell proliferation activities [31, 33]. Flavonoids are one of the large family of polyphenolic compounds which plays a vital role to decrease the levels of blood glucose and lipid and also contribute to enhance human immunity [34, 35].

The larger molecular weight polyphenolic component present in plants, which is associated with proteins are tannins [36]. Tannins are abundantly seen in leaves. They are considered as antinutrients and secondary antioxidants. Tannins possess special property to act as an antioxidant where they chelate metal ions such as Fe^{2+} and retards oxidation [37]. On the basis of quantitative analysis of polyphenolic content in the leaves, Ethyl acetate, Ethanol and aqueous extracts were taken from antioxidant studies. Among the tested plant extracts, ethanolic extract showed strong radical scavenging activity surpassing the activity of standard antioxidants. An antioxidant is a molecule (or an ion, or a relatively stable radical) that is capable of slowing or even preventing the oxidation of other molecules [38].

There are several antioxidant mechanisms through which antioxidant act by scavenging of reactive oxygen species and nitrogen free radicals. The simple, stable and accurate method used to study the radical scavenging activity is the stable DPPH assay. The ability of the extracts containing antioxidant to annihilate the DPPH radical indicates the scavenging activity of the drug. [39]. The significant role of Nitric oxide rely in the regulation of various physiological processes in the cells. Since it is a free radical product, excessive production of nitric oxide may be associated with several diseases. [40, 41]. The ethanolic extract efficiently scavenged the nitrite being formed and showed a good inhibitory activity against the NO radical generated than ethyl acetate and aqueous extracts. Hydrogen peroxide catalyzes the oxidation of essential thiol (- SH) groups and the weak nature of H_2O may directly inactivate few enzymes that play a crucial role in cell survival.H_2O_2 may result in toxicity as it enters into the cell, where it can react with Fe^{2+} and Cu^{2+} ions and result in the formation of hydroxyl radical. Thus the removal of hydrogen peroxide

becomes essential for cells as a line of defensive mechanism. [42]. The ethanolic extract of *Alternanthera sessilis* exhibited better decomposition of H_2O_2 radical than other extracts in a concentration dependent manner. Hydroxyl radical causes severe damage to the cells as it is the most centered oxygen species. The competition between deoxyribose and extract for hydroxyl radicals is measured as percentage of inhibition. The present study gives a lucid result that the ethanolic extract showed better hydroxyl scavenging activity than ethyl acetate and aqueous extracts [43]. The origin of reactive oxygen species may occur from superoxide radical [44, 45]. The major damage to a cell takes place through oxidative stress. The contributor to produce powerful and dangerous hydroxyl radicals and singlet oxygen is superoxide radical. [44, 46].The ability of the plant extract and the standard compound ascorbic acid to quench superoxide radicals from reaction mixture can be deduced that the plant extract is more potent scavenger of superoxide radical.

The antioxidants available from the natural sources provide enormous health benefits and they contribute to prevent the oxidative stress related diseases, due to the attack of free radicals on key biomolecules of the cell like lipids and nucleic acids [47]. The reducing ability of colourless ferric complex to a coloured ferrous complex is carried out by an electron donating antioxidant colorimetric ally. The obtained result showed that there was an increased ferric reducing power in ethanol and aqueous extracts than ethyl acetate and standard. Therefore, it could be determined from the study that electrons may be donated to free radicals to maintain integrity and stability in the biological system [48, 49]. The extract of *Alternanthera sessilis* is found to scavenge all free radicals and hence this study revealed that the ethanol extract was found to exhibit potent antioxidant activity when compared to the ethyl acetate and aqueous extracts.

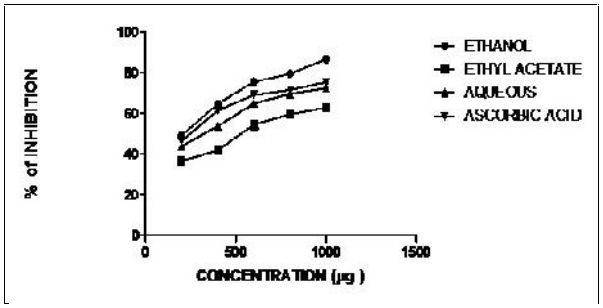

Fig 1: DPPH radical scavenging activity

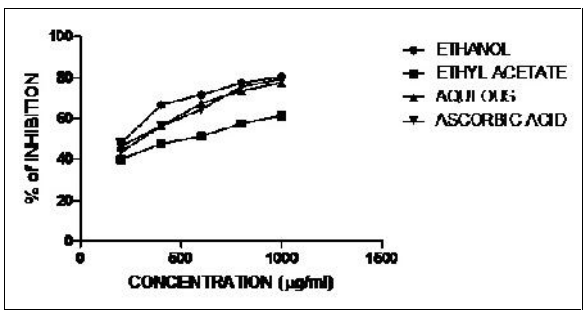

Fig 2: Nitric oxide radical scavenging activity

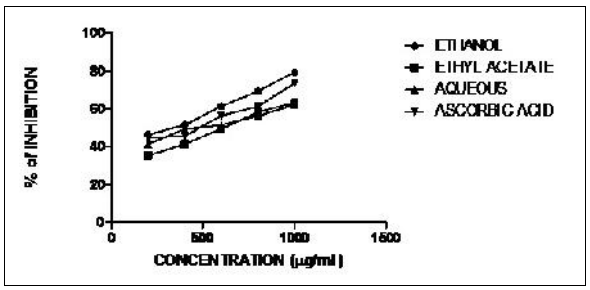

Fig 3: Hydrogen peroxide radical scavenging activity

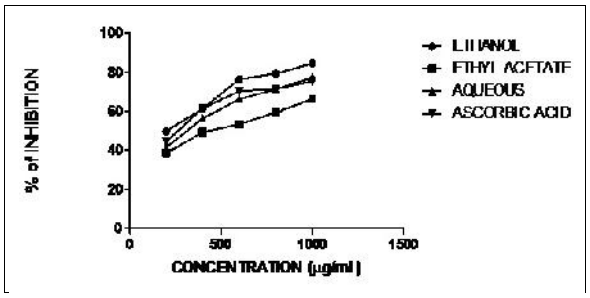

Fig 4: Hydroxyl radical scavenging activity

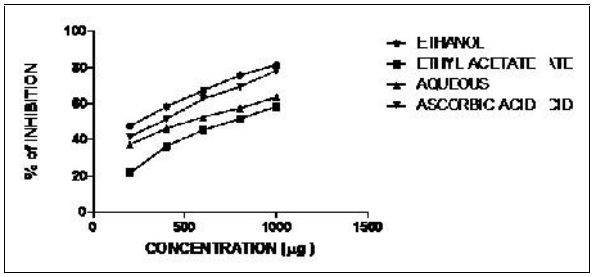

Fig 5: Superoxide radical scavenging activity

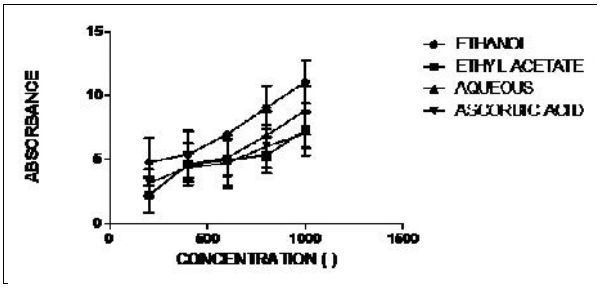

Fig 6: Reducing power activity of different extracts

4. Conclusion

The antioxidant activity was correlated with the amount of total polyphenolic content present in the respective extracts in each assay. The present study demonstrates that the ethanolic extract of *Alternanthera sessilis* leaves, which contains high content of polyphenolic compounds exhibit high antioxidant activity and free radical scavenging activity. These results revealed that this plant extract could be an important source of natural antioxidant. Increasing intake of dietary antioxidants may contribute to enhance immunity and also to maintain a healthy lifestyle. The plant Alternanthera *sessilis* would be a good source of antioxidants and this vegetable can be included in the daily food regime. The investigation would be further extended to assess the antioxidant activity of the extract in vivo to study their clinical importance.

Table 1: Amount of total phenol, flavonoid and tannin content in *A.sessilis*

S. No.	PARAMETER	CONCENTRATION (mg/g of extract)		
		Ethyl acetate	Ethanol	Aqueous
1	Total Phenol mg CAE/g of extract	107.8 ± 1.46	501.63 ± 1.41	208.83 ± 1.34
2	Total Flavonoid mg QUE/g of extract	24.65 ± 1.62	41.04 ± 1.07	32.81 ± 1.33
3	Total Tannins mg GAE/g of extract	106.06 ± 1.05	378.08 ± 1.03	218.90 ± 1.19

Table 2: Antioxidant activity [IC 50 (µg /mL)] of different extracts of Alternanthera sessilis leaves and standards and FRAP (µ M (Fe II) equivalent) method

S.No.	Free radical scavenging method	50 % inhibition concentration (IC_{50}) in µg			
		Ethyl acetate	Ethanol	Aqueous	Ascorbic acid Standard
1	DPPH	550 ± 4.24	202.5 ± 3.53	326 ± 6.36	249.5 ± 7.77
2	Nitric oxide	537 ± 2.82	229 ± 7.07	283.5 ± 4.94	337.1 ± 2.12
3	Hydrogen peroxide	642.1 ± 6.36	343.8 ± 5.65	460 ± 3.53	512.6 ± 9.19
4	Hydroxyl	438.8 ± 4.24	240.8 ± 3.53	336.6 ± 2.82	280.5 ± 4.94
5	Super oxide	799.6 ± 1.41	249.5 ± 2.12	539.6 ± 0.707	424 ± 2.82
6	FRAP	11.08 ± 1.69	7.35 ± 1.41	8.88 ± 1.90	7.03 ± 1.69

Conflict of Interest: We declare no conflict of interest.

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