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Phytochemical Analysis and Antibacterial Activities of *Pimpinella tirupatiensis* against *Bacillus subtilis*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa*

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Abstract: ***The human culture directly or indirectly was influenced by plants from the time immemorial. The progress of human civilization was accompanied by the use of plants and nature throughout the ages. Plants are fundamental to almost all life on earth providing protection and sustenance to other organisms. Plants which are nature's precious gift to mankind have health improving properties. Nature has served as a rich repository of medicinal plants for thousands of years and an improve number of modern drugs have been isolated from natural sources notably of plant origin. Herbal medicine, based on their traditional uses in the form of powders, liquids or mixtures, has been the basis of treatment for various ailments in India since ancient times. There are numerous references for the use of plants as therapeutic agents both in the works of Indian medicine and other works of antiquity. Plants have played a major role as the basic source for the establishment and enhancing the economy of developing countries like India. To elevate the medicinal properties, root tubers of Pimpinella tirupatiensis were collected from different localities of tirumal hills, Eastern Ghats, Andhra Pradesh, India. The methanolic extracts of root tubers were qualitatively screened for phytochemicals by using standard procedures which revealed the presence of various important bioactive chemical entities. Antibacterial activity of the methanolic extracts of root tubers were evaluated against Bacillus subtilis, Klebsiella pneumoniae and Proteus vulgaris, Pseudomonas aeruginosa. The methanolic extracts of root tubers have exhibited significant broad spectrum antibacterial activities.***

Keywords: Herbal medicine, Therapeutic agents, Phytochemicals, Root tubers

1. Plant Material Collection

During the exploration of ethno-medico-botanical survey, the root tubers of *Pimpinella tirupatiensis* were collected from different localities of Tirumal hills, Eastern Ghats, Andhra Pradesh, India. The root tubers were thoroughly washed with water and sufficient quantity of samples collected and chopped off into small fragments and shade dried. The dried samples were ground to make coarse powder (each 1 kg) and stored in polythene containers at room temperature. These samples were used for the detection of various secondary metabolites. Phytochemical screening was done following standard procedure adopted by Harborne (1973) and Gibbs (1974).

Table 1-a: Preliminary Phytochemical screening of *Pimpinella tirupatiensis* (Root tubers - Methanolic Extract)

Alkaloids	+
Flavonoids	+
Indoles	-
Leucoantho-cyanins	+
Steroids	+
Carbohydrates	+
Phenols	+
Steroidal nucleus	-
Saponins	+
Tannins	+
Proteins	+
Lignins	+
Methylenedioxy functional compounds	+

2. Screening for selective secondary constituents:

A. Phenolic compounds

a) Extractive procedure

The phenolic constituents were extracted by following the method given by Bate Smith (1954) and Ibrahim and Towers (1960). About 25g of healthy plant material was macerated with 100ml of 2N Hydrochloric acid.(HCL). The homogenate was digested on a boiling water bath for about half-an-hour. The contents were cooled and filtered through Whatman No.1 filter paper. The filtrate was extracted repeatedly with peroxide free diethyl ether. All the extracts were pooled and concentrated to 100ml and was treated three times with 25ml of 5% anhydrous sodium carbonate solution. The pooled carbonate solution was adjusted to pH 2.0 with Conc. Hydrochloric acid.

The acidified fraction at pH 2.0 was extracted with equal volumes (25ml) of fresh diethyl ether thrice. The combined other extracts were washed with distilled water repeatedly till all traces of hydrochloric acid was removed. The ether soluble water was removed by freezing the extract. The ether was evaporated to dryness on water bath at 98^0C and the resulting phenolic compound residue was dissolved in 1ml of 95% ethanol. This was stored at low temperature in dark container for ready use.

b) Identification

About 2g of fresh weight equivalent to the final alcoholic extract was spotted on 23 x 29 cm whatmann No.1 Chromatographic filter paper with the help of micro pipette. The origin of the spot area was dried immediately with the help of hair-drier. Two dimensional ascending Chromatographic technique was adapted with benzene-acetic acid-water (60:70:30 v/v/v, upper layer) in the direction and sodium formate-formic acid-water (10:1:200 w/v/v) in the second direction. The Chromatographic chambers were saturated with the above solvent systems one day before the development of the Chromatograms at 22 to 24^0c. The sheets after development were removed from the chambers and dried at room temperature. The dried sheets were observed under Ultra violet light and fluorescent regions were marked. The papers while exposing to Ammonia vapours were also observed under UV light and new fluorescent spots were marked.

The separated phenolic compounds on the Chromatograms were identified by comparison of R_f values and individual spot colours of Chromogenic sprays. The final confirmation was made with authentic samples by co-chromatography.

Table 1b: Phenols

Digallic acid	-
Gallic acid	-
Ellagic acid	-
Aesculetin	+
Cis-p-coumaric acid	+
iso-chlorogenic acid	+
Chlorogenic acid	+
Caffeic acid	-
Protocatechuic acid	+
Gentistic acid	+
Scopoletin acid	+
Phloretic acid	+
ρ-Hydroxy benzoic acid	+
α-Resorcyclic acid	+
β-Resorcyclic acid	+
trans-ρ-coumaric acid	+
Vanillic acid	+
Pcoumarylquinic acid	-
Cis-ρ-coumaric acid	+
Melilotic acid	+
Cis-Ferulic acid	+
trans-Ferulic acid	+
Coumarin	+
Salycyclic acid	+
Cinnamic acid	+
Syringic acid	+

Table 1c: R_f values and colour reactions of Phenolic compounds on Paper Chromatograms

Compound	R_f values in solvent		U.V fluorescence		Silphanilic reagent	P-Nitraniline reagent
	1	**2**	**Without NH_3**	**With NH_3**		
Digallic acid	0.29	0.69	None	Blue violet	Violet	Violet
Gallic acid	0.45	0.66	violet	Violet	Violet	Violet
Ellagic acid	0.02	0.28	None	Blue green	Blue green	Blue green
Aesculetin	0.28	0.33	white blue	Very bright blue	Brown	Grey
Cis-p-coumaric acid	0.42	0.77	Light blue	Deep blue	Dark brown	Blue
iso-chlorogenic acid	0.01	0.31	Faint blue	Duckegg green	Light orange	Brown
chlorogenic acid	0.01	0.76	Faint blue	Duckegg green	Orange	Brown
Caffeic acid	0.06	0.38	Blue	Bright blue	Buff	Light brown
Protocatechuic acid	0.05	0.58	None	None	Buff	Light brown
Gentistic acid	0.14	0.68	Bright blue	Yellow	Buff	Blue white
Scopoletin acid	0.30	0.51	Bright blue	Very bright blue	Yellow	Brown
Phloretic acid	0.66	0.77	None	None	Yellow	Purple white
ρ-Hydroxy benzoic acid	0.38	0.68	None	None	Bright yellow	Light pink
α-Resorcyclic acid	0.16	0.83	None	None	Brown	Orange brown
β- Resorcyclic acid	0.28	0.75	None	None	Dark brown	Brown
trans-ρ-coumaric acid	0.50	0.45	Light blue	deep blue	Light brown	Blue
Vanillic acid	0.78	0.57	None	None	Orange	Purple
P–coumarylquinic acid	0.97	0.64	None	None	Light yellow	Yellow
Cis-ρ-coumaric acid	0.42	0.77	Light blue	deep blue	Dark brown	Blue
Melilotic acid	0.51	0.76	None	None	Orange yellow	Purple
Cis-Ferulic acid	0.89	0.65	Light blue	Blue	Purple	Blue green
trans-Ferulic acid	0.80	0.31	Light blue	Blue	Purple	Blue green
Coumarin	0.99	0.66	None	None	Yellow	Violet
Salycyclic acid	0.93	0.31	Dark blue	Dark violet	Yellow	Pink
Cinnamic acid	0.96	0.04	None	None	Yellow	Green
Syringic acid	0.92	0.48	None	None	Red	Dark blue

B. Flavonoids Compounds

a) Extractive procedure

The flavonoid compounds were extracted according to the method given by Markham (1982). About 5g of powdered plant material was extracted in two steps. First 18ml of Methanol and 2 ml of water (9:1) were added, shaken well and kept for one day at room temperature. The supernatant solution of the extract was taken and transferred to another test tube. Secondly Methanol and water of 5ml each (1:1) was added to the remaining residue, stirred well and the mixture left for 24 hours. The two extracts were combined, mixed well and filtered through cotton wool. Later the filtrate was evaporated to about 1/3 the original volume till

most of the methanol was removed. The resultant aqueous extract was extracted with chloroform repeatedly for 3 times. The solvent extracted aqueous chloroform layer was evaporated to dryness under vaccum in a rotary evaporator. The dried residue was saturated with 1 ml of 95% alcohol and stored in a dark place at low temperature.

b) Identification

The extracts containing flavonoids were separated in suitable quantities on Whatmann No.1 Chromatographic filter paper(23x29 cm) adopting the ascending technique using the solvent system Iso-propyl alcohol **:** Ammonia (25%) **:** Water(8:1:1:v/v/v) and n-butanol:acetic acid: water(4:1:5v/v/v) (top layer was used).The dried Chromatograms were examined under UV lamp and the fluorescent spots were marked. The papers while exposed to Ammonia Vapours were also observed under UV lamp and the new fluorescent regions were marked. The flavonoid compounds were identified by comparing the R_f values and colours with those of the authentic samples on the Chromatograms. Co-Chromatographic studies were conducted with authentic markers to confirm the identification.

C. Chromogenic spray reagents

Diazotized sulphanilic acid reagent (Markham, 1982) :0.3% solution of sulphanilic acid in 8% HCl (25ml) was mixed with 5% Sodium nitrite solution(1.5ml) just before use. The Chromatographic sheets were sprayed with this mixture and with a 20% solution of anhydrous Sodium carbonate.

1% Alcoholic ferric chloride:1 g of Ferric chloride was dissolved in alcohol and made up to 100ml.

1% Alcoholic Aluminium chloride:1g of Aluminium chloride was dissolved in alcohol and made up to 100ml, just before spraying of the chromatographic sheets.

Table 2a: Flavonoids

Quercetin	+
Rutin	+
Myricetin	+
Luteolin	+
Apigenin	+
Orientin	-
Vitexin	+

Table 2b: R_f values and colour reactions of Flavonoids on Paper Chromatograms

Compound	R_f values in solvent		U.V. fluorescence		Sulphanilic Reagent	1% Alcoholic Ferric Chloride	1% alcoholic Aluminium Chloride
	1	2	Without NH3	With NH3			
Quercetin	0.26	0.64	yellow	Light yellow	Bright yellow	Green	Yellow
Rutin	0.03	0.57	Orange brown	Yellow	Green	Olive	Grey yellow
Myricetin	0.07	0.43	Yellow	Bright yellow	Light yellow	Olive	Grey yellow
Luteolin	0.44	0.78	Dull yellow	Yellow	Light red	Pale green	Pale yellow
Apigenin	0.61	0.91	Red brown	Red brown	Pink	Pale green	None
Orientin	0.78	0.31	Yellow	Yellow green	Grey	None	None
Vitexin	0.91	0.42	Dull yellow	yellow	Bright red	none	None

Solvents: 1. Iso-propyl alcohol **:** Ammonia(25%) **:** Water (8:1:1)
2. n-butanol**:** Acetic acid **:** Water(4:1:5)

C. Amino acids

a) Extractive procedure

Amino acids were extracted following the method of Das Chowdary *et al.*, (1967). About 5g of plant material was cut into small pieces and plunged immediately into a round bottomed flask containing 20ml of 80% Ethanol. It was refluxed for 30 minutes over a boiling water bath. The boiled material was ground in mortar using additional volumes of 10ml ethanol 80% (v) and centrifuged at 2000 rpm. The supernatant was collected and the Ethanol removed in vaccum. The aqueous extract was passed through Dowex 50W-X8 (H^+ from 20-50 mesh) column (1x10cm). the amino acids were eluted from the column with 50ml of 2N Ammonium hydroxide. The elute was evaporated to dryness and the residue was saturated with 95% Ethanol (1ml).

b) Identification

The extracts containing amino acids were seperated on Whatmann No.1 Chromatographic filter paper(23x28 cm) employing two-dimensional ascending technique using the solvent system:

1) Sec. butanol**:** Formic acid **:** Water(75:13:12v/v/v)-1st direction.

2) Buffer saturated phenol (1:2v/v)-2nd direction(6.3 g of Sodium citrate and 3.7g of Potassium dihydrogen phosphate were dissolved in 100ml of distilled water. To this 200ml of distilled phenol was added)

The amino acids were identified based on comparision of R_f values, colours and with those of authentic samples by co-chromatographic studies.

C) Chromogenic spray reagents

Ninhydrin: It was prepared by dissolving 200 mg of Ninhydrin in 98ml of n-butanol with 2ml of glacial acetic acid.

Table 3a: Amino acids

Aspartic acid	-
Arginine	+
Asparagine	+
α-Alanine	+
β-Alanine	+
2-Aminobutyric acid	-
Cystaine	+
Cystine	+
Glutamic acid	+
Glutamine	+
Glycine	-
Histidine	+
Isoleucine	+

Leucine	+
Lycine	-
γ-Methylene glutamic acid	-
γ-Methylene glutamine	-
Norleucine	-
Ornithine	+
Phenylalanine	-
Proline	+
Serine	+
Threonine	-
Tryptophan	-
Valine	+
Tyrosine	-

Table 3b: R_f values and colour reactions of Amino acids on Paper Chromatograms

Compound	R_f values in solvent		Colour with Ninhydrin
L-Aspartic acid	0.05	0.10	Light violet
DL-Arginine	0.10	0.60	Violet
DLAsparagine	0.19	0.41	Violet
α-Alanine	0.26	0.28	Light violet
β-Alanine	0.42	0.77	Deep violet
DL-2-Aminobutyric acid	0.55	0.70	Light violet
L-Cystaine	0.38	0.61	Deep violet
L-Cystine	0.23	0.53	Violet
L-Glutamic acid	0.17	0.20	Violet
L-Glutamine	0.14	0.82	Violet
L-Glycine	0.09	0.38	Violet
L-Histidine	0.08	0.52	Violet
DL-Isoleucine	0.68	0.82	Violet
L-Leucine	0.79	0.83	Deep violet
L-Lycine	0.79	0.83	Deep violet
γ-Methylene glutamic acid	0.20	0.68	Violet
γ-Methylene glutamine	0.30	0.71	Violet
DL-Norleucine	0.96	0.68	Violet
DL-Ornithine	0.04	0.27	Light violet
DL-Phenylalanine	0.60	0.90	Light violet
L-Proline	0.39	0.89	Yellow
DL-Serine	0.20	0.23	Deep violet
DL-Threonine	0.28	0.35	Deep violet
DL-Tryptophan	0.50	0.76	Violet
DL-Valine	0.59	0.77	Deep violet
L-tyrosine	0.52	0.65	Light violet

Solvents: 1. Sec. butanol **:** Formic acid **:**Water(75**:**13**:**12)
2. Buffer**:** Phenol(1:2)

D. Anthocyanidins

a) Extractive procedure

The anthocyanidin constituents were extracted according to the method described by Harborne (1973).

About 5g of plant material was immersed in 20ml of 2N HCl in a boiling test tube and heated for 30-40 minutes at6 100^0c. The extract was cooled and filtered. The filtrate was washed twice with 20 ml of ethyl acetate to remove flavones. The remaining aqueous extract was further heated at 80^0c for 3 minutes to remove the last traces of ethyl acetate. The pigment was re-extracted again with a small volume of Iso-amyl alcohol. The Ethyl acetate extract was evaporated to dryness on a boiling water bath. The anthocyanidin in the residue was diluted with 1ml of 1% Methanolic HCl and preserved at low temperature in dark for future use.

b) Identification

The extracts containing anthocyanidins was separated on 23 x 29 cm Whatmann No.1 Chromatographic filter paper. Uni-dimensional Chromatographic ascending technique was employed with Conc. Hydrochloric acid, Formic acid, Water (2**:**5**:**3 v/w/v) and n-butanol **:** Acetic acid **:** water (4:1:5). The Chromatograms developed from the said solvent systems were taken out from the glass chambers and dried at room temperature. The fluorescent regions of dried papers were marked under UV light. The R_f values and colours of the spots were determined by comparing with those of the authentic markers on the Chromatograms.

Cyanidin	+
Petunidin	-
Delphinidin	-

Table 4b: R_f Values and Colours of Anthocyanidins on Paper Chromatograms

Compound	R_f values in solvent		Visible colour	U.V. fluorescence	
	1	**2**		**without NH_3**	**with NH_3**
1	0.38	0.68	Magenta	Pink	blue green
2	0.26	0.50	Purple	Mauve	Mauve
3	0.15	0.40	Purple	Mauve	blue green

Solvents : 1. Conc. Hydrochloric acid **:** Formic acid **:** Water(2**:**5**:**3)
2. n-butanol **:** Acetic acid **:** Water(4**:**1**:**5).

E. Lipids

a) Extractive procedure

Lipids were extracted following the method adopted by Hoppe and Heitefuss (1974). A solvent mixture consisting of 30ml Chloroform, 60ml of Methanol and 20 ml Water(1**:**2**:**0.8 v/v/v) was taken and allowed to boil. About 5g of plant material was homogenized in the above solvent mixture. The contents were filtered and taken separately. The residual mixture was treated with 70ml Methanol and the filtrate taken. The residue was again washed with 100ml Chloroform. All the above filtrate mixtures were taken in a separating funnel and 90ml of water added. The mixture now contains Chloroform**:** Methanol**:** Water in the ratio of 2**:**2**:**1.8 respectively and the mixture were allowed to settle. The lower lipid layer containing chloroform was separated and transferred into a breaker. The upper water layer was treated with 50ml of Chloroform successively for three times. All the extracted Chloroform layers were evaporated to dryness in a vacuum by rotary evaporation maintained at 40^0C. The residue was treated with 2ml of Benzene to remove the traces of chlorophyll if present. Now the final lipid residue was dissolved in 2 ml of Chloroform and stored at low temperature in dark until use.

b) Identification

Thin layer plates were prepared by spreading a slurry of silicagel-G (50g in 100ml distilled water) to 105 mm thickness over thin glass plates. The glass plates were air dried and stored at room temperature. Before using, the plates were heated at 110^0c for 30 minutes in a hot air oven for activation.

Using micropipette, one gram equivalent of lipid extract was taken and potted on TLC plates. The spotted areas were allowed for immediate dryness with the help of a drier. The

dried plates were run in uni dimensional ascending Chromatography by using TLC glass chambers. The chambers were saturated with developing solvents one day before the plates were developed.

Solvent systems

1) Chloroform**:** Methanol**:** Acetic acid**:** Water (170**:**25**:**25**:**3 v/v/v/v)
2) Acetone**:** Benzene**:** Water(91**:**30**:**8 v/v/v).

The plates were placed in airtight tanks. The developed plates were removed and dried at room temperature and exposed to Iodine vapours to visualize all the lipid compounds.

The TLC plates were sprayed with 25% Sulphuric acid reagent with the help of an automizer for the clear detection of various lipid layers. The lipids were identified by comparision of R_f values, color and with those of authentic samples by Co-Chromatographic studies.

c) Chromogenic spray reagent

The TLC plates were sprayed with 25% Sulphuric acid and heated to 230^0c for 15 minutes, and the colour observed. Glycolipids gave red brown while phospholipids gave bright, red and other lipids gave pale brown colour spots on white background.

Table 5 a: Lipids

Phosphatidyl serine	-
Phosphatidyl inositol	+
Phosphatidyl Clioline	+
Phosphatidyl ethanolamine	-
Digalactosyl diglyceride	+
Phosphatidyl glycerol	+
Sulphoquinovosyl diglyceride	+
Monogalactosyl diglyceride	+
Steryl glycoside	+

Table 5b: Rf values and colour reaction of lipids on thin layer Chromatograms

Compound	R_f values in solvent	Colour with iodine vapours	Colour with H_2SO_4 (25%)
Phosphatidyl serine	0.09	+	+
Phosphatidyl inositol	0.13	+	+
Phosphatidyl Clioline	0.18	+	+
Phosphatidyl ethanolamine	0.23	+	+
Digalactosyl diglyceride	0.33	+	+
Phosphatidyl glycerol	0.41	+	+
Sulphoquinovosyl diglyceride	0.57	+	+
Monogalactosyl diglyceride	0.81	+	+
Steryl glycoside	0.87	+	+

Solvent : $CHCl_3$: MeOH : HOAc : H_2O (170:25:25.3).

Physicochemical studies

For several years, the crude drugs were identified by comparison only with the standard descriptions available. But recently, due to advancement in the field of Pharmacognosy, various techniques are followed to the standardization of crude drugs. Among such techniques, ash and extractive values are considered very important in promoting the quality and in establishing the Pharmacopeial standards of the drug.

Substandard quality of many drugs is due to either faulty collection or incorrect storage. When the constituents of a drug cannot be readily estimated by any other means, ash and extractive values are very useful especially for their evaluation. To prove its acceptability as a drug, studies of ash and extractive values are basically required in determining the quality and purity of selected crude drug.

Materials and Methods

The physical constants like ash and extractive values were determined by standard methods (Anonymous, 1985 and Kokate, 1991).

3. Experimental Studies

A. Determination of ash values

The object of ashing crude drugs is to remove all traces of organic matter which may otherwise interfere in an analytical determination. The inorganic salts naturally occurring in drug or adhering to it or deliberately added to as a form of adulteration are removed by gradual incineration of the drug. Thus the residue remaining after incineration of the powdered drug is known as ash content. The ash values are a criterion to judge the identity or purity and quality of the crude drug.

a) Determination of total ash

Total ash usually consists of Phosphates, Silicates and Carbonates of Potassium, Calcium and Magnesium. About 2 gm of the air dried powdered drug was accurately weighed and taken in a tared silica crucible, which was previously ignited and weighed. The powdered drug was spread as a fine even layer at the bottom of the crucible. The crucible containing the drug was gradually incinerated in a muffle furnace by increasing the temperature. It was heat dull red hot until free from carbon or converted into ash. Later the crucible was allowed to cool and kept in desiccators. The residue was weighed the percentage of total ash calculated with reference to the air dried sample. The residue was weighed the percentage of total ash calculated with reference to the air dried sample. The procedure was repeated till constant result was obtained.

b) Determination of acid insoluble ash

Acid insoluble ash is a part of total ash insoluble in dilute Hydrochloric acid. The acid insoluble ash content is determined and recommended for certain drugs may be coated with dirt and sand. The total ash obtained as described above was treated with 25ml of 10% Hydrochloric acid and boiled for 5 minutes. The insoluble matter was filtered and collected on ashless filter paper (Whatman No.42) and the paper washed with hot water. The insoluble ash was ignited and weighed in a tared silica crucible. The acid insoluble ash of the drug was repeated to get constant results.

c) Determination of water soluble ash

The total ash obtained from the above process was boiled with 25 ml of distilled water for 5 minutes. The insoluble ash was filtered through ash less filter paper (Whatman No.42). The residue was once again washed with water and ignited up to temperature not exceeding 450^oc and converted into ash. The difference between the weight of total ash and

insoluble matter represent the weight of water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried drug.

B. Determination of extractive values

Extractive values indicate the nature of the constituents present in a crude drug. Based on the diversity in chemical nature and properties of the contents of the drug, various solvents were used for the determination of extractives. The solvents used for extraction dissolve appreciable quantity of substances desired. The methods used to find out the extractive values were as follows.

a) Determination of alcohol soluble extract

Alcohol is an ideal solvent frequently employed to determine various chemical contents like Tannins, Resins etc. Generally, Ethyl alcohol is used for determination of alcohol-soluble extractive. Dilute Alcohol a may also be used in some cases, depending upon solubility of constituents in a crude drug. In the present work, the author used 100 ml of 90% Alcohol for determining crude drug was macerated with 100 ml of 90% Alcohol in a stoppered flask for 24 hours. It was shaken frequently every 6 hours and allowed to stand to 18 hours. Then the extract was filtered rapidly through filter paper, taking precaution against loss of alcohol, about 25 ml of the filtrate was evaporated to dryness in a tared flat bottomed shallow dish, transferred to the oven and dried at 105^{o}C and weighed. The percentage of alcohol soluble extract was calculated with reference to the air dried drug.

Table 6: Ash value of Root tubers

S.No.	Plant species	Part(s)	Total ash (%)	Acid insoluble ash (%)	Water insoluble ash (%)
1.	***Pimpinella tirupatiensis***	Tuber	9.50	3.00	1.75

Table 7: Extractive value of the root tubers

S.No.	Species	Part(s)	90% alcohol soluble extract (% w/w)	Water soluble extract(%w/w)	Chloroform soluble extract(%w/w)	Pet.Ether soluble extract(%w/w)
1	*Pimpinella tirupatiensis*	Tuber	17.92	12	2.4	0.9

Antibacterial Activity

The presence of antimicrobial substances in the higher plants provide a source of inspiration for novel drug compounds as plants derived medicines have made significant contribution towards human health. The antimicrobial compounds from plants may inhibit microbial growth by different mechanism than those presently used antimicrobials and may have a significant clinical value in treatment of resistant microbial strains. Therefore, random screening of plants for active chemicals is as important as the screening of ethnobotanically targeted species (Principle, 1989)**.**

Materials and Methods

Collection of medicinal plants: Selected medicinal plant, *Pimpinella tirupatiensis* has been used in the present study and it was collected from Tirumala hills, Eastern Ghats, Andhra Pradesh, India.

Bacterial Cultures: The bacterial cultures were procured from the Department of Microbiology, Sri Venkateswara Institute of medical Sciences (SVIMS), Tirupati, Andhra Pradesh, India. Pathogenic Gram positive bacteria such as *Bacillus subtilis, Klebsiella pneumoniae*, and Gram negative bacteria *Proteus vulgaris, Pseudomonas aeruginosa* were maintained on nutrient agar slants at 4^{0}C until further used for experimental studies**.**

Preparation of the medium

Nutrient Agar media (P.H 7.0)

For the preparation of 1 lt of Nutrient Agar media ingredients like 1.5 g of beef extract, 1.5 g of yeast extract, 5 g of peptic digest of animal tissue and 5 g of sodium chloride were weighed and added in 500 ml of distilled water and heated with agitation to dissolve the constituents. Finally, the volumes were made upto 1 lt. Before the addition of agar (15 g) the pH of the medium was adjusted to 7.0 by adding few drops of 0.1 N NaOH or HCl using digital pH meter (Elico Pvt. Ltd., Hyderabad). These were then sterilized by autoclaving at 15 lbs pressure at 120^{0} C for 15 min; cooled to 40^{0} C and approximately 20 ml of medium was poured to each 90mm sterilized petridish.

Preparation of inoculum:

Bacteria: 18 hrs old bacterial broth cultures were used as inoculae after adjusting its population to 10^{6} CFU/ ml (Colony Forming Units) using 0.9% (w/v) sterile saline by the method described by Forbes *et al*.,(1990).

Preparation of plant extract: The methanolic extracts of the four selected medicinal plants were prepared by dissolving 10gm of fine powder of each medicinal plants separately in 100 ml of methanol solvent. The contents were kept in orbitary shaker for 48 h. Then the extract was filtered and it is dried in hot air oven at 40^{0} C. Then the extract was stored under refrigeration at 4^{0} C for further studies**.**

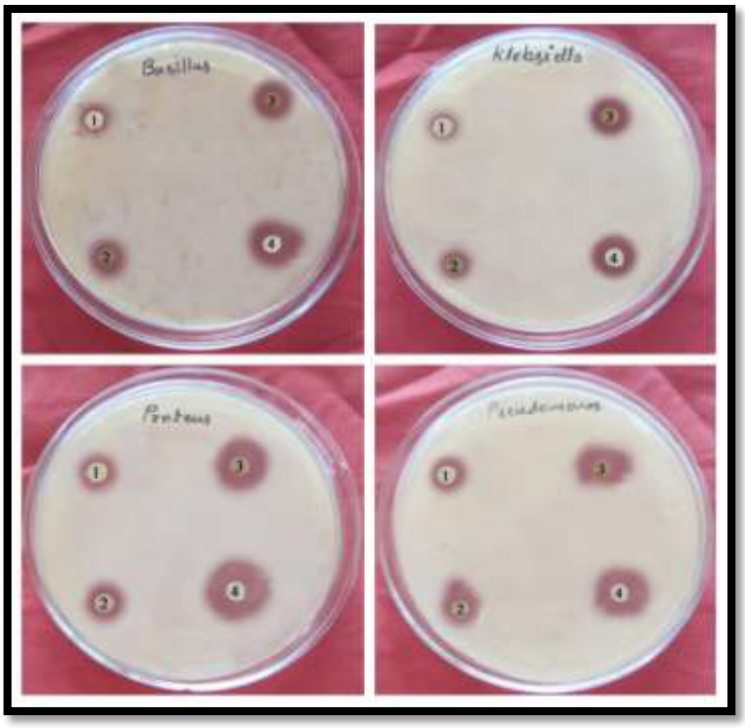
Preparation of sterile disc: Whatman's No.3 filter paper was punched into 5 mm disc form and they sterilized, each sterile disc was incorporated individually with 20 – 60 mg/ml of extracts using micropipette. Precautions were taken to prevent the flow of the solvent extract from the discs to the outer surface. The condensed extracts were applied in small quantities on discs and they were allowed to dry in air. Then they were stored at 4^{0} C.

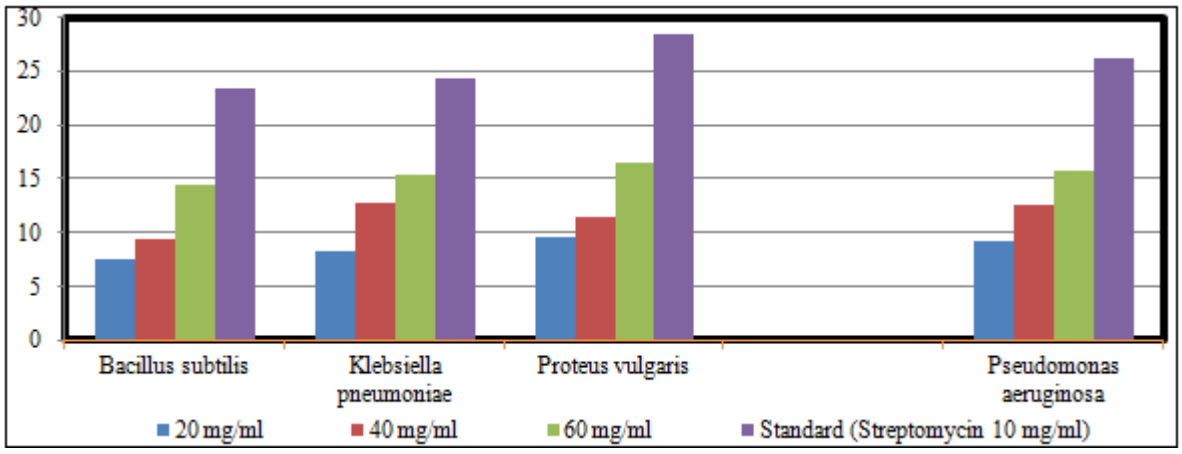
Assay of antimicrobial activity using Disc diffusion method: The antimicrobial activity was performed by employing the disc diffusion method adopted by Bauer *et al.,* (1966) and Cruickshank (1968). The 20 ml of sterilized Muller Hinton Agar was poured into sterile petriplates, after solidification, 100 µl of fresh culture of human pathogens were swabbed on the respective plates. Sterile discs of Whatman No. 1 filter paper of about 6 mm were prepared.

The discs were kept over the agar plates using sterile forceps at various concentrations (20, 40, and 60 mg/ml). Streptomycin 10mg/ml containing disc used as standard control. The plates were incubated for 24 h at 37^0 C and the diameter of the inhibition zones was recorded. The assessment of antimicrobial activity was based on measurement of inhibition zones formed around discs. A zone of inhibition around the disc indicates that the compounds, which diffused into the agar from the disc, inhibited the growth of the organism.

Antibacterial activity of *Pimpinella tirupatiensis*:

S. No	*Name of the microorganism*	*20 mg/ml*	*40 mg/ml*	*60 mg/ml*	*Standard (Streptomycin 10 mg/ml)*
1.	*Bacillus subtilis*	7.5 ± 0.45 mm	9.4 ± 0.6mm	14.52 ± 0.89mm	23.45 ± 0.65mm
2.	*Klebsiella pneumoniae*	8.2 ± 0.24mm	12.8 ± 0.89mm	15.34 ± 0.59mm	24. 4 ± 0.53mm
3.	*Proteus vulgaris*	9.5 ± 0.23mm	11.35 ± 1.2mm	16.5 ± .64mm	28.53 ± 0.74mm
4.	*Pseudomonas auriginosa*	9.2 ± 0.54mm	12.5 ± 0.16mm	15.82 ± 0.44mm	26.25 ± 0.36 mm





4. Results

Present investigation, antibacterial activity of *Pimpinella tirupatiensis* on pathogenic bacterial strains, the plant exhibited significant antibacterial activity. Methanol root extract of *Pimpinella tirupatiensis* at the concentration 60 mg/ml, *P.vulgaris* (16.5mm) was more susceptible, followed by *P. aeruginosa* (15.8mm), *K.pneumonia* (15.3mm), and *B.subtilis* (14mm).

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