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In vitro antioxidant and radical scavenging activity of different extracts of *Pouzolzia indica*

Indranil Kumar Yadav[1], Hari Pratap Singh[1] D.A. Jain[1], Anas Ahmed[2] and Durga Indranil[3]

[1]Institute of Pharmacy, Bhagwant University, Ajmer- 305004, India.

[2]Department of Pharmacognosy, MCOPS, Manipal-576104, India.

[3]Dr. B.R. Ambedkar Pooja College of Pharmacy, Gorakhpur- 273013, India.

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ABSTRACT

Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. A great number of aromatic, medicinal, spice and other plants contain chemical compounds exhibiting antioxidant properties. In the present study the antioxidant activity and radical scavenging activity of aqueous and alcoholic extracts of *Pouzolzia indica*, traditionally used by Indian population as folk remedies was evaluated.

Introduction

It has been established that oxidative stress is among the major causative factors in the induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others[1]. In living systems, free radicals are generated as part of the body's normal metabolic process, and the free radical chain reactions are usually pro-duced in the mitochondrial respiratory chain, liver mixed function oxidases, through xanthine oxi-dase activity, atmospheric pollutants and from transitional metal catalysts, drugs and xenobiotics.In addition, chemical mobilization of fat stores under various conditions such as lactation, exercise, fever, infection and even fasting, can result inincreased radical activity and damage. Free radi-calsor oxidative injury now appears the fundamentalmechanism underlying a number of human neurologic and other disorders. Oxygen free radical can initiate peroxidation of lipids, which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes, and play a role in the long-term complication of diabetes[2-6].

The most effective way to eliminate free radicals which cause the oxidative stress is with the help of antioxidants. Antioxidants are those substances which possess free radical chain reaction breaking properties. Antioxidants, either exogenous or endogenous, whether synthetic or natural, can be effective in preventing free radical formation by scavenging them or promoting their decomposition and suppressing such disorders[1, 7].

Plants are potent biochemical factories and have been components of phytomedicine since times immemorial; man is able to obtain from them a wondrous assortment of industrial chemicals. Plants based natural constituents can be derived from any part of plant like bark, leaves, flowers, roots, fruits, seeds, etc i.e. any part of the plant may contain active components[8]. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. The medicinal actions of plants are unique to particular plant species or groups are consistent with this concept as the combination of secondary products in a particular plant is taxonomically distinct.Recently there has been an upsurge of interest in the therapeutic potential medicinal plants as antioxidants in re-antioxidants in reducing oxidative stress-induced tissue injury[9]. Among the numerous naturally occurring antioxidants; ascorbic acid, carotenoids and phenolic compounds are more effective. They are known to inhibit lipid peroxidation (by inactivating lipoxygenase), to scavenge free radicals and active oxygen species by propagating a reaction cycle and to chelate heavy metal ions.

The present study evaluated the potential antioxidant activity and radical scavenging activity of of different extracts of *Pouzolzia indica* by using *in vitro* method.

Experimental:

Collection of the plant material:

The aerial parts of *Pouzolzia indica* (Family: Urticaceae) were collected from Manipal, Udupi District, Karnataka, India, in the month of September-November and authenticated by botanist Dr. Gopalakrishna Bhat, Professor of Botany, Poorna Prajna College, Udupi, India. A herbarium specimen bearing voucher No. PP. (555) has been deposited in the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal University. All other chemicals were used of AR grade.

Preparation of successive solvent extracts[10, 11]:

a) Ethanolic extract:

The marc left after acetone extraction was dried and extracted with (95%) ethanol by hot extraction process

Tele:

E-mail addresses: neel_pharma@rediffmail.com

(Soxhlet). After completion of extraction, the solvent was removed by distillation, concentrated and then stored in a dessicator until further use.

b) Aqueous extract:

The marc left after ethanol extraction was dried and extracted with chloroform: water (1:99) by maceration process for 7 days. After completion of extraction, the solvent was removed by distillation, concentrated and then stored in a dessicator until further use.

The various extracts obtained by successive solvent extraction were divided in to two portion for anti-snake venom studied and phytochemical investigations.

Phytochemical analysis of different extracts[10, 11]:

Different chemical tests were carried out for both extracts of *P. indica* to identify the presence of various chemical constituents like alkaloids, saponins, carbohydrates, glycosides, steroids, fixed oils and fats, tannins and phenolic compounds, proteins and amino acids, gums and mucilage and flavonoids.

***In vitro* antioxidant study**

Scavenging Of 1,1- Diphenyl-2-Picryl Hydrazyl (DPPH) Free Radical[12, 13]

The DPPH free radical scavenging activity was assessed according to Sreejayan and John *et al*. method. To the ethanolic solution of DPPH (1 mM) an equal volume of the test compound dissolved in methanol was added at various concentrations (2-1024 µg/ml) in a final volume of 1.0 ml. An equal amount of methanol was added to the control. After 20 min, absorbance was recorded at 517 nm. The percentage of scavenging was calculated according to the following equation:

$$\% \text{ Scavenging} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

Scavenging of ABTS Free Radical[12, 13]

ABTS scavenging assay was carried out following a modified method. Briefly, ABTS radical cation (ABTS) solution was produced by reacting ABTS stock solution with 2.45 mmol/L potassium persulfate in the dark for 12 h and adjusting the absorbance to 0.70±0.02 at 734 nm. For the photometric assay, 3 ml of the ABTS solution and 100 µL samples solution were mixed and measured immediately after 6 min at 734 nm (absorbance did not change significantly up to 10 min). The ABTS free radical scavenging rate was calculated in the following formulation:

$$\% \text{ Scavenging} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

O-Phenanthroline Method Reduction of Ferric Ions[12, 13]

The reducing power of *P. indica* was determined according to the method previously described (Sreejayan 1996, John *et al*. 1984).

The reaction mixture containing O-Phenanthroline (0.5 mg), Ferric chloride (200 µ M), and drug at various concentrations ranging from 2 µg to 1000 µg/ml in a final volume of 5 ml was incubated for 10 minutes at ambient temperature. The absorbance at 510 nm was recorded. Ascorbic acid was added instead of test compound and Absorbance obtained taken as equivalent to 100% reduction of all ferric ions. Blank was carried out without drug.

Superoxide dismutase Scavenging Activity (Alkaline DMSO method)[14, 15]

The scavenging of Superoxide dismutase activity was determined according to Govindarajan *et al*. 2003 method. In this method, 1 ml alkaline DMSO and 0.2 ml NBT 20 mM (50 mg in 10ml phosphate buffer pH 7.4) was added to the 0.5 ml of different concentration of extract. The absorbance was measured at 560 nm. Ascorbic acid was used as a standard. The % scavenging was calculated by following equation:

$$\% \text{ Scavenging} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

Spontaneous lipid peroxidation method[14, 15]

The *in vitro* lipid peroxidation assessed according to Govindarajan *et al*. 2003 method. This study requires homogenate rat brain. Albino rats (180-200g) of either sex were used for the study. After decapitation, the brain was removed carefully. The tissue was immediately weighed and homogenated with cold 1.15% KCl to make 10% homogenate. This homogenate was immediately used for the *in vitro* lipid peroxidation study.

The 0.5ml of rat brain homogenate was added to the 1 ml of various concentrations of the drug. Then the mixture was incubated for 30 min. The peroxidation was terminated by the addition of 2 ml of TBA-TCA –HCl reagent (15% w/v Trichloroacetic acid, 0.375%w/v thiobarbituric acid and 0.25N hydrochloric acid). The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent ppt was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the supernatant was measured at 535 nm. The % scavenging was calculated by following equation:

$$\% \text{ Scavenging} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

Total antioxidant activity[16, 17]

The total antioxidant activity of *P. indica* was determined according to Shirwaikar *et al.* 2004, Prieto *et al.* 1999 method. In this method, 0.1ml of extract (10mg/ml) dissolved in water added to 1 ml of the reagent containing 0.6 M sulphuric acid, 28 mM Sodium Phosphate and 4 mM Ammonium Molybdate and incubated at 95^{o}C for 90 min, cooled to room temperature, measured the absorbance at 695 nm. The antioxidant activity was expressed as the number of equivalents of ascorbic acid.

Results and discussion:

Aqueous and ethanolic extracts of *P. indica* were investigated for their potential antioxidant activity. The average value of extractive and fluorescence analysis was shown in Table 1.

The obtained extracts were subjected to phytochemical screening for its constituents by standard methods and the results were tabulated in Table 2. Preliminary phytochemical studies revealed the presence of alkaloids, carbohydrate, Phenolic compounds, gum and mucilage.

In vitro tests of alcoholic and aqueous extract of aerial part *P. indica*, evaluated for its antioxidant property revealed DPPH, ABTS free radical, total antioxidant, super oxide (NBT alkaline DMSO), and spontaneous lipid peroxidation effect.

The antioxidant reacts with stable free radical, DPPH and converts it to 1,1-diphenyl–2-picryl hydrazine. The ability to scavenge the stable free radical DPPH was measured by decrease in the absorbance at 517 nm. The ethanol and water extracts of *P. indica* exhibited a significant dose dependent

inhibition of DPPH activity (Table 3). A concentration-dependent assay was carried out with these extracts and the results are presented in Fig.1, but the alcoholic extract has more prominent scavenging activity rather than aqueous extract.

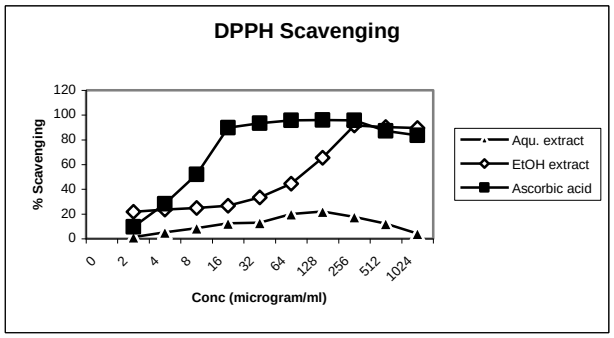

Fig. 1: DPPH radical scavenging activity of *P. indica*

ABTS free radical is another synthetic radical and more versatile than DPPH, and the ABTS model can be used to assess the scavenging activity for both the polar and non-polar samples. From the results (Figure 2), ethanol and water extracts of *P. indica* were able to scavenge ABTS free radical in a dose-dependent manner. However, radical scavenging ability of ethanolic extract was stronger than that of aqueous extract (Table 4).

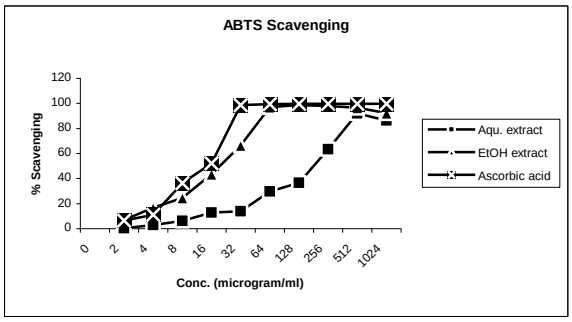

Fig. 2: ABTS Free radical scavenging activity of *P. indica*

The reducing ability of a compound generally depends on the presence of reductants which have been exhibited antioxidative potentialby breaking the free radical chain, donating a hydrogen atom. The presence of reductants (i.e. antioxidants) in *P. indica* leaves and flowers extracts causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Figure 3 shows the reductive capabilities of the *P. indica* leaves and flowers extracts compared to ascorbic acid. The reducing power of *P. indica* leaves and flowers extracts was very potent and the power of the extract was increased with quantity of sample (Table 5).

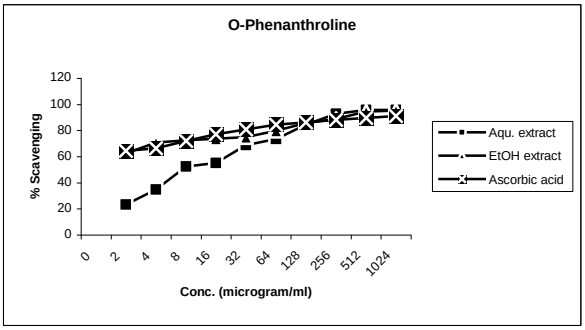

Fig. 3: Reduction of Ferric Ions by *P. indica* extracts (O-Phenanthroline Method)

Alkaline DMSO was used as super oxide generating system. The generated super oxide will react with NBT to give coloured diformazan. Diformazan being insoluble in water slowly precipitates out. Therefore, the spectral measurement must be done immediately after the reaction is carried out. In the presence of scavenger, reduction of NBT will occur which is measured at 560 nm. The ethanol and water extracts of *P. indica* exhibited prominent scavenging activity in dose dependent manner (Fig.4), but the aqueous extract showed somewhat more scavenging rather than ethanolic extract. As the concentration was increased the scavenging activity also increased, but at higher doses it fell down (Table 6). Ascorbic acid was used as a standard.

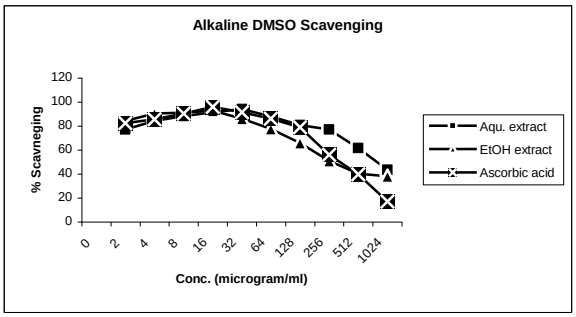

Fig. 4: Superoxide Dismutase Scavenging Activity of *P. indica*

Malondialdehye, formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction. Malondialdehyde reacts with thiobarbituric acid to form TBARS to give red color species, which is measured at 535 nm. The result of lipid peroxydation assay of extracts of *P. indica* was shown in Table 7. It indicated the profound lipid peroxydation activity of water extracts rather than ethanolic extract (Fig.5)

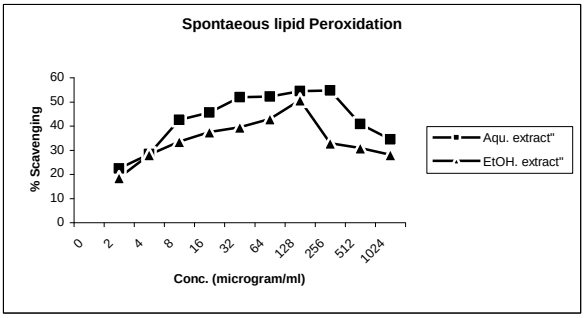

Fig. 5: Spontaneous Lipid Peroxidation by *P. indica* extracts

The result of total antioxidant activity of extracts of *P. indica* was shown in Table 8. it indicated that alcoholic extract (10mg/ml) is having activity equivalent to 0.365 mg/ml (365µg/ml) of ascorbic acid and aqueous extract (10mg/ml) is having activity equivalent to 0.175 mg/ml (175µg/ml) of ascorbic acid.

Conclusion:

It has been reported that reactive oxygen species contribute to various pathophysiological conditions and endogenous defense mechanisms have evolved to offer protection in these conditions. An increase in the antioxidant reserves of the organism can reduce oxidative stress and some of the plant derived agents may help to reduce it. Determination of the natural antioxidant compounds of plant extracts will help to develop new drug candidates for antioxidant therapy. The plants

may be considered as good sources of natural antioxidants for medicinal uses such as against aging and other diseases related to radical mechanisms. All methods have proven the effectiveness of the plant extracts compared to the reference standard antioxidant ascorbic acid. Further investigation on the isolation and identification of antioxidant component(s) in the plant may lead to chemical entities with potential for clinical use.

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Table 1: Average value of extractive and Fluorescence Analysis of extracts of *P. indica*

S.No.	Solvent	Colour and Consistency	Average value of Extractive (w/w)	Fluorescence Analysis	
				White light	UV light (254 nm)
1	Ethanol (95%)	Reddish brown (sticky semi solid)	6.3 %	Reddish brown	Light green
2	Water	Dark brown (non-sticky solid)	2.1 %	Dark brown	Green

Table 2: Phytochemical screening of *P. indica*

Test	Ethanol extract	Water extract
Alkaloids	+	-
Carbohydrates	+	+
Phytosterols	-	-
Fixed oils and fats	-	-
Saponins	-	-
Phenolic compounds and tannins	+	-
Gums and Mucilage	-	+

Table 3: DPPH radical scavenging activity of *P. indica*

S. No.	Conc. µg/ml	Aqueous extract		EtOH extract		Ascorbic acid	
		Abs	% Sca.	Abs	% Sca.	Abs	% Sca.
1.	2	0.661	1.36	0.524	21.79	0.441	9.56
2.	4	0.635	5.22	0.512	23.58	0.350	28.24
3.	8	0.612	8.66	0.503	24.92	0.234	52.08
4.	16	0.587	12.39	0.492	26.57	0.050	89.76
5.	32	0.583	12.99	0.446	33.43	0.033	93.27
6.	64	0.537	19.85	0.372	44.41	0.021	95.73
7.	128	0.523	21.94	0.231	65.52	0.019	95.92
8.	256	0.553	17.46	0.057	91.56	0.020	95.81
9.	512	0.558	12.24	0.064	90.38	0.063	87.17
10.	1024	0.643	4.03	0.071	89.37	0.080	83.58
11.	Control	0.670	-	0.670	-	0.487	-

Table 4: ABTS Free radical scavenging activity of *P. indica*

S. No.	Conc. µg/ml	Aqueous extract		EtOH extract		Ascorbic acid	
		Abs	% Sca.	Abs	% Sca.	Abs	% Sca.
1.	0.0	0.2502		0.2137		0.3637	
2.	2	0.2498	0.15	0.1987	7.01	0.3403	6.43
3.	4	0.2429	2.91	0.1783	16.56	0.3240	10.92
4.	8	0.2346	6.2	0.1613	24.52	0.2316	36.32
5.	16	0.2180	12.86	0.1212	43.28	0.1741	52.13
6.	32	0.2155	13.86	0.0716	66.49	0.0050	98.63
7.	64	0.1763	29.53	0.0061	97.14	0.0029	99.42
8.	128	0.1588	36.53	0.0029	98.64	0.0020	99.45
9.	256	0.0920	63.22	0.0050	97.66	0.0018	99.50
10.	512	0.0203	91.88	0.0076	96.44	0.0012	99.67
11.	1024	0.0343	86.29	0.0169	92.06	0.0015	99.58

Table 5: Reduction of Ferric Ions (O-Phenanthroline Method)

S. No.	Conc. µg/ml	Aqueous extract		EtOH extract		Ascorbic acid	
		Abs	% Sca.	Abs	% Sca.	Abs	% Sca.
1.	0.0	0.0105		0.0105		0.0206	
2.	2	0.0137	23.36	0.028	62.5	0.0580	64.48
3.	4	0.0161	34.78	0.036	70.83	0.0612	66.34
4.	8	0.0221	52.49	0.038	72.44	0.0737	72.05
5.	16	0.0234	55.13	0.040	73.75	0.0903	77.19
6.	32	0.0337	68.84	0.042	75.00	0.1070	80.74
7.	64	0.0394	73.35	0.052	79.80	0.1329	84.50
8.	128	0.0680	84.56	0.073	85.62	0.1506	86.12
9.	256	0.1466	92.84	0.094	88.83	0.1752	88.24
10.	512	0.2479	95.76	0.193	94.56	0.2000	89.70
11.	1024	0.2510	95.82	0.218	95.18	0.2294	91.02

Table 6: Superoxide Dismutase Scavenging Activity (Alkaline DMSO Method)

S. No.	Conc. µg/ml	Aqueous extract		EtOH extract		Ascorbic acid	
		Abs	% Sca.	Abs	% Sca.	Abs	% Sca.
1.	0.0	1.231		1.231		1.231	
2.	2	0.281	77.17	0.193	84.32	0.218	82.29
3.	4	0.195	84.15	0.111	90.48	0.178	85.54
4.	8	0.114	88.30	0.107	91.30	0.119	90.33
5.	16	0.104	91.55	0.087	92.93	0.050	95.95
6.	32	0.072	94.15	0.170	86.19	0.105	91.47
7.	64	0.148	87.97	0.275	77.66	0.170	86.19
8.	128	0.235	80.90	0.420	65.88	0.259	78.96
9.	256	0.281	77.17	0.604	50.93	0.535	56.13
10.	512	0.471	61.73	0.735	40.29	0.741	39.80
11.	1024	0.697	43.38	0.760	38.26	1.020	17.14

Table 7: Spontaneous Lipid Peroxidation Method

S. No.	Conc. µg/ml	Aqueous extract		EtOH extract	
		Abs	% Sca.	Abs	% Sca.
1.	0.0	0.5015		0.4212	
2.	2	0.3890	22.43	0.3429	18.58
3.	4	0.3590	28.41	0.3029	28.08
4.	8	0.2886	42.52	0.2820	33.47
5.	16	0.2732	45.52	0.2634	37.46
6.	32	0.2411	51.94	0.2551	39.43
7.	64	0.2395	52.24	0.2403	42.94
8.	128	0.2285	54.43	0.2076	50.71
9.	256	0.2274	54.65	0.2828	32.85
10.	512	0.2971	40.75	0.2911	30.88
11.	1024	0.3286	34.47	0.3029	28.08

Table 8: Total Antioxidant Activity of *P. indica* extracts

S. No.	Sample	Conc. mg/ml	Absorbance
1	Ascorbic acid	0.10	0.3060
		0.20	0.5790
		0.30	0.9034
		0.40	1.1833
		0.50	1.5040
2	EtOH extract	10.0	1.1042
3	Aqueous extract	10.0	0.5308