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Der Pharmacia Lettre, 2016, 8 (17):202-206
(http://scholarsresearchlibrary.com/archive.html)

Der Pharmacia Lettre
Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

In vitro antioxidant activities, total flavonoids and total phenolic content of ethanolic extract from the plant of *Pleiospermium alatum*

***Ramprasad and Madhusudhan**

Department of Pharmacy, Annamalai University, Annamalai Nagar-608 002, Tamil Nadu, India

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ABSTRACT

To evaluate the in vitro antioxidant activities and to estimate total phenol and flavonoids of the crude ethanolic extract from the bark and leaf of the plant Pleiospermium alatum. The shade dried powder ofbark and leaf was extracted with ethanol by using soxhlet extractor and crude extract was used for in vitro antioxidant activity by DPPH, nitric oxide, iron chelating, hydroxyl radical, super oxide radical scavenging, total antioxidant and FRAP assay methods. Total phenols and flavonoids also estimated. The results revealed that P. alatumethanolic extract have higher antioxidant property and contains large amount of phenols and flavonoids. This study helps to predict it is a better source of natural antioxidant and contains large amount of phenols and flavonoids which can be used as drugs and further investigation may lead to the development of drug formulation.

Keywords: Ethanolic extract, *In vitro* antioxidant, *Pleiospermium alatum*, Total flavonoids.

INTRODUCTION

Pleiospermium alatum.; Synonyms, *L. heyneana*.. *Pleiospermium alatum*(Wall exight&Arn)Swingle belongs to the family Rutaceae. It is widely grown in India, as a common weed. It is considered as valuable medicinal herb in traditional systems of medicine in India. Action diuretic, slightly aperient. It is used as a diuretic in calculous affections, also for chronic obstruction of liver and bowels [1]. A smaller var., found in western Uttar Pradesh, Rajasthan, Saurashtra and the Deccan Penninsula, is equated with *P. alatum*.

However, no data are available in the literature on the antioxidant activity of *P. alatum*. Therefore we undertook the current investigation to examine antioxidant activities, total flavonoids and total phenolic contents of ethanolic extract from bark and leaf of the plant *P. alatum* through various *in vitro* models.

MATERIALS AND METHODS

2.1. Collection and preparation of plant material

The bark and leaf of the fresh plants *Pleiospermium alatum* was collected from the natural habitats of Kayathar, Thoothukkudi district, Tamil Nadu, India. Taxonomic identification was made from Botanical Survey of Medical Plants Unit Siddha, Government of India, Palayamkottai. The samples were washed thoroughly in running tap water to remove soil particles and adhered debris and finally washed with sterile distilled water. The bark and leaf of the plants were shade dried and ground into fine powder. The powdered materials were stored in air tight polythene bags until use.

2.2. Plant sample extraction

The powder samples of *Pleiospermium alatum* were extracted with ethanol at temperature between 60-65˚C by hot continuous percolation method in Soxhlet apparatus [2]for 24 hrs. The extract was concentrated by using a rotary evaporator and subjected to freeze drying in a lyophilizer till dry powder was obtained.

2.3. Evaluation of antioxidant activities by i*n vitro* techniques

DPPH photometric assay

The effect of ethanolic extract on DPPH radical was assayed using the Mensor method [3]. A methanolic solution of 0.5mL of DPPH (0.4mM) was added to 1 mL of the different concentrations of plant extract and allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol without the extracts served as the positive control. After 30 min, the absorbance was measured at 518 nm and converted into percentage radical scavenging activity as follows.

$$Scavenging activity(\%) = \frac{A_{518}\ \text{Control} - A_{518}\ \text{Sample}}{A_{518}\ \text{Control}} \times 100$$

Where A_{518} control is the absorbance of DPPH radical+ methanol; A_{518} sample is the absorbance of DPPH radical+ sample extract/ standard.

Nitric oxide radical scavenging activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Garrat method [4]. The reaction mixture (3mL) containing 2 mL of sodium nitroprusside (10mM), 0.5 mL of phosphate buffer saline (1M) were incubated at 25^0C for 150 mins. After incubation, 0.5 mL of the reaction mixture containing nitrite was pipetted and mixed with 1 mL of sulphanilic acid reagent (0.33%) and allowed to stand for 5 min for completing diazotization. Then 1 mL of naphthylethylenediaminedihydrochloride (1% NEDA) was added, mixed and allowed to stand for 30 mins. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions which can be estimated by the use of GriessIlosvay reaction at 540 nm.

Iron chelating activity

The Benzie and strain method [5] was adopted for the assay. The principle is based on the formation of *O*-Phenanthroline-Fe^{2+} complex and its disruption in the presence of chelating agents. The reaction mixture containing 1 mL of 0.05% O-Phenanthroline in methanol, 2 mL ferric chloride (200µM) and 2 mL of various concentrations of ethanolic extracts ranging from 125 to 1000µg was incubated at room temperature for 10 min and the absorbance of the same was measured at 510 nm. EDTA was used as a classical metal chelator. The experiment was performed in triplicates.

Hydroxyl radical scavenging activity

This was assayed as described by Elizabeth and Rao method [6]. The assay is based on quantification of degradation product of 2-deoxy ribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+} -Ascorbate –EDTA –H_2O_2system (Fenton reaction). The reaction mixture contained 0.1 mL deoxyribose (2.8mM),0.1 mL EDTA (0.1 mM), 0.1 mL H_2O_2 (1mM), 0.1 mL ascorbate (0.1mM), 0.1 mL KH_2PO_4-KOH buffer, pH 7.4 (20mM) and various concentrations of ethanolic extract from *P. alatum* in a final volume of 1 mL. The reaction mixture was incubated for 1 hour at 37^0 C. Deoxyribose degradation was measured as TBARS and the percentage inhibition was calculated.

Superoxide radical scavenging activity

Superoxide radical (O_2^-) was generated from the photoreduction of riboflavin and was deducted by nitro blue tetrazolium dye (NBT) reduction method. Measurement of superoxide anion scavenging activity was performed based on the method described by Winterbourne[7]. The assay mixture contained sample with 0.1mL of Nitro blue tetrazolium (1.5 mM NBT) solution, 0.2 mL of EDTA (0.1M EDTA), 0.05 mL riboflavin (0.12 mM) and 2.55 mL of phosphate buffer (0.067 M phosphate buffer). The control tubes were also set up where in DMSO was added instead of sample. The reaction mixture was illuminated for 30 min and the absorbance at 560 nm was measured against the control samples. Ascorbate was used as the reference compound. All the tests were performed in triplicate and the results averaged. The percentage inhibition were calculated by comparing the results of control and test samples.

Total antioxidant activity (Phosphomolybdic acid method)

The antioxidant activity of the ethanolic extract of *P. alatum*was evaluated by the transformation of Mo (VI) to Mo (V) to form phosphomolybdenum complex [8]. An aliquot of 0.4 mL of ethanolic extract was mixed in a vial with 4 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vials were capped and incubated in a water bath at 95^0C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was read at 695 nm against a blank. The antioxidant activity was expresses relative to that of ascorbic acid.

FRAP assay

A modified method of Benzie and Strain [5] was adopted for the FRAP assay. The stock solutions included 300 mM acetate buffer, pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mMHCl and 20 mM $FeCl_3.6H_2O$. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $FeCl_3.6H_2O$. The temperature of the solution was raised to 37^0 C before using. Ethanolic extract of *P. alatum* (0.15 mL) was allowed to react with 2.85 mL of FRAP solution for 30 min in the dark condition. Readings of the coloured product (Ferrous tripyridyltriazine complex) were measured at 593 nm. The standard curve was linear between 200 and 1000 µM $FeSO_4$. Results were expressed in µM (Fe (II) /g dry mass and compared with that of ascorbic acid.

Total flavonoids

The measurement of total phenol is based on method [9].To 0.2g of the ethanolic extract of *P. alatum* was ground with ethanol-water in 2 different ratios namely 9:1 and 1:1 respectively. The homogenate was filtered and these 2 ratios were combined. This was evaporated to dryness until most of the ethanol has removed. The resultant aqueous extract was extracted in a separating funnel with hexane or chloroform. The solvent extracted aqueous layer was concentrated 0.5 mL of aliquot of extract was pipette-out in a test tube. 4 mL of the vanillin reagent (1% vanillin in 70% conc. H_2SO_4) was added and kept in a boiling water bath for 15 mints. The absorbance was measured at 360 nm. A standard was run by using catechol (110 µg/mL).

Total phenol

The measurement of total phenol is based on method [10]. To 0.25g of ethanolic extract of *P. alatum*, added 2.5 mL of ethanol and centrifuged at 2^oC for 10 mins. The supernatant was preserved. Then, the ethanolic extract was re-extracted with 2.5 mL of 80% ethanol and centrifuged. The pooled supernatant was evaporated to dryness. Then, added 3 mL of water to the dried supernatant. To which added 0.5 mL of Folins phenol reagent and 2 mL of sodium carbonate (20%). The reaction mixture was kept in boiling water bath for 1 min. The absorbance was measured at 650 nm in a spectrophotometer.

RESULTS AND DISCUSSION

DPPH is a stable free radical at room temperature often used to evaluate the antioxidant activity of several natural compounds. The percentage of DPPH radical scavenging activity of ethanolic extract of *P. alatum* exhibited a maximum DPPH scavenging activity of 62.68% whereas for rutin (standard) was found to be 69.83% at 1000 µg/mL presented in Table 1. The IC_{50} of the ethanolic extract and rutin were found to be 530µg/mL and 480µg/mL respectively.

The percentage of nitric oxide free radical scavenging activity of ethanolic extract of *P. alatum* shown maximum activity was 56.25% and standard (ascorbate) was found to be 62% at 1000 µg/mL presented in Table 1. The IC_{50} of the ethanolic extract and standard (ascorbate) were found to be 480µg/mL and 410µg/mL better antioxidant is respectively.

Table 1: Effect of ethanolic extract of *P. alatum* on DPPH assay&Nitric oxide free radical scavenging method

S.No	Concentration (µg/mL)	% of activity(±SEM)*		% of activity(±SEM)*	
		Sample (Ethanolic extract)	Standard (Rutin)	Sample (Ethanolic extract)	Standard (Ascorbate)
1	125	20.46±0.052	18.85 ± 0.076	30.61±0.10	27.63±0.076
2	250	29.65±0.076	22.08 ± 0.054	42.25±0.12	49.53 ±0.054
3	500	45.45±0.068	52.21 ± 0.022	51.35±0.11	55.12±0.022
4	1000	62.68±0.021	69.83 ± 0.014	56.25±0.12	62.00±0.014
		IC_{50} = 530 µg/mL	**IC_{50} = 480 µg/mL**	**IC_{50} = 480 µg/mL**	**IC_{50}=410 µg/mL**

**All values are expressed as mean ±SEM for three determinations*

Iron binding capacity of the ethanolic extract of *P. alatum* and the metal chelator EDTA at various concentrations were examined and the values were presented in table 2. Maximum chelating of metal ions at 1000µg/mL for ethanolic extract and EDTA was found to be 86.95% and 97.90% respectively. The IC_{50} value of ethanolic extract and EDTA were recorded as 435µg/mL and 65µg/mL respectively.

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins [11].The percentage of hydroxyl radical scavenging activity of ethanolic extract of *Pleiospermium alatum* was presented in Table 2. The ethanolic extract of *P. alatum* was exhibited a maximum hydroxyl radical scavenging activity of 63.20 % whereas for ascorbate (standard) were found to be 55.23 % at 1000 µg/mL. The IC_{50} of the ethanolic extract and ascorbate were found to be 565µg/mL and 410µg/mL respectively.

Table 2: Effect of ethanolic extract of *P. alatum* on Iron chelating method & Hydroxyl radical scavenging activity

S.No	Concentration (µg/mL)	% of activity(±SEM)*		% of activity(±SEM)*	
		Sample (Ethanolic extract)	Standard (EDTA)	Sample (Ethanolic extract)	Standard (Ascorbate)
1	125	20.18 ± 0.044	58.68 ± 0.007	36.80 ± 0.036	26.87 ± 0.076
2	250	35.46 ± 0.022	65.87 ± 0.018	44.36 ± 0.040	30.30 ± 0.054
3	500	56.32 ± 0.012	83.83 ± 0.012	55.35 ± 0.044	60.64 ± 0.022
4	1000	86.95 ± 0.010	97.90 ± 0.019	63.20± 0.034	55.23 ± 0.014
		IC_{50} = 435 µg/mL	**IC_{50} = 65 µg/mL**	**IC_{50} = 565 µg/mL**	**IC_{50} = 410 µg/mL**

**All values are expressed as mean ±SEM for three determinations*

Percentage scavenging of superoxide anion examined at different concentrations of ethanolic extract of *P. alatum* were depicted in table 3. The maximum scavenging activity of ethanolic extract of *P. alatum* and quercetin at 1000 µg/mL was found to be 76.15% and 98.01% respectively. Superoxide scavenging ability of plant extract might primarily be due to the presence of flavanoids[12]. The IC_{50} value of ethanolic extract and quercetin were recorded as 210µg/mL and 60µg/mL respectively.

The percentage of total antioxidant activity of ethanolic extract of *P. alatum*presented in Table 3. The ethanolic extract of *P. alatum* exhibited a maximum total antioxidant activity of 68.33 % at 1000 µg/mL whereas for ascorbate (standard) was found to be 55.23 % at 1000 µg/mL. The IC_{50} of the ethanolic extract and ascorbate were found to be 530µg/mL and 410µg/mL respectively.

Table 3: Effect of ethanolic extract of *P. alatum* on superoxide anion scavenging activity &Total antioxidant activity

S.No	Concentration (µg/mL)	% of activity(±SEM)*		% of activity(±SEM)*	
		Sample (Ethanolic extract)	Standard (Quercetin)	Sample (Ethanolic extract)	Standard (Ascorbate)
1	125	42.54 ± 0.046	73.81 ± 0.006	22.62 ± 0.028	26.87 ± 0.076
2	250	54.08 ± 0.022	91.31 ± 0.011	34.98 ± 0.026	30.30 ± 0.054
3	500	67.91± 0.044	92.99 ± 0.024	48.06 ± 0.020	60.64 ± 0.022
4	1000	76.15± 0.030	98.01 ± 0.012	68.33 ± 0.042	55.23 ± 0.014
		IC_{50} = 210 µg/mL	**IC_{50} = 60 µg/mL**	**IC_{50} = 530 µg/mL**	**IC_{50} = 410 µg/mL**

**All values are expressed as mean ±SEM for three determinations*

Table 4: Effect of ethanolic extract of *P. alatum*on FRAP assay

S.No	Concentration (µg/mL)	% of activity(±SEM)*	
		Sample (Ethanolic extract)	Standard (Ascorbate)
1	125	40.56 ± 0.032	72.04 ± 0.014
2	250	59.22 ± 0.030	82.05 ± 0.034
3	500	65.98 ± 0.022	86.04 ± 0.026
4	1000	76.68 ± 0.044	98.07 ± 0.041
		IC_{50} = 170 µg/mL	**IC_{50} = 50 µg/mL**

**All values are expressed as mean ±SEM for three determinations*

The antioxidant potential of *Pleiospermium alatum* was ascertained from FRAP assay based on their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). The reducing ability of the ethanolic extract of *P. alatum* and ascorbate at various concentrations were examined and the values are presented in Table 4. The maximum reducing

ability at 1000µg/mL for ethanolic extract and ascorbate were found to be 76.68% and 98.07% respectively. The IC_{50} values of ethanolic extract and ascorbate were recorded as 170µg/mL and 50µg/mL respectively.

Flavonoids present in food of plant origin are also potential antioxidants [13,14]. Most beneficial effects of flavonoids are attributed to their antioxidant and chelating abilities [15, 16]. The total amount of flavonoids content of ethanolic extract of *P. alatum* was presented in Table 5.

Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups [17]. Phenolic compounds are known as powerful chain breaking antioxidants [18]. The total amount of phenolic content of ethanolic extract of *P. Alatum* was presented in Table 5.The ethanolic extract of bark and leaf of the plant *P. Alatum* was found higher content of phenolic components.

Table 5: Total flavonoids & total phenolic content of ethanolicextract of *P. alatum*

S.No	Extract	Total flavonoids content (mg/g ±SEM)*	Total phenol content (mg/g of Catechol) ± SEM)*
1	Ethanolic extract of *Pleiospermium alatum*	1.964 ± 0.040	2.462 ± 0.030

**All values are expressed as mean ±SEM for three determinations*

CONCLUSION

From the results obtained in the present study, it was concluded that ethanolic extract of *Pleiospermium alatum*, exhibits high antioxidant and free radical scavenging activities, which contains large amounts of phenolic compounds. These *in vitro* assays indicate that this plant extracts is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. Therefore, further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract and *in vivo* antioxidant activity of this extract needs to be assessed prior to clinical use.

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