

In Vitro Antioxidant Activity of *Pterocarpus marsupium* Roxb. Leaves

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ABSTRACT

The leaves of *Pterocarpus marsupium* Roxb. (Fabaceae) was extracted in methanol and subjected to determination of total phenolic content. The plant yielded high phenolic content in the leaves (260 ± 0.021 mg gallic acid/g of dry plant material). The methanol extract was then screened for antioxidant activity using 1,1-diphenyl-2-picryl hydroxyl (DPPH) quenching assay, nitric oxide radical inhibition activity, scavenging capacity towards hydroxyl ion radicals, superoxide scavenging activity and ferric reducing activity power (FRAP) using established procedures. The data obtained in the present study showed a high level of antioxidant activity. A positive correlation was found between total phenolic content and the antioxidant activities of the extract. These findings indicate that phenolic components may have antioxidant capacity, as reported in other plants.

Keywords: DPPH, FRAP, hydroxyl ion, nitric oxide, superoxide, total phenolics

Abbreviations: DPPH, 1,1-diphenyl-2-picryl hydroxyl; FRAP, ferric reducing activity power

INTRODUCTION

Free radicals have been implicated with a variety of pathological processes, including cancer, diabetes, atherosclerosis, neurodegenerative disorders and arthritis. Antioxidants of plant origin are believed to help protect the cells from free radical damage. Epidemiological studies have indicated the role of fruits and vegetables for the reduction of these chronic diseases (Lieu 2003). These benefits are thought to result from the antioxidant components of plant origin, vitamins, flavonoids, and carotenoids (Pietta 2000; Rice-Evans 2001; Prior 2003). Therefore, several studies in recent years showed that polyphenols in plants scavenge active oxygen species and effectively prevent oxidative cell damage (Vagi *et al.* 2005). Hence, considerable attention has already been focused on the isolation, characterization and utilization of natural antioxidants from medicinal plants as potential disease preventing agents.

Pterocarpus marsupium Roxb., a member of Fabaceae, is a tall deciduous tree that grows up to 30 m height. It is used in the treatment of diabetes, bleeding piles, dysentery and in all skin inflammations (Nadkarni 1976). Despite several bioactivity studies on *P. marsupium* (Pandey and Sharma 1975; Jahromi and Ray 1993; Manickam 1997; Vats *et al.* 2002; Joshi *et al.* 2004; Mankani *et al.* 2005), the *in vitro* antioxidant activities have not been reported. In the view of the above, the present investigation was attempted to evaluate the total phenolics content, and to examine the potential antioxidant activities by means of DPPH radical quenching test, hydroxyl ion scavenging assay, nitric oxide scavenging activity, inhibition of superoxide ion assay and ferric reducing activity power (FRAP) of methanol extracts of *P. marsupium*.

MATERIALS AND METHODS

Chemicals

The experiments were performed using a scanning mini spectrophotometer (Elico, India). All chemicals used including the solvents, were of analytical grade. 1,1-diphenyl-2-picryl hydrazyl (DPPH), butylated hydroxyl toluene (BHT) and catechin were purchased from Sigma Chemical Co. folin ciocalteu reagent, gallic acid, trichloroacetic acid (TCA), ferric chloride, ascorbic acid, sodium nitroprusside, sulfanilamide, orthophosphoric acid, naphthyl ethylene diamine dihydrochloride, EDTA, ferrous ammonium sulfate, phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium chloride (NBT) and tripyridyl-triazine (TPTZ) were purchased from SD fine Chemicals.

Plant material

The leaves of *P. marsupium* were collected from Sadivayal ($10^{\circ} 56''$ N and $76^{\circ} 43''$ E), situated at the foot of Siruvani Hills in Western Ghats of India during March 2006. The plant was authenticated by Botanical Survey of India (Southern circle), Coimbatore. A voucher specimen was deposited at the herbarium of Karpagam University, Coimbatore.

Preparation of extract

The leaves were washed in tap water, shade dried and powdered. The powder was exhaustively extracted with methanol in the ratio of 1:7 (w/v) for 24 h by using Soxhlet apparatus. The extract was completely evaporated using rotary flash evaporator (Buchi type). Different concentrations of extracts were prepared from the resultant crude methanol extract to determine *in vitro* antioxidant assays. The yield of the crude methanol extract is given in **Table 1**.

Table 1 Antioxidant profile of the *P. marsupium* methanol extract and standards[#]

Sample	IC ₅₀ (µg mL ⁻¹)*			
	DPPH assay	Nitric oxide test	Hydroxyl radical assay	Super oxide assay
Methanol extract	42 ± 0.002 c	64 ± 0.013 a	41 ± 0.002 b	147 ± 0.01 a
Catechin ¹	ND	62.2 ± 0.04 b	16 ± 0.013 a	30.3 ± 005 c
BHT ²	26 ± 0.005 a	46 ± 0.002 d	19 ± 0.01 c	ND

[#]Each value in the table was obtained by calculating the average of three experiments (n=3) Mean ± Standard Deviation

* IC₅₀ value is defined as the concentration of the extract necessary to inhibit the radical concentration by 50%

ND - Not Determined

¹ Catechin - Reference Standard

² BHT- Reference Standard

Values in a column with different superscripts indicate significantly different at P>0.05

Analytical methods

1. Determination of total phenolics

The phenolic content was determined by a UV spectrophotometer using folin-Ciocalteu method (Sadasivam and Manickam 1992). An aliquot of the sample extract (0.1 ml) was mixed with distilled water (3 ml) and 0.5 ml of Folin-ciocalteu reagent was added. After 3 min 2 ml of 20% sodium carbonate was added and mixed thoroughly. The tubes were incubated in a boiling water bath for exactly 1 min. It was then cooled and the absorbance was measured at 650 nm using spectrophotometer (Elico Scanning mini spec SL 177, India) against the reagent blank. The results are the mean (n=3) ± standard deviation and expressed as gallic acid equivalent (mg GAE/g).

2. Antioxidant activity determination

The antioxidant activity of methanol extract of the leaves was determined by DPPH radical quenching assay, nitric oxide scavenging activity, hydroxyl ion scavenging, superoxide radical scavenging activity and FRAP.

3. DPPH radical scavenging assay

The DPPH radical scavenging activity was studied following the method of Blois (1958) with slight modifications. Briefly, various concentrations of samples were mixed with 5 ml of 0.1 mM methanol solution of DPPH and vortexed. The tubes were allowed to stand at room temperature for 20. The control was prepared as above without any extract, and methanol was used for the baseline correction. Changes in the absorbance of the samples were measured at 517 nm. Mean values were obtained from triplicate experiments. The percentage of decolorization was calculated according to the following formula:

$$\% \text{ Decolorization} = (\text{Control}_{\text{OD}} - \text{Sample}_{\text{OD}} / \text{Control}_{\text{OD}}) \times 100$$

Butylated hydroxyl toluene (BHT) was used as reference standard. A percentage inhibition versus concentration curve was plotted and the concentration of the extract required for 50% inhibition of radicals was expressed as IC₅₀ values (µg mL⁻¹).

4. Nitric oxide scavenging activity

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 Griess Illosvoy reaction. Nitric oxide generated from sodium nitroprusside was measured by the Griess reagent (Green *et al.* 1982). Stock solution (10 mM) of sodium nitroprusside was prepared in PBS (phosphate-buffered saline) (pH 7.4). Various concentrations of the extract and sodium nitroprusside (1 mM) in PBS in a final volume of 3 ml were incubated at 25°C for 150 min. After incubation, 0.5 ml of the solution was removed and diluted with 0.5 ml of the Griess reagent (1% sulfanilamide, 2% orthophosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was read at 546 nm. Percentage inhibition was calculated as:

$$\text{Nitric oxide scavenging activity (\%)} = (A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$$

where A_{control} is the absorbance of the control and A_{sample} is the absorbance of the test.

Catechin and BHT were used as positive controls.

5. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the *P. marsupium* extract was measured according to the method of Klein *et al.* (1991). Various concentrations of extracts was mixed 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). 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. After incubation the reaction was terminated by the addition of 1.0 ml of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0 g of ammonium acetate, 3.0 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the color formed was measured spectroscopically at 412 nm against reagent blank. Catechin and BHT were used as positive controls.

The percentage hydroxyl radical scavenging activity (HRSA) is calculated by the following formula:

$$\% \text{ scavenging} = 1 - (\text{difference in absorbance of sample} / \text{difference in absorbance of blank}) \times 100$$

6. Superoxide radical scavenging activity

The superoxide radical scavenging activity was assessed by the method of Yu *et al.* (2006) with a slight modification. Superoxide anion radicals generated in the phenazine methosulfate-reduced form of nicotinamide adenine dinucleotide (PMS-NADH-NBT) system by oxidation of NADH were assayed by the reduction of NBT. In this experiment, Superoxide anion radicals were generated in 1.25 ml of Tris -HCL buffer 16 mM, pH 8.0 containing 0.25 ml of NBT 300 M, 0.25 ml of NADH 468 M and extract/solution (20, 40, 60, 80, 100 or 120 g/ml). Then, 0.25 ml of PMS (60 M) solution was added to the mixture to start the reaction. The reaction mixture was incubated at room temperature for 5 min and the absorbance was read at 560 nm by spectrophotometer against a blank. Catechin was used as a control. The scavenging activity of the superoxide anion radicals was calculated by the following equation:

$$\% \text{ of scavenging} = [(A_0 - A_1) / A_0] \times 100]$$

where A₀ is the absorbance of the control (blank without extract) and A₁ is the absorbance in the presence of the extract/standard.

The plot of scavenging activity of superoxide anion radical was done and the IC₅₀ value (concentration of sample to scavenge 50% of superoxide anion radicals) was obtained.

7. Ferric reducing-antioxidant power assay (FRAP)

The reducing capacity of the extract was measured using FRAP assay described by Benzie and Strain (1996). FRAP reagent (1.8 ml) was mixed 0.2 ml of test sample, then incubated at 37°C for

10 min in a water bath. The FRAP reagent contained 20 mM TPTZ solution 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 0.3 M acetate buffer with pH 3.6. After incubation the absorbance were measured immediately at 593 nm. The calibration curve was plotted with OD vs concentration of FeSO_4 in the range of 0–1 mM and the total antioxidant activity was expressed as $\mu\text{mol Fe (II)}/100 \text{ g}$.

Statistical analysis

The experimental results concerning the study were mean standard deviation of three parallel measurements. Linear regression analysis was used to calculate the IC_{50} values. One-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were carried out to determine significant differences ($P < 0.05$) between the means of assays by SPSS (version 10 for Windows 98, SPSS Inc.).

RESULTS AND DISCUSSION

The identification and investigation on antioxidants from medicinal plants is a fast expanding field of research and several antioxidants have been investigated such flavonoids and other phenolic compounds. As mentioned in the introduction, the link between degenerative diseases and natural sources, particularly the antioxidant composition of the latter, has been recognized, leading to the production of dietary supplements. In the view of the upsurging interest in the health benefits of the medicinal plants, we examined total phenolics and evaluated the antioxidant effects of crude methanolic extract of *P. marsupium* leaves.

Analysis of total phenolics

The total phenolic content of *P. marsupium* leaves was performed based on Folin-Ciocalteu method. The leaves were found to contain high amounts of phenolics content. The amount of phenolic concentration was equal to $260 \pm 0.021 \text{ mg}$ of gallic acid equivalents (GAE) of dry material. The high antioxidant potential is attributed to its high phenolic content. Polyphenols are present in a variety of plants utilized as important components of both human and animal diets (Bravo 1998; Chung *et al.* 1998; Crozier *et al.* 2000). There is a strong evidence on the preventive effects of phenolics on age related chronic diseases (Boyer and Liu 2004; Kroon and Williamson 2005).

Antioxidant activities

Antioxidant quality is measure of the effectiveness of the antioxidant(s) present as a pure compound or a mixture (Vinson 2005). Therefore, the methanolic extract of *P. marsupium* were tested for their antioxidant properties in a range of in vitro assays to determine their potency to scavenge ROS. From this study, we found that methanolic leaf extract of *P. marsupium* exerted compatible inhibitory activity against free radicals.

DPPH radical scavenging activity

The DPPH assay constitutes a quick and low cost method, which has frequently been used for the evaluation of the antioxidative potential of various natural products (Molyneux 2003; Prior 2003). The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and radical, which results in the scavenging of the radical by hydrogen donation. This is visualized as a discoloration from purple to yellow. In the present study, the extract of the leaf exhibited a concentration dependent antiradical activity (Fig. 1) by quenching DPPH radical with an IC_{50} value of $42 \pm 0.002 \mu\text{g mL}^{-1}$ (Table 1). The quenching of DPPH radical by hydrogen donating antioxidants is characterized by a rapid decline in the absorbance at 517 nm. The results of this study infer that the methanol extract reduces the radical to the corresponding hydrazine when it reacts with the hydrogen ions

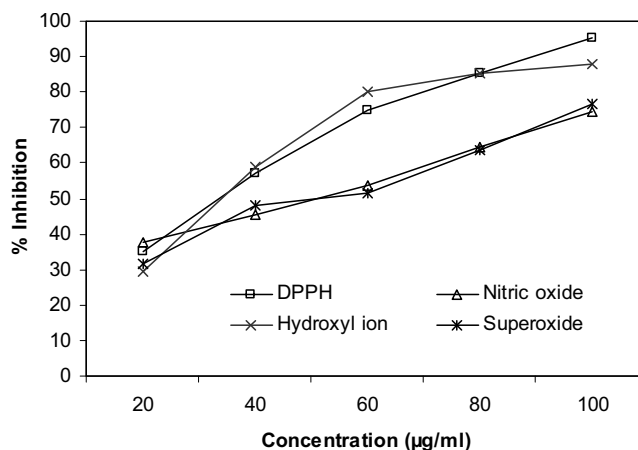


Fig. 1 Effects of different concentrations of methanolic leaf extract of *P. marsupium* on DPPH radical ($y=14.841x \pm 25.003$ $r^2=0.966$), nitric oxide ($y=9.279x \pm 27.421$ $r^2=0.995$), hydroxyl radical ($y=14.335x \pm 25.301$ $r^2=0.851$) and superoxide ion ($y=10.572x \pm 22.576$ $r^2=0.974$). Results are Mean \pm STD of three experiments ($n=3$).

released from the samples which contain antioxidant principles (Sánchez-Moreno 2002).

Nitric oxide scavenging activity

The extent of nitric oxide radical (NO) scavenged was determined by the decrease in intensity of pink coloured chromophore at 540 nm. From the results obtained, it was found that *P. marsupium* exerted marked inhibitory potential against nitric oxide generation (Fig. 1). From the results obtained, it is inferred that the extract possessed antiradical activity with an IC_{50} value of $64 \pm 0.013 \mu\text{g mL}^{-1}$ (Table 1). NO scavenging potential of the leaf extract is comparable with that of catechin ($\text{IC}_{50} = 62.2 \pm 0.04$). The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract inhibits nitrite formation by directly competing with oxygen in the reaction with NO. The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO^-) (Huie and Padmaja 1993). NO is a secondary messenger and plays an important role in the control of blood pressure. However, it is also implicated in several pathological conditions. The present study proved that the extract studied has potent NO scavenging activity.

Hydroxyl radical scavenging activity

Hydroxyl radical (OH^\cdot), the most reactive free radical has the capacity to conjugate with nucleotides in DNA, cause strand breakage, and lead to carcinogenesis, mutagenesis and cytotoxicity (Naidu *et al.* 2008). When *P. marsupium* methanolic extract was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction (Fig. 1) with an IC_{50} value of $41 \pm 0.002 \mu\text{g mL}^{-1}$ (Table 1). The OH ion scavenging results indicate that extract of the aerial part scavenged OH radicals and percentage inhibition was proportional to the concentration of the extract.

Superoxide scavenging activity

Superoxide radical is the main source for the formation of other ROS such as hydroxyl radical and H_2O_2 (Pietta 2000). Result of the superoxide anion scavenging activity of *P. marsupium* methanolic extract measured by PMS-NADH-NBT superoxide generating system is illustrated in Fig. 1. The scavenging effect of extract at a concentration of $100 \mu\text{g mL}^{-1}$ is 76.67% with an IC_{50} value of $147 \pm 0.01 \mu\text{g mL}^{-1}$ (Table 1). In the PMS-NADH-NBT system superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The decrease of absorbance at

Table 2 FRAP value of *P. marsupium* methanol extract.¹

Concentration $\mu\text{g mL}^{-1}$	FRAP ($\mu\text{m Fe}^{2+}/100\text{ g dry matter}$)
20	65.40 \pm 0.44 a
40	93.81 \pm 1.24 a
60	115.97 \pm 0.86 c
80	130.90 \pm 0.54 d
100	160.82 \pm 0.12 b

¹ Data expressed as mean \pm SE of three individual experiments (n = 3) for all three assays

Values in a column with different superscripts indicate significantly different at P>0.05

Table 3 Multiple correlation analysis.

Parameters	TPC	DPPH	NO	OH	SO	FRAP
TPC	1					
DPPH	0.966	1				
NO	0.995	0.937	1			
OH	0.851	0.964	0.801	1		
SO	0.974	0.937	0.973	0.820	1	
FRAP	0.989	0.968	0.979	0.863	0.979	1

TPC - total phenol content; DPPH - diphenyl picryl hydrazyl; NO - nitric oxide scavenging; OH - hydroxyl radical scavenging; FRAP - ferric reducing antioxidant power assay; SO - superoxide scavenging

560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture.

Ferric reducing antioxidant power (FRAP)

The FRAP assay measures the antioxidant effect of any substance in the reaction medium as reducing ability. Antioxidant potential of the leaf extract was estimated from the ability to reduce ferric-tripyridyl-triazine (TPTZ-Fe) (III) to TPTZ-Fe (II) complex. The Ferric Reducing Antioxidant Power (FRAP) method was developed to measure the ferric reducing ability of plasma at low pH (Benzie and Strain, 1996). An intense blue color is formed when the Fe-TPTZ complex is reduced to the ferrous form and the absorption at 593 nm was recorded. The data presented in **Table 2** shows the power of methanol extract to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) was found fair with FRAP values ranged from 65.40 \pm 0.44 to 160.82 \pm 0.12 $\mu\text{m Fe}$ (II)/100 g dry matter of extract for MeOH extract.

Multiple correlation analysis

In this present study, the association between antioxidant activities and total phenolic content (TPC) was observed to be statistically significant based on multiple regression analysis (**Table 3**). A significant linear correlation was established between TPC and corresponding inhibitory efficacy on DPPH radical by the extract ($r^2 = 0.966$). A linear positive correlation was observed between TPC and superoxide anion scavenging ($r^2 = 0.974$), NO scavenging ($r^2 = 0.995$), hydroxyl ion radical scavenging ($r^2 = 0.851$) and FRAP ($r^2 = 0.989$) of the methanol extract of *P. marsupium*. Many supportive reports emphasize the positive correlation between phenolic content and antioxidant efficacy (Kukic *et al.* 2006; Buřičová and Réblová 2008; Canadano-*vić*-Brunet *et al.* 2008; Suaib *et al.* 2009). A positive correlation between antioxidant activity and polyphenol content was found, suggesting that the antioxidant capacity of the plant extracts is due to a great extent to their polyphenols (Ng *et al.* 2000; Kiselova *et al.* 2006). Direct correlation among the antioxidant tests were also demonstrated by linear regression method. A strong correlation was found between FRAP and other assays (**Table 3**). These correlations confirmed the antioxidant activity of phenolic content of the tested extract. Similar positive correlation among the antioxidant methods was reported by Liu *et al.* (2009). However, Hinneburg *et al.* (2006) found no correlation between the FRAP and DPPH assays on extracts from culinary herbs and spices.

CONCLUSIONS

Recent years have seen an exponential increase in research antioxidant properties of medicinal plants. If it is accepted that higher intakes of natural antioxidants containing phenolics are associated with long-term health benefits, then the results presented in this paper offer possible avenues toward health promotion by identifying those compounds. The results presented in this report indicate that *P. marsupium* leaf extract efficiently attenuated oxidative stress via its anti-oxidant properties. Thus, *P. marsupium* leaf extract might be useful in the development of raw materials of medicine. Additional work is therefore necessary to fractionate the extract further to elicit a better understanding of how each chemical fraction contributes to the overall antioxidant activity and to determine whether the unique mixture of plant phenolics contributes to a synergistic antioxidant activity.

ACKNOWLEDGEMENTS

The authors Dr. J. M. Sasikumara and P. Pavai express their deep sense of gratitude to Karpagam University Management for its support and motivation. We thank Dr. Gopalan for his help in the identification of plant specimen.

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