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Original Article

In vitro Anti-oxidant Activity in Methanolic Extracts of five *Pogostemon* Species

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ABSTRACT

Objectives: The Oxidation damages are induced by highly reactive oxygen species in human body. Antioxidants terminate the inactive of these radicals and reduce the risk. In this study, we compared the antioxidant properties of five *Pogostemon* species. **Methods:** The *in vitro* antioxidant activity was evaluated by DPPH, ABTS, reducing power assay, ferrous ion chelating activity, Hydroxyl radical scavenging activity (HRSA) and ferric reducing antioxidant power (FARP) assay. The antioxidants compound like total phenols and total flavonoid were also evaluated in these plants. **Results:** The presence of high amount of phenols and flavonoid in these plants, the metanolic extract's antioxidant can strongly scavenge DPPH and ABTS radical with significant IC_{50} value range. Other modals of experimented where have potential antioxidant activity. **Conclusions:** These *Pogostemon* species had significant antioxidant agent, it could be an excellent source of antioxidants with great explore as therapeutic agent.

Key words: Antioxidant activity, *Pogostemon*, Metanolic extracts, Antioxidants, Therapeutic agent.

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INTRODUCTION

Plants have provided with all in terms of shelter, clothing, food, flavours and fragrances and medicine properties. Plants have formed the basis of complicated traditional medicine systems that have been in continuation for thousands of years to provide new remedies. The Lamiaceae contains about 236 genera and 6,900 to 7,200 species,[1] in this *Pogostemon* is one of the important genera and a large widespread genus having 114 species in India. Both roots and leaves are mostly used by tribal people in this genus like the fresh root or poultice of the leaves is applied on the snake bites particularly Phursa (*Echis carinatus*) snake bite, uterine haemorrhage, leaf extract used as an insect repellent, treatment of food poison, vomiting and stomach troubles, respiratory tract infection and pollen and nectar used as a source of Panagol honey in Maharashtra.[2]

The superoxide anion (O_2) hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) are the form of active oxygen and free radicals. These are constantly formed in human body through normal metabolic action. Hence, this leads to a variety of biochemical and physiological lesion, often consequent in metabolic destruction and cell death.[3] The synthetic antioxidants were creating the genotoxic effect[4] and other chronic disease.[5] Therefore, the antioxidant properties of secondary metabolites have formed the foundation of many applications in a preservation process as pharmaceuticals, alternative medicine and natural therapies.[6] Here the studies to explore the five *Pogostemon* species which have been scientific conform for their potential medicinal values.

MATERIALS AND METHODS

Preparation of plant extracts

Pogostemon vestitus, *P. mollis, P. speciosus, P. wightii* and *P. nilagiricus were colocted* belongs to the family Lamiaceae were collected from natural forest of Western Ghats of Coimbatore, Tamil Nadu. The specimen was identified by Dr. M. Murugesan, Scientist B, Botanical Survey of India, Shillong. Fresh plant materials were washed under running tap water, air-dried and powdered. About 50 g of coarsely powdered plant materials (50 g/250 ml) were extracted in a soxhlet extractor for 8-10 hrs, with methanol. The extract obtained was then concentrated using a vacuum evaporator and weighted. For stock solutions, 1 mg/ml of methanolic extract was dissolved in dimethyl sulfoxide (DMSO).

Determination of Total phenols

Total phenolics were quantified and expressed as Gallic acid equivalents according to a method proposed by Singleton *et al.*[7] About 3.9 ml of distilled water and 0.5 mL of Folin-ciocalteau reagent were added to 0.1ml of the extract in a tube and incubated at room temperature for 3 min after which 2 mL of 20% sodium carbonate was added and kept in a boiling water bath for 1 min. Phenols react with phosphomolybdic acid in the Folin-ciocalteau reagent in alkaline medium and produce a blue coloured complex (molybdenum blue) that can be estimated colorimetrically at 650 nm. The total phenol content of the extract was calculated and expressed as Gallic Acid Equivalent (GAE) mg g^{-1} extract.

Determination of Flavonoids

Total flavonoid content was estimated by the aluminium chloride colorimetric assay.[8] An aliquot (1 mL) of extract and standard solution of Catechin (100 mg mL^{-1}) was added to 10 mL volumetric flask containing 4 mL of distilled water. To this 0.3 mL of 5% $NaNO_3$ was added. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. After 1 min, 2 mL of 1M NaOH was added and the total volume was made up to 10 mL with distilled water. The solution was mixed well and the absorbance was measured against reagent blank at 510 nm. The value of optical density was used to calculate the total flavonoid content present in the sample. The mean of the three values were expressed as milligrams of Rutin equivalents (mg g^{-1}) extract on a dry weight basis.

Free radical scavenging activity (2, 2-diphenyl-1-picryl-hydrazyl [DPPH•])

DPPH• the free radical scavenging activity of methanolic extract of *P. mollis*, *P. vestitus*, *P. nilagiricus*, *P. wightii* and *P. speciosus* were measured by the method of Blois.[9] 0.2 mM solution of DPPH• in methanol was prepared, and 100 µl of this solution was added to various concentrations of methanolic extract at the concentrations of 50, 100, 150, 200

and 250 µg/ml. After 30 minutes, absorbance was measured at 517 nm. Butylated hydroxytoluene (BHT) was used as a standard material. All the tests were performed in triplicate and percentage of inhibition was calculated by comparing the absorbance values of the control and test samples.

$$\text{Percentage of inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Free radical scavenging activity (2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid [ABTS•+])

The total antioxidant activity of the samples was measured by ABTS•+ radical cation decolorization assay followed by the method of Re *et al.*[10] ABTS•+ was produced by reacting 7 mM ABTS•+ aqueous solution with 2.4 mM potassium persulfate in the dark for 12-16 hrs at room temperature. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. Prior to assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30°C to give an absorbance of 0.70±0.02 at 734 nm. Then, 2 ml of diluted ABTS•+ solution was added to the sample concentration at 20 µl (1 mg/ml). After 30 minutes of incubation at room temperature, the absorbance was recorded at 734 nm and percentage of inhibition was calculated. Trolox was used as a reference standard. Triplicates were performed.

Reducing power assay

Reducing power activity was determined according to the method of Yildirim *et al.*[11] Different concentrations of methanolic extract (100, 200, 300, 400, 500 µg/ml) of the study species were mixed with 1 ml of 200 mM sodium phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferric cyanide followed by incubation at 50°C for 20 min. After adding 1 ml of 10% trichloroacetic acid, the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was taken out and mixed with 2 ml of distilled water and 0.5 ml of 1% ferric chloride. All the tests were performed in triplicates, and ascorbic acid was used as a reference standard.

Ferrous ion chelating activity

The chelating of ferrous ions by methanolic extract was estimated by the method of Singh and Rajini.[12] Different concentrations of methanolic extracts (100, 200, 300, 400 and 500 µg/ml) were mixed with 100 µl of 2 mM ferrous sulfate solution and 300 µl of 5 mM ferrozine. The mixture was incubated at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. Ethylene diamine tetra acetate (EDTA) was used as standard. All the tests were performed in triplicate and percentage of inhibition was calculated by using the formula,

$$\text{Percentage of inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Hydroxyl radical scavenging activity (HRSA)

The scavenging activity for the sample extract on hydroxyl radical was measured according to the method of Klein *et al.*[13] 20 µg concentration of the extract was added with 1.0 ml of iron–EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1.0 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) sequentially. The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. The reaction was terminated by the addition of 1.0 ml of ice-cold trichloroacetic acid (17.5% w/v). Then, 3.0 ml of nash reagent (75.0 g of ammonium acetate, 3.0 ml of glacial acetic acid, and 2.0 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at laboratory temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the color formed was measured spectrophotometrically at 412 nm against the reagent blank. Results were compared with the activity of standard antioxidant BHT. The % HRSA was calculated using the following formula:

$$\text{HRSA (\%)} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

Statistical analysis

The statistics were performed by one-way analysis of variance (ANOVA) test and the significance of the difference between means was determined by Duncan's Multiple Range Test at ($p<0.05$) significant level.

RESULTS AND DISCUSSION

Antioxidant agents are closely associated to the prevention of degenerative diseases, such as cardiovascular and neurological illnesses, oxidative stress malfunctions and cancer.[14] Therefore, the ability to scavenge free radicals is an important mechanism to treat patients suffering from degenerative diseases. Hence, the synthetic antioxidants have been used to prevent the oxidation, but their safety has been questioned due to toxicity issue.[15] At the same time antioxidant agents must be low cast and lower side effect, due to this cause the natural product (plant origin) are the best option for mankind.

Total phenols and Total flavonoids

The aromatic, phenol and flavonoid contents shown strong correlation in antioxidant activity, these compounds can undergo redox reactions and therefore scavenge free radicals.[16] The total phenolic contents of the examined plant extracts are presented in Table 1. In this experiment, the highest phenolic content was found *P. vestitus* (2.8/100 g), followed by *P. mollis* (2.5/100 g) and *P. speciosus* (2.3/100 g). The lowest values of phenols were found in *P. wightii* (1.97/100 g) and *P. nilagiricus* (1.6/100 g) respectively. The antioxidant activity of these species correlated well with phenolic compounds. This agreed with the Lamiaceae species, which possessed the strong free radical scavenging activity due to presence of Phenols.[17] The Flavonoid molecules can act as proton donors and excellent radical scavenging activity due to the presence of hydroxyl groups.[18] The concentrations of flavonoids in *P. mollis*, *P. vestitus*, *P. nilagiricus*, *P. wightii* and *P. speciosus* were determined spectrophotometrically. The total flavonoids contents from the examined plants methanol extracts were presented in Table 1. The highest flavonoid content was identified in the *P. mollis*. Hence few reports showed the Lamiaceae species have significant antioxidant capacity by the flavonoids.[17]

DPPH radical scavenging activity

The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The absorbance decreases as a result of a colour change from purple to yellow as the radical is scavenged by antioxidants through donation of hydrogen to form the stable DPPH-H molecule. The DPPH radical scavenging effect percentage accelerated with increase in the concentration of the extract (100-500 µg mL^{-1}). This study confirmed better antioxidant potential by DPPH radical scavenging method in methanolic extracts of *P. mollis* (253.8 µg mL^{-1}), *P. vestitus* (IC_{50} value 279.32 µg mL^{-1}), *P. nilagiricus* (IC_{50} value 370.37 µg mL^{-1}), *P. wightii* (IC_{50} value251.25 µg mL^{-1}) and *P. speciosus* (IC_{50} value 337.83 µg mL^{-1}) (Table-2). Similarly, appreciable antioxidant and radical scavenging activities were found in *P. quadrifolius*[19] and in some Lamiaceae members.[20]

ABTS$^{\bullet+}$ radical scavenging activity

ABTS$^{\bullet+}$ assay measures the relative antioxidant ability to scavenge the radical ABTS$^{\bullet+}$and is an excellent tool for determining the antioxidant activity of hydrogen donating antioxidants and chain breaking antioxidants. In the present study *P. mollis*, *P. vestitus*, *P. nilagiricus*, *P. wightii* and *P. speciosus* were presented markedly higher ABTS$^{\bullet+}$ radical scavenging activity at 20 µg mL^{-1} concentration (Table 3). The results confirmed that at all the plants showed a higher inhibitory potential. This correlated with the finding of Wojdylo *et al*.[20] in Lamiaceae members.

Ferrous ion chelating assay

Iron is essential for oxygen transport in respiration, activity of enzymes and extremely reactive metal, catalyzes oxidative changes in lipids, proteins and other cellular components.[21] Chelating agents may inhibit radical generations by stabilizing transition metals consequently reducing free radical damage. The metal chelating ability of the methanolic extracts was measured by Ferrozine combines with ferrous ions forming a red coloured complex which absorbs at 562 nm.[22] The metal chelating activity was determined and it exponentially increases with increase in the concentration of the extract from 100 to 500 µg mL^{-1}. The percentage of inhibition of the metal chelation was varying, while chelating activity in the methanol extracts of all five species were present in Table 4. Apparently the methanolic extracts of study plants exhibited excellent chelating ability for ferrous ions and might afford protection against oxidative damage. Where, the chelating agent's forms σ bond with a metal, are effective as secondary antioxidants, because they reduce the redox potential there by stabilizing the oxidized form of the metalion.[23]

Table 1: Estimation of Total phenol and Total phenol

S.No.	Plants	Total phenol /100 g	Total flavonoid /100 g
1	Pogostemon mollis	2.5	16.3
2	Pogostemon vestitus	2.8	14.3
3	Pogostemon nilagiricus	1.6	14.2
4	Pogostemon wightii	1.97	15.8
5	Pogostemon speciosus	2.3	12.4

Table 2: DPPH radical scavenging activity

Concentration	% of inhibition (µg/mL)				
	Pogostemon mollis	Pogostemon vestitus	Pogostemon nilagiricus	Pogostemon wightii	Pogostemon speciosus
100	31.48	44.85	21.008	55.35	22.16
200	45.21	54.58	31.39	59.87	29.66
300	65.1	62.41	41.53	70.65	52.52
400	81.32	68.23	49.46	76.75	60.44
500	88.98	74.64	68.11	81.06	67.57
IC_{50} (µg/mL)	253.8	279.32	370.37	251.25	337.83

Table 3: ABTS radical scavenging activity

Concentration (µg/mL)	Concentration of ABTS (µmol/g)				
	Pogostemon mollis	Pogostemon vestitus	Pogostemon nilagiricus	Pogostemon wightii	Pogostemon speciosus
20	4661.5±40.3	4249.8±4.7	3592.7±2.9	4846.8±3.0	3541.7±3.5
40	1675.7±23.2	1795.5±11.7	1032.2±0.5	2017.1±2.6	1214.5±0.5
60	653.6±3.9	678.4±0.0	489.0±0.3	1008.7±0.2	371.0±0.4
80	281.8±12.7	166.2±3.9	100.6±1.2	516.3±0.1	101.2±0.1

Table 4: Ferrous ion chelating activity

Concentration	% of inhibition (µg/mL)					
	EDTA	Pogostemon mollis	Pogostemon vestitus	Pogostemon nilagiricus	Pogostemon wightii	Pogostemon speciosus
100	42.799	32.974	69.31	29.2	14.26	49.93
200	46.43	39.03	53.56	35.12	26.37	39.03
300	68.775	44.01	41.99	50.47	49.93	55.85
400	73.485	60.026	58.68	58	51.68	65.54
500	80.484	64.064	61.1	63.79	61.37	61.37

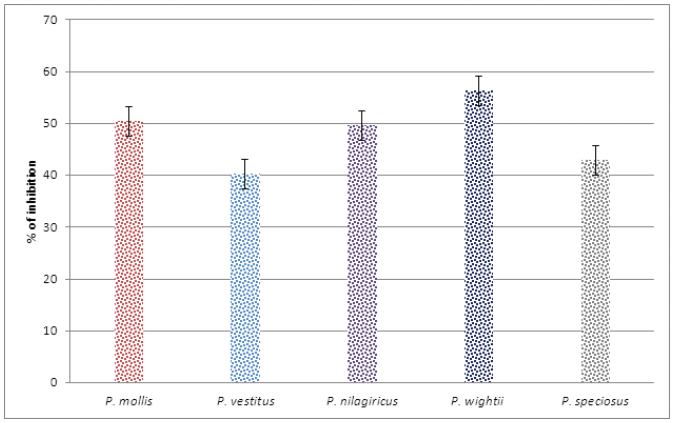

Figure 1: Hydroxyl radical scavenging activity

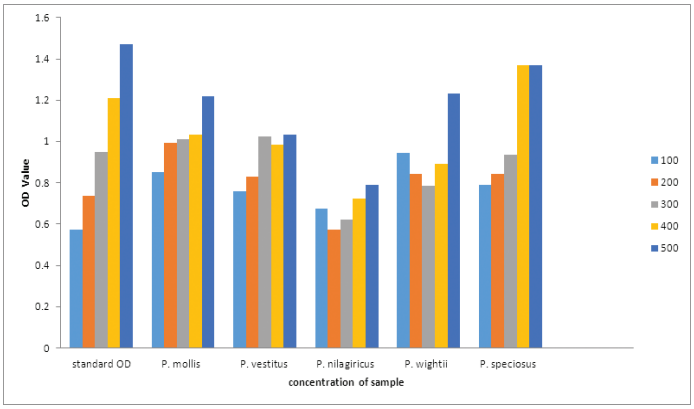

Figure 2: Reducing power assay

Hydroxyl radical scavenging activity (HRSA)

Hydroxyl radical is considered as extremely reactive in biological systems and has been implicated as highly damaging species in free radical pathology, capable of damaging bio-molecules of cells. Hydroxyl radical scavenging capacity of the extract is directly related to its antioxidant activity. It was carried out by measuring the competition between deoxyribose and the extract for hydroxyl radicals generated. In the present study, the hydroxyl radical scavenging activity for the methanolic extracts of *P. mollis*, *P. vestitus*, *P. nilagiricus*, *P. wightii* and *P. speciosus* were found to be 50.45± 0.07, 40.3± 0.06, 49.51± 0.03, 56.34± 0.04 and 42.91± 0.0.1 respectivly (Figure 1).

Ferric reducing antioxidant power (FARP) assay

Reducing power activity is often used to evaluate the ability of natural antioxidant to donate electron.[24] FRAP assay depends upon the ferric tripyridyltriazine (Fe (III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe (II)-TPTZ) by a reductant at low pH. Fe (II) - TPTZ has an intensive blue color and can be monitored at 593 nm.[25] There is a direct correlation between antioxidant activities and reducing power of certain plant extracts.[26] In this study, the methanol extracts exhibited significant reducing power activity in the plants extracts, the reductive capabilities for the methanolic extracts of *P. mollis*, *P. vestitus*, *P. nilagiricus*, *P. wightii* and *P. speciosus* were presented in (Figure 2). According to the results, it can be said that methanolic extracts of plants have been significant activities on the reducing power activity.

CONCLUSION

The all five *Pogostemon* species were subjected to screening for their possible antioxidant activity by DPPH and ABTS•+ scavenging assay, Reducing power assay, Ferrous ion chelating activity and Hydroxyl radical scavenging activity. To the best of our knowledge, this is the better antioxidant activity report of the five Lamiaceae plant extracts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATION USED

DMSO: Dimethyl sulfoxide; **GAE:** Gallic Acid Equivalent; **DPPH•:** (2, 2-diphenyl-1-picryl- hydrazyl); **ABTS•+:** 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid; **HRSA:** Hydroxyl radical scavenging activity and FARP- Ferric reducing antioxidant power.

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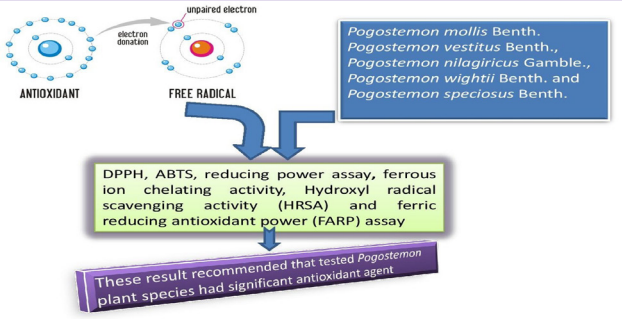
PICTORIAL ABSTRACT



SUMMARY

- The Oxidation damages are induced by highly reactive oxygen species in human body. Antioxidants terminate the inactive of these radicals with reduce the risk. In this study, we compared the antioxidant properties of five *Pogostemon* species through the *in vitro* antioxidant activity assays with evaluation of antioxidants compound total phenols and total flavonoid. The presence of high amount of phenols and flavonoid, the metanolic extract's antioxidant can strongly scavenge DPPH and ABTS radical with significant IC50 value range. Other modals of experimented where have potential antioxidant activity. These *Pogostemon* species had significant antioxidant agent, it could be an excellent source of antioxidants with great explore as therapeutic agent.