Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(7):801-806

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

In vitro antimicrobial and antioxidant activities of *Pimpenella tirupatensis* leaf methanolic and ethyl acetate extracts

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ABSTRACT

In-vitro antimicrobial and antioxidant activities of Pimpenella Tirupatensis Methanol and Ethyl acetate leaf extracts concentrations ranging from 50 to 150 µg/mL were analyzed on different clinical bacterial strains (Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Salmonella typhimurium, Proteus mirabilis, Bacillus subtilis, and Proteus vulgaris), using agar disc diffusion method and broth dilution method (MIC and MBC determination) for antimicrobial activity and DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, Nitric oxide scavenging assay, Ferrous reducing antioxidant power (FRAP) for antioxidant activity. The extracts showed maximum inhibitory effect against K. pneumonia, Proteus mirabilis and Pseudomonas aeruginosa, with no activity against S. typhimurium and Proteus vulgaris among the bacterial strains. The antioxidant activity showed that the extracts exhibited scavenging effect in concentration-dependent manner on superoxide anion radicals and hydroxyl radicals leading to the conclusion that the plant has got a broad spectrum antimicrobial and antioxidant activity and could be a potential alternative for treating various diseases.

Key words: Antioxidant, Antimicrobial, Ethyl acetate, Methanol, *Pimpenella tirupatensis*

INTRODUCTION

Plants have been used as healers and health-rejuvenators since time immemorial. The use of plant products in the form of local medicines dates back to 4000-5000 B.C. Even today, plants play an important role in the health care of about 80% of the world population and is estimated that more than half of the drugs under clinical use at present owe their origin to plants [1]. Plants are utilized as therapeutic agents since time immemorial in both organized (Ayurveda, Unani) and unorganized (folk, tribal, native) form [2]. Nowadays, multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious diseases e a situation that forced scientists to search for new antimicrobial sub-stances [3]. Reactive Oxygen Species (ROS) including free radicals such as (O_2, OH) and non-free radicals (H2O2) along with different forms of active oxygen are involved in diverse physico-chemical processes in the body [4] playing a critically important role in the pathogenesis of different diseases, such as neurodegenerative disorders [5], diabetes, cancer [6], cardiovascular diseases, atherosclerosis [7], liver cirrhosis [8], cataracts and inflammation [9, 10].

Antimicrobial activity of ethyl acetate and hexane extracts of *Pimpinella tirupatiensis* root shown better results. Active fractions were analysed through GCMS for the presence of important phytochemicals and revealed the presence of totally 25 compounds including alkaloids, flavonols, flavones and volatile oils of which 24 were known compounds.

Cardio protective activity of ethanolic extracts of *Pimpinella tirupatiensis* leaves was evaluated against cardio toxicity induced by doxorubicin in albino rats. Where, *Pimpinella tirupatiensis* showed marked activity and good

recovery from cellular damage was recorded and active fractions analysed for presence of phytochemicals through GCMS gave the reports of presence significant amounts of alkaloids, flavonols and flavones which are potent antioxidants by nature [11].

Aqueous extracts of *Pimpinella tirupatiensis* leaf also exhibited very good antioxidant activity
Showing significant values for DPPH followed by Nitric oxide, Hydrogen peroxidase and reducing power. Petroleum ether and ethanol extracts of *Pimpinella tirupatiensis* leaf showed better antimicrobial activity [12].

As the plant species are proceeding towards extinction a sense of urgency is required plants have to be investigated a new for their antimicrobial usefulness.

EXPERIMENTAL SECTION

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

Extraction of plant material:
The leaves of the *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 antimicrobial activity.

Determination of antimicrobial activity:
Test organisms
The test organisms used in this study were combination of gram +ve and gram –ve pathogenic bacteria. *Escherichia coli* (MTCC 7410), *Staphylococcus aureus* (MTCC 7443), *Salmonella typhimurium* (MTCC 98), *Bacillus subtilis* (MTCC 511), *Klebsiella pneumonia* (MTCC 3384), *Proteus mirabilis* (MTCC 425), *Proteus vulgaris* (MTCC 744), and *Pseudomonas aeruginosa* (MTCC 2295) were procured from IMTECH, Chandigarh. The bacterial cultures were maintained on Mueller-Hinton agar slants at 4˚C with a subculture period of 15 days. Each bacterial strain was reactivated from the stored slants to Mueller-Hinton broth and cultured overnight at 37˚C before the antimicrobial assay.

Antibacterial susceptibility assay
The antimicrobial activities of extracts were determined by two methods including disc diffusion test and broth dilution assay. Sterile disc of 5 mm diameter was saturated with 20 µl of the different concentrations of extract solution ranging from 50 to 150 (µg/mL). The paper discs were dried and placed on the surface of the inoculated agar plates. Plates were kept for 1 h in refrigerator to enable prediffusion of the extracts into the agar. Then the inoculated plates with test microorganisms were incubated at 37^0C for overnight to allow bacterial growth.

Amikacin was used as positive control. The antibacterial activities of the extracts were evaluated by measuring the inhibition zones.

Determination of MIC and MBC
The MIC and MBC of various leaf extracts was determined by broth micro-dilution method and modified according to the lab conditions.

Determination of anti-oxidant activity:
DPPH radical-scavenging activity
DPPH radical-scavenging activity of *Pimpinella tirupatiensis* in various organic solvent systems was determined as described by Burits and Bucar, [13]. 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) and Quercetin/BHT (250 µg/ml) in ethanol were added to 4 ml of 0.004% methanolic solution of DPPH. After incubation for 30 min at room temperature in the dark, absorbance was measured against a blank. Tests were carried out in triplicate. The ability of extracts Quercetin and BHT to scavenge DPPH radical was calculated using following equation.

Ferric reducing activity (FRAP assay)

The FRAP assay was done according to the method of Benzie and Strain 1999[14], with some modifications. 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 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. Ferrous sulphate was used as a positive control.

Nitric oxide radical scavenging activity

The method of Rai *et al* 2006 [15] based on spontaneous generation of nitric oxide (NO•) from sodium nitroprusside (SNP)-buffered solution was used to assess NO• scavenging ability of *Pimpinella tirupatiensis* in various organic solvent systems. Briefly, 0.5 ml of SNP (10 mM) in phosphate buffered-saline was mixed with 0.5 ml of *P. tirupatiensis* extracts (0.05 – 1.0 mg/ml) and incubated in the 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. Results were expressed as a percent of scavenged nitric oxide with respect to negative control. Ascorbic acid was used as positive control. All analyses were done in triplicate.

RESULTS AND DISCUSSION

Antimicrobial activity:

The antimicrobial activities of different concentrations (ranging from 50 to 150 µg/ml) of crude methanol and ethyl acetate extracts of *P.tirupatensis* (Table 1) were determined against different bacterial strains and recorded as inhibition zone diameter (IZD), measured in 'mm' with, amikacin as positive control for bacteria. The Ethyl acetate extract showed high IZD against *Pseudomonas aeruginosa* (11 mm), *Proteus* mirabilis (11 mm), *and Bacillus subtilis* (10 mm).whereas methanol extracts showed low IZD against among all the test organisms. The MIC and MBC values in ethyl acetate extracts showed low values as compared with methanol extracts against all the test organisms, that indicates the ethyl acetate extracts was highest inhibitory action in broth micro dilution method.

Table1: Antimicrobial activity of *p.tirupatensis* leaf extracts (µg/mL)

Extract	Test Organisms	Inhibition zone diameter (mm)			
		50	100	150	Amikacin
Leaf - Methanol	*Pseudomonas aeruginosa*	6±0.57	8±0.54	9±0.57	23±0.40
	Proteus vulgaris	6±0.53	7±0.67	8±0.57	24±0.26
	Salmonella typhi	6±0.54	6±0.57	6±0.57	23±0.43
	Proteus mirabilis	-	-	8±0.57	24±0.416
	Klebsiella pneumoniae	-	8±0.57	10±1.0	26±0.473
	Staphylococcus aureas	-	-	9±0.57	24±0.45
	Bacillus subtilis	-	-	8.4±0.53	25±0.153
	Escherichia coli	6±0.50	8±0.20	9 ±0.43	24±0.49
Leaf - Ethyl acetate	*Pseudomonas aeruginosa*	-	6±0.20	11±0.37	23±0.40
	Proteus vulgaris	-	-	-	24±0.26
	Salmonella typhi	-	-	-	23±0.43
	Proteus mirabilis	-	-	11±0.60	24±0.416
	Klebsiellapneumoniae	-	-	7±0.32	26±0.473
	Staphylococcus aureas	-	-	6±0.55	24±0.45
	Bacillus subtilis	6±0.11	7±0.26	10±0.36	25±0.153

Antioxidant Activity:

The antioxidant activity of both the extracts as measured by the ability to scavenge DPPH free radicals was compared with the standards/ascorbic acid. The results (Table: 3) for the DPPH assay revealed that both the methanol and ethyl acetate extracts exhibited significant antioxidant activity. The highest % of inhibition was shown by methanol extract with a maximum of 56% inhibition compared to the positive control (ascorbic acid 68%) at 100 µg/ml followed by ethyl acetate (52 %) (Fig: 1). The highest % of inhibition was exhibited by methanol extract (table:4) (45%) at 100 µg/ml concentration followed by ethyl acetate (42%) as determined by Nitric oxide anion

scavenging(Fig:2). The highest absorbance was showed in methanol extract (0.154) with maximum of (1.914) absorbance compared to positive control (ferrous sulphate) at 593 nm by FRAP method (table: 5).

Table 2: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of *p.tirupatensis* leaf extracts (µg/mL) against test organisms

Test organisms	Methanol		Ethyl acetate		Amikacin	
	MIC	MBC	MIC	MBC	MIC	MBC
Escherichia coli	125	250	125	250	7.8	15.6
Salmonella typhi	250	125	125	250	15.6	31.2
Proteus vulgaris	125	250	500	1000	7.8	15.6
Proteus mirabilis	250	500	125	250	7.8	15.6
Pseudomonas aeruginosa	125	250	125	250	7.8	15.6
Klebsiella pneumoniae	125	250	250	500	7.8	15.6
Staphylococcus aureus	125	250	62.5	125	3.9	7.8
Bacillus subtilis	125	250	500	1000	7.8	15.6

Table: 3 DPPH scavenging of Methanol, and Ethyl acetate extracts of *pimpinella tirupatiensis* Leaf compared to that of ascorbic acid.

Concentration (µg/ml)	Standard (Ascorbic acid)	Methanol	Ethyl acetate
20	41.19±0.26	16.97±0.45	19.62±0.47
40	48.39±0.54	30.12±0.65	28.03±0.28
60	55.38±0.69	39.42±0.25	40.22±0.62
80	60.36±0.12	48.87±0.69	46±0.36
100	68.64±0.37	56.15±0.54	52.49±0.12

Pharmaceutical and scientific communities have recently received the attention of the medicinal plants, as the herbal remedies prepared from the whole plant are generally safe with fewer side effects if used in the proper therapeutic dosages [16]. The antimicrobial activity of both the plant extracts against the different clinical strains of bacteria supported the scientific validity of the plant being used traditionally as a medicine. The inhibition of a maximum of eight bacterial strains by ethyl acetate extract may be attributed to the presence of soluble phenolic and polyphenolic compounds in the extract. The results are in confirmation with a recent study of Bandh et al. [17] in which it was shown that the methanol extract of Nepeta cataria inhibited the growth of all the bacterial and fungal test organisms, suggesting that the antimicrobial activity of the extract may be related to some phenolic components.

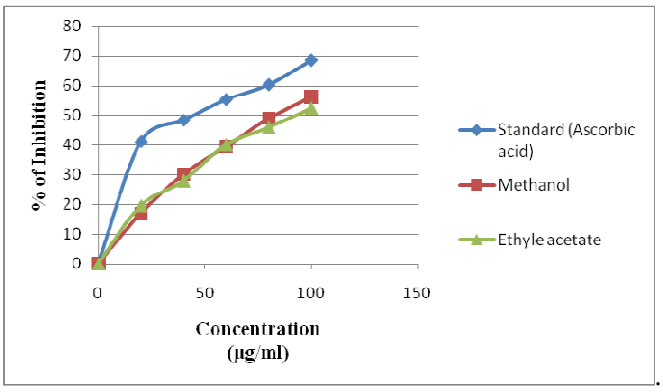

Figure: 1 DPPH radical scavenging activity of methanol and ethyl acetate Leaf extracts of *P. tirupatensis* at various concentrations

Table: 4. Nitric oxide anion scavenging of Methanol and Ethyl acetate extracts of *P.tirupatiensis* - leaf compared to that of ascorbic acid

Concentration (µg/ml)	Standard (ascorbic acid.)	Methanol	Ethyl acetate
20	40.19±0.23	30.21±0.26	28.14±0.65
40	44.6±0.36	33±0.48	31.35±0.54
60	49.26±0.25	37±0.56	35.28±0.69
80	54.59±0.14	40±0.46	39.17±0.74
100	60.25 ±0.39	45±0.65	42.14±0.36

The lack of antibacterial activity in some of the concentrations of the extract is not surprising as a number of plant extracts have been found ineffective against certain test or-ganisms at lower concentrations and may be attributed to the presence of lesser amounts of the antimicrobial compounds. The MIC and MBC values supported the results

obtained in the antibacterial screening, showing clearly that the ethyl acetate extract was most potent than methanol extract as the MIC values were lower than the MBC values, similar to the results of Karou et al. [18]. George et al. [19] explained that the observed differences to be due to the fact that while synthetic antibiotics are in a pure form, crude plant extracts contains some impure substances that may be inert and do not have any antibacterial activities.
The property of plant extracts to scavenge the free radicals has been evaluated using separate assays for each type of reactive oxygen species. The mechanism of an antioxidant action in-vitro involves direct inhibition of the generation of reactive oxygen species, or the scavenging of free radicals. Thus, it is clear that a single method cannot give a comprehensive prediction of antioxidant efficacy of the extracts. So, use of more than one method is recommended [20].

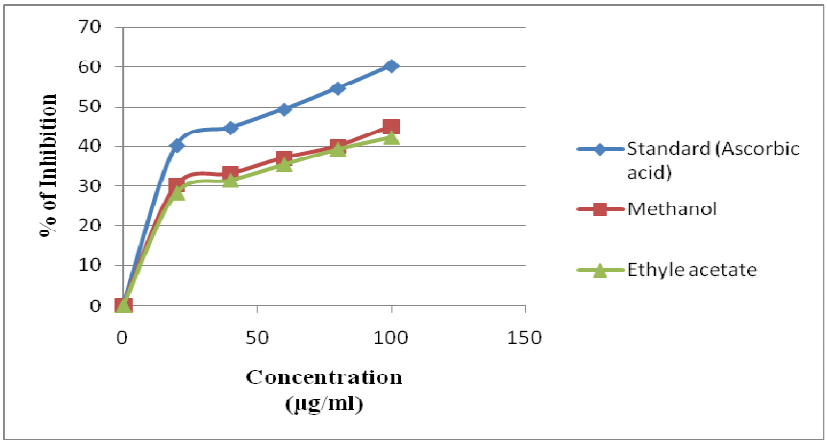

Figure: 2 Nitric oxide scavenging activity of methanol and ethyl acetate Leaf extracts of *P. tirupatensis* at Various concentrations Compared with standard (Ascorbic acid)

Table: 5 Ferric reducing antioxidant power of Methanol and Ethyl acetate extracts of *P. tirupatiensis*-leaf compared to that of ferrous sulphate.

Concentration(mg/mL)	Standard	Methanol	Ethyl acetate
10	0.236±0.002	0.033±0.003	0.014±0.003
20	0.466±0.002	0.056±0.005	0.035±0.003
30	0.793±0.003	0.085±0.003	0.055±0.003
40	1.094±0.002	0.107±0.003	0.064±0.002
50	1.135±0.002	0.109±0.001	0.075±0.003
60	1.583±0.002	0.150±0.009	0.096±0.002
70	1.914±0.003	0.154±0.004	0.114±0.002

The DPPH free radical scavenging activity is due to the neutralization of DPPH free radical by the plant extract, either by transfer of hydrogen or of an electron [21]. Due to strong DPPH scavenging property of ascorbic acid it is used as a standard anti-oxidant. The results show that methanol extract of *P.tirupatensis* may have hydrogen donors thus scavenging the free radical DPPH, with highest scavenging activity than the ethyl acetate leaf extract, which may be attributed to the total phenolic compounds.

The potential of antioxidant and antimicrobial activities of the essential oils and extracts from many plants are of great interest in both fundamental science and food industry, since their possible use as natural additives emerged from a growing tendency to replace synthetic antioxidants by natural ones.

CONCLUSION

The study revealed the presence of bio active components in the plant of *P.tirupatensis*. The phytoconstituents of this plant may be responsible for the usefulness in the management and treatment of wounds and other diseases. These results warrant at least in part the properties and attributed to the plant. That from above it is clear that it will be applicable as a potential source of antioxidant for future researches.

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