Research Article

Evaluation of Total Phenolics, Flavonoids, and Antioxidant Activity of Leaf Extracts of *Pimpenella tirupatensis*

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ABSTRACT

In the present study our goal was to investigate the total phenolic and flavonoid content of various leaf extracts of *Pimpinella tirupatiensis* and their antioxidant activity. Total phenolic content (TPC) of leaf extracts ranged from 96.08±1.8 to 391.08 ±2.2 (mg GAE/gm) and total flavonoids content (TFC) ranged from 9.264±1.7 to 189.16±2.3 (mg QT/gm). Antioxidant activity of various leaf extracts was evaluated by DPPH (1, 1-diphenyl-2-picryl-hydrazyl), FRAP (Ferric Reducing Ability of Plasma), Nitric oxide scavenging, and Reducing power assays. The IC_{50} values based on DPPH (77±3.0) and Nitric oxide (107±3.0) assays of leaf acetone extracts were lower and showed potential antioxidant properties. A significant but marginal positive correlation was found between TPC and IC_{50}, TFC and IC_{50} values. The high level of total phenolic and flavonoid content in acetone leaf extract indicated high antioxidant activities. The results obtained from the present study, point out that the acetone extract of *P. tirupatiensis* is a potential source of natural free radical scavengers.

Keywords: DPPH, FRAP, Reducing Power, Total phenolics content, antioxidant activity.

INTRODUCTION

Free radicals are highly reactive molecules that contain one or more unpaired electrons. They either donate or take electrons from other molecules in an attempt to pair with their electrons and generate more stable species. High amount of free radicals in living beings has been known to cause various problems like asthma, cancer, cardiovascular diseases, liver diseases, muscular degeneration, and other inflammatory processes, resulting in the so-called oxidative stress[1]. Imbalance between oxidants and antioxidants is defined as oxidative stress which causes damage to all types of biomolecules like protein, nucleic acid, DNA, and RNA [2]. Hence, the balance between reactive species or free radicals and antioxidants is believed to be a critical concept for maintaining a healthy biological system. Antioxidants act as free radical scavengers, reducing agents, quenchers of singlet oxygen molecule, and activators for antioxidative enzyme to suppress the damage induced by free radicals in biological system.

Despite the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care. The antioxidants present in plants help to stabilize or deactivate free radicals, often before they attack targets in biological cells. Recently interest in naturally occurring antioxidants has considerably increased for use in food, cosmetic and pharmaceutical products, as they possess multifacetedness in their multitude and magnitude of activity and provide enormous scope in correcting imbalance[3,4].

P.tirupatiensis belonging to family Apiaceae (local name-kondakothimera) is a rare and endemic medicinal plant, restricted to the Seshachalam hills of the Eastern Ghats, India[5]. The tubers of *P. tirupatiensis* are used for stomachache, scorpion sting, gastric ulcers, skin diseases and as an aphrodisiac by the Adivasi tribes (Erukalas, Nakkalas, Sugali, Yanadis) inhabiting in the area. The boiled tubers are also used as food[6].

Cardio protective activity of ethanolic extracts of *P. tirupatiensis* leaves was evaluated against cardio toxicity induced by doxorubicin in albino rats. Where, *P.tirupatiensis* showed marked activity and good recovery from cellular damage[7]. Significant amounts of alkaloids, flavonols and flavones were reported in the active fractions of *P.tirupatiensis* [8].

Antimicrobial activity of *P. tirupatiensis leaf* acetone *extracts* was reported, against *Proteus mirabilis* at different concentrations[9].

The present study was designed to explore the antioxidant activity of leaf extracts & relative content of total phenolics & flavonoids in leaf extracts of *P.tirupatiensis.*

MATERIALS AND METHODS

Plant materials

The Plant material of *P.tirupatiensis* used in the present study was collected from Seshachalam forest from Tirupati & identification (voucher no: 1208) has been done by Prof. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati, India.

Extraction of plant material

The leaves of *P.tirupatensis* were dried in the shade at room temperature and ground to powder. Fifty grams of

powdered plant material was extracted in 200ml of each solvent (Acetone, Ethyl Acetate, Chloroform, Methanol and Hexane) separately and kept on orbital shaker for 48 hrs. The extracts were filtered through whatmann filter paper after 48hrs and concentrated using rota evaporator under reduced pressure to yield the residue. These extracts were further used to evaluate their total poly-phenolic content, total flavonoid content, and antioxidant activity.

Determination of total polyphenolic content

Total Polyphenolic content of leaf extracts of *P.tirupatiensis* in various organic solvent systems was determined using Folin–Ciocalteau reagent and modified according to the lab conditions[10]. Different *P. tirupatiensis* extracts were made up to 2.0 ml with distilled water followed by addition of 1.0 ml of 10% Folin–Ciocalteau reagent. After vortexing briefly, 1.0 ml of 10% sodium carbonate was added and mixture was allowed to stand at room temperature for an hour. Following incubation, absorbance was measured at 760 nm using UV-Vis spectrophotometer (Shimadzu, Japan). The total concentration of Polyphenolic compounds in extracts was measured as Gallic acid equivalent (GAE) and expressed as mg GAE/g of dry extract. Experiments were performed in triplicate.

Determination of total flavonoid content

Total flavonoid contents were measured with the aluminium chloride colorimetric assay and was modified according to the lab conditions[11]. Leaf extracts of *P.tirupatiensis* in various organic solvents at a concentration of 500 µg/mL were used. Different concentrations of Quercetin (10-100 µg/mL) were used as standard. To the above mixture, 0.3ml of 5% $NaNO_2$ was added. After 5 minutes, 0.3ml of 10% $AlCl_3$ was added, followed by addition of 1 ml of 1 M NaOH after 6 min, the total volume was made up to 5 ml with distilled water. The solution was mixed well and the absorbance was measured at 510 nm. Total flavonoid content of the extracts was measured as Quercetin equivalent and expressed as mg QT/g of extract dry weight. Experiments were performed in triplicate.

Determination of anti-oxidant activity

DPPH radical-scavenging activity

DPPH radical-scavenging activity of *P.tirupatiensis* in various organic solvent systems was determined as described by Burits and Bucar[12]. The capacity of extracts to scavenge lipid-soluble 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical, which results in bleaching of purple colour exhibited by stable DPPH radical, is monitored at an absorbance of 517 nm. Briefly, 1.0 ml of extracts (0.25 – 0.5 mg/ml) was incubated for 30 min at room temperature in the dark, and absorbance was measured against a blank. The Scavenging activity was observed by bleaching of DPPH solution from violet color to light yellow and ascorbic acid was used as control. Tests were carried out in triplicate.

Ferric reducing activity of plasma (FRAP assay)

The FRAP assay was done according to the method of Benzie and Strain with some modifications[13]. This method is based on reduction of TPTZ-Fe^{3+} complex to TPTZ-Fe^{2+} form in the presence of antioxidants. The stock solutions included acetate buffer (300 mM, pH 3.6), 2, 4, 6-tripyridyl s-triazine (TPTZ) solution (10 mM in 40 mM HCl) and ferric chloride ($FeCl_3.6H_2O$) solution (20 mM). The fresh working FRAP solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution and 2.5 ml $FeCl_3$ solution. Extracts were made up to 2.0 ml with distilled water and 1.0 ml of FRAP solution was added. An intense blue colour developed which was measured at 593 nm after an incubation period of 20 min. The absorbance was related to absorbance changes of a ferrous sulphate solution (0 – 250 mg) tested in parallel. All results were based on two separate experiments and antioxidant capacity was expressed as mg $FeSO_4$/ mg of dry extract. Butylated Hydroxy Toluene (BHT) was used as a positive control.

Reducing power

The reducing power of extracts was evaluated according to the method described by Yen and Chen[14]. Different concentrations of leaf extracts in various organic solvent systems (250 and 500 µg/ml) were incubated with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [K_3Fe (CN) 6] at 50°C for 20 min. The reaction was terminated by adding 2.5 ml of 10% TCA solution and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (1.0 ml) was mixed with 2.5 ml of distilled water and 1.0 ml of 0.1% ferric chloride ($FeCl_3$) solution and the absorbance was measured at 700 nm after incubation at room temperature for 10 min. BHT was used as positive control. Experiments were performed in triplicate.

Nitric oxide radical scavenging activity

This method based on spontaneous generation of nitric oxide (NO•) from sodium nitroprusside (SNP)-buffered solution[15], was used to assess NO• scavenging ability of *P.tirupatiensis* leaf extracts in various organic solvent systems. Briefly, 0.5 ml of SNP (10 mM) in phosphate buffered-saline was mixed with 0.5 ml of extracts (0.05 – 1.0 mg/ml) and incubated in dark at room temperature for 2.5 h. A control was set up as above, but sample was replaced with same amount of water. After incubation, 1.0 ml of sulfanilic acid reagent (0.33 % sulfanilic acid in 20 % glacial acetic acid) was added to 0.5 ml of reaction mixture. After 5 min, reaction mixture was incubated further with 1.0 ml 0.1 % naphthylethylenediamine dihydrochloride (NEDD) for 30 min at 25 °C. Absorbance of chromophore formed was read at 540 nm. Ascorbic acid was used as positive control. All analyses were done in triplicate.

Statistical analysis

All the experiments were performed in triplicate, and all the data were presented as 'mean ± standard deviation'. Correlation R^2 analysis and IC_{50} values were calculated by the GraphPad Prism version 7. P-value less than 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Yield of extracts

The yields of various solvent extracts from *P. tirupatiensis* leaves were shown in Table 1. For leaves, the highest yield was obtained with acetone extract (9.0±0.7), followed by methanol extract (6.0±1.3), Ethyl acetate extract (2.0±1.4), Chloroform extract (1.0±1.2), and Hexane extract (1.0±0.6), the solvents with high polarity are effective for extraction of natural antioxidants[16-18]. Results showed that the leaf in acetone extracts gave the highest yield.

Total phenolic content (TPC)

Total phenolic content was estimated by using Folin-Ciocalteu reagent. TPC of the various leaf extracts of *P. tirupatiensis* was solvent dependent and expressed as milligrams of gallic acid equivalents (GAE). The TPC values of *P. tirupatiensis* leaf extracts in various solvents were listed in table 1.The TPC values for various leaf extracts varied widely, ranging from 96.08±1.8 to 391.08 ±2.2 (mg GAE/gm dry wt of extract) expressed as gallic acid equivalents as shown in figure 1.

Acetone leaf extract exhibited highest total phenolic content (391.08 ±2.2) (Fig-1). Compounds containing phenols in their structures are commonly recognized as antioxidants because they can serve as free radical scavengers[19]. Various studies showed that plants with high phenolic content have significant antioxidant capacity[20-25]. As a result, plants, such as dietary spices and medicinal herbs, are common natural sources of antioxidants.

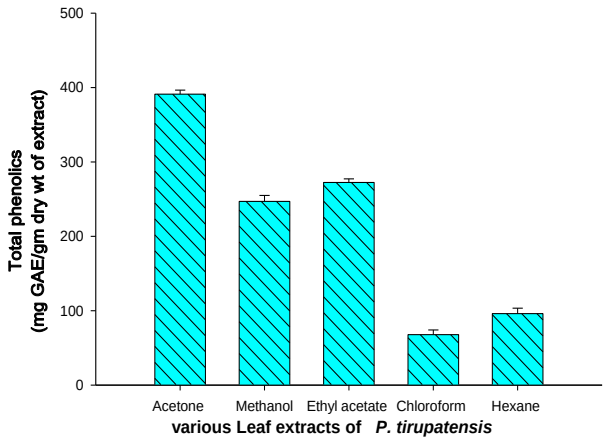


Figure 1: Total phenolic content of various Leaf extracts of *P. tirupatensis.*

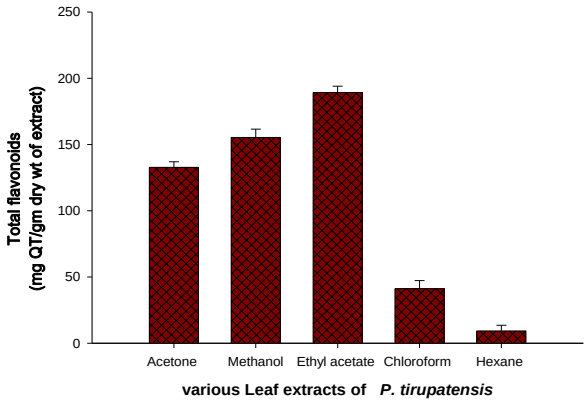


Figure 2: Total flavonoid content of various Leaf extracts of *P. tirupatensis*

Total flavonoid content (TFC)

Total flavonoid content was estimated by using aluminum chloride colorimetric assay. TFC of various leaf extracts of *P. tirupatiensis* was solvent dependent and expressed as milligrams of Quercitin equivalents (QT) .The TFC values of various leaf extracts were listed in table 1.The TFC values of leaf extracts varied widely, ranging from 9.264±1.7 to 189.16±2.3 (mg QT/gm dry wt of extract) expressed as Quercitin equivalents (figure 2). Ethyl acetate leaf extract exhibited highest total flavonoid content (189.16±2.3). Flavonoids are naturally occurring in plants and are thought to have positive effects on human health. Studies on flavonoid derivatives have shown a wide range of antibacterial, antiviral, anti-inflammatory, anticancer, and anti-allergic activities[26, 27]. Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals implicated in several diseases[28].

DPPH scavenging activity

The DPPH (1, 1-Diphenyl-2-picrylhydrazyl radical) assay have been widely used to determine the free radical-scavenging activity of various plants and pure compounds. Scavenging effect of samples on DPPH radical, were of the following order of *P. tirupatiensis* leaf acetone > leaf methanol > leaf ethyl acetate > leaf chloroform > leaf hexane extracts (Fig 3A). The IC_{50} (IC_{50} value is the concentration of the sample required to inhibit 50% of radical) values of *P. tirupatiensis* leaf extracts were calculated and listed in the table 2. IC_{50} values were of the following order ascorbic acid (45.71±2.0), acetone (77±3.0) and methanol (80.1±2.0) leaf acetone extract showed better scavenging activity among them. Usually, higher total phenol and flavonoids contents lead to better DPPH-scavenging activity[29]. The electron donation ability of natural products can be measured by (DPPH) purple-coloured solution bleaching[30]. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourizes the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical

scavenging activity of the compound under test[31]. In the present study among all the extracts tested acetone, methanol and ethyl acetate showed significantly higher inhibition percentage which is positively correlated with total phenolic content (table 3).

Reducing Power Assay

Reducing power of various leaf extracts was of the order (Fig 3B) leaf acetone > leaf methanol > leaf chloroform > leaf ethyl acetate > leaf hexane extracts. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermedia tes of lipid per oxidation processes[32]. Therefore, various leaf extracts *P. tirupatiensis* were electron donors and can react with free radicals to convert them to more stable products and terminate radical chain reaction.

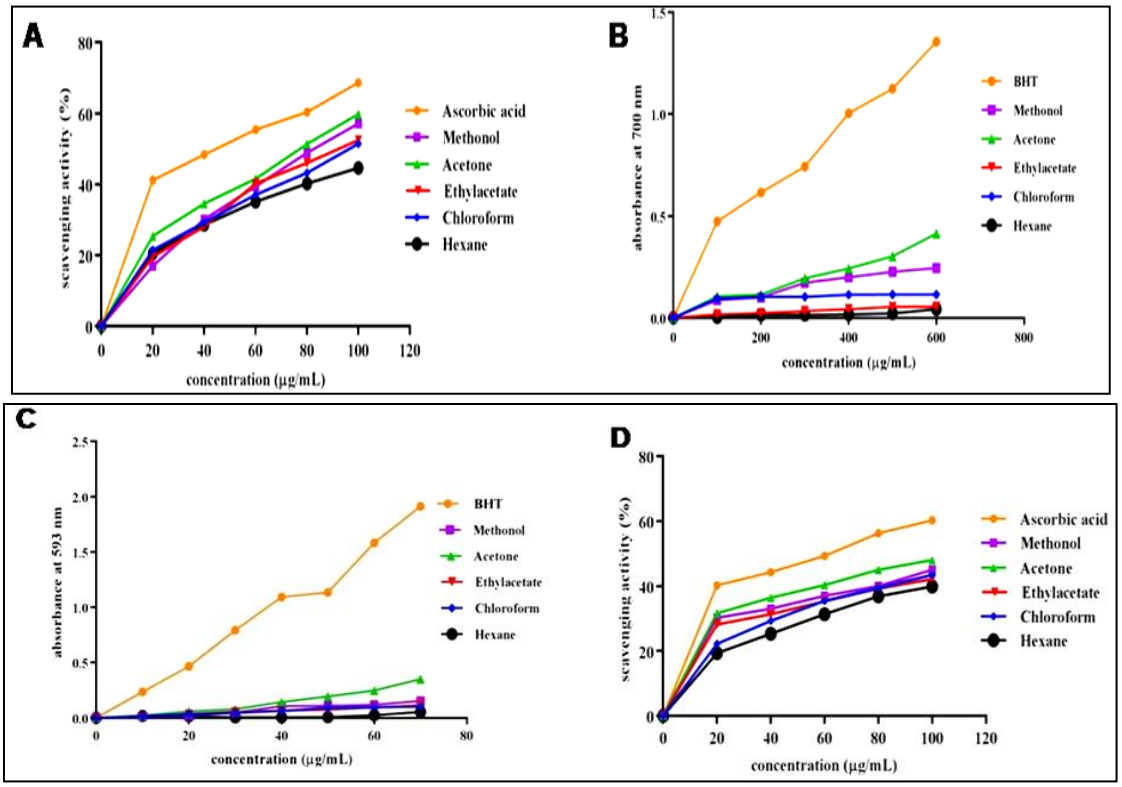
FRAP assay

Reduction of ferric tripyridyl complex to ferrous (2, 4, 6-tripyridyl-s triazine) ie: ferric (III) (colorless) to ferrous (II) (blue) was monitored by measuring absorbance at 593 nm. In the present study free radical scavenging activity of *P. tirupatiensis* leaf extracts was determined by the FRAP. Figure 3C showed free radical scavenging activity of *P. tirupatiensis* leaf extracts and were of the following order leaf acetone > leaf methanol > leaf ethyl acetate > leaf chloroform > leaf hexane extracts. Schafer and Buettner reported that higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferric ion, where antioxidants are the reducing agent[33]. The absorption readings were related to the reducing power of the electron donating antioxidant present in the extract. Hence FRAP assay can categorize the reducing power and the antioxidant potential.

Table 1: Total phenolics, Total flavonoids and extraction yield of various Leaf extracts of *P. tirupatensis*.

Plant extracts	Total phenolics (mg gallic acid equivalent /gm dry wt of extract)	Total flavonoids (mg Quercetin equivalent /gm dry wt of extract)	Extraction yield (%)
Acetone extract	391.08 ±5.4	132.76±4.2	9.0±0.7
Methanol extract	246.96±7.9	155.32±6.3	6.0±1.3
Ethyl acetate extract	272.32±4.9	189.16±4.9	2.0±1.4
Chloroform extract	67.84±6.2	41.12±6.1	1.0±1.2
Hexane extract	96.08±7.3	9.264±4.3	1.0±0.6

Each value in the table is represented as mean ± SD (n = 3).



Each value represents mean ± SD (n=3) (A) DPPH radical scavenging activity (B) Reducing power assay (C) Ferric reducing activity (D) Nitric oxide scavenging activity.

Figure 3: (A-D) Antioxidant activities of various Leaf extracts of *P. tirupatensis* at different concentrations.

Nitric oxide scavenging

NO scavenging capacity is determined by the decrease in the absorbance at 540 nm, induced by antioxidants. In order to estimate the antioxidant potency through NO scavenging by the test samples, the change of optical density of NO was monitored. Figure 3D showed comparative NO scavenging activity of extracts, and were of the following order leaf acetone > leaf chloroform > leaf methanol > leaf hexane > leaf ethyl acetate extracts. IC_{50} values were of the following order ascorbic acid (59.2±0.7) IC_{50} value (µg/mL), acetone (107±3.0), chloroform (121.2±3.0), and methanol (131.2±1.0) leaf acetone extract showed better scavenging activity among tested extracts (Table 2).

Table 2: Radical scavenging activities of various Leaf extracts of *P. tirupatensis*

IC $_{50}$ Value (µg/mL) of radical scavenging		
Plant extracts/ chemical	**DPPH radical**	**Nitric oxide radical**
Acetone extract	77±3.0	107±3.0
Methanol extract	80.1±2.0	131.2±1.0
Ethylacetate extract	90±4.0	142.2±2.0
Chloroform extract	96±3.0	121.2±3.0
Hexane extract	114±1.0	134.2±2.0
Ascorbic acid	45.71±2.0	59.2±0.7

Each value in the table is represented as mean ± SD (n = 3)

Table 3: Correlations between the IC_{50} values of antioxidant activities and phenolic and Flavonoid Content of *P. tirupatensis*.

Assay	Correlation R^2	
	Phenolics	**Flavonoid**
IC_{50} of DPPH radical scavenging ability	0.64979*	0.611718*
IC_{50} of Nitric oxide radical scavenging ability	0.15954	0.061197

* indicates significance at $P < 0.05$.

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological processes [34]. Excess concentration of NO is associated with several diseases[35].Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as NO_2, N_2O_4, N_3O_4, NO_3^- and NO_2 are very reactive[36]. These compounds are responsible for altering the structural and functional behavior of many cellular components. Phenolic and flavonoid compounds have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals[37, 38]. The nitric oxide scavenging activity of flavonoids and phenolic compounds are known[39-43]. We can consider that these constituents might be responsible for the observed nitric oxide scavenging activity. In the present study among all the extracts tested acetone, methanol and chloroform showed significantly higher inhibition percentage.

CONCLUSION

The results of this study indicated that *P. tirupatiensis* leaf acetone extract has a high antioxidant activity among all tested extract. A positive relationship between antioxidant activities and total phenolic content was also observed. The high level of total phenolic and flavonoid content in acetone leaf extract indicated high antioxidant activities. This relationship was also reported in previous studies on other plants[44, 45]. Further work is required to establish the components in phenolics and flavonoids that may have contributed to the high antioxidant activities so far observed.

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