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Phytochemical Investigation and *In Vitro* Antimicrobial Activity of *Centella asiatica* (L.) Urban. A potent Antijaundice medicinal plant

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Abstract

Centella asiatica (L.) is also known as Indian penny wort is a small creeping herbaceous plant belongs to the plant *Apiaceae*. This plant has been referred in the ancient traditional Indian ayurvedic medicine system about 3000 year's age. This herb is antidiabetic, antitumor and antidiuretic used in the treatment of jaundice, asthma, bronchitis, dropsy, kidney troubles and leprosy diseases. An investigation was carried out to study the antibacterial activity of *Centella asiatica* of five different solvent extracts from leaf, stem and roots against selected laboratory bacterial pathogens such as *Bacillus subtilis*, *Staphylococcus aurius, Pseudomonas aurigenosa*, *Escherichia coli*, *Klebsiella pnemoniae* and *Salmonella typhi* by agar diffusion method. Zone of inhibition was measured (mm) were compared with standard antibiotics such as kanamycin, cefotoxime, penicillin. The organic solvents such as ethanol, methanol, petroleum ether, chloroform and aqueous extracts were employed. Among all the extracts, methanolic extracts of leaf has showed maximum antibacterial activity against *Bacillus subtilis*, *Salmonella typhi*. Phytochemical screening methods were also done to identify the major secondary metabolites in the species such as glycosides, terpenoids, alkaloids, phenolics and tannins. In addition to that the comparative analysis of phenols and tannins of both in conventional plants and in *in vitro* propagated plants were also traced.

Keywords: *Centella asiatica* (L.), antibacterial activity, agar diffusion, phytochemical constituents, zone of inhibition.

Introduction

Medicinal plants are being the most reliable source of curative drugs used as traditional medicines and folk medicines. Medicinal plants are plants containing inherent active ingredients used to cure disease or relieve pain. The idiom "phyto medicine" determines the parts of plants (leaves, flowers, seeds, roots, barks, stems, etc.) used for preparing medicines [1]. They are primary sources of obtaining antimicrobial drugs [2]. Now a days herbal medicines using as better remedies for their lesser side effects, better adoptability with a economical affordability [3]. *Centella asiatica* (L.) is a small creeping herb known as brahmi in unani medicine this plant is used as cordiotonic, nervine tonic etc. The leaves are said to be useful in the treatment of jaundice and in some chronic disorders [4]. It is used to cure liver disinfection and poor digestion. An antioxidant, anti tumorogenic, anti inflammatory, anti diabetic properties were reported [5]. Secondary metabolites were produced by plants to carry out different functions as defense and pollination. But their antioxidant, antimicrobial properties were created interest and widely exploited for the benefit of mankind [6]. The present study was designed to find out the antimicrobial property of leaves, stem and roots. The study was accordance with the fact that aerial parts and methanolic extracts of leaves of *Centella asiatica* (L.) [7,8]. In view of the wide ranging medicinal value of *Centella asiatica* as mentioned in Ayurvedic literature or else, it is essential that more clinical and pharmacological trials are needed to investigate the unexploited potential of this plant. However World Health Organization (WHO) also has recognized the importance of traditional herbal medicine and has been active in creating strategies [9].

Materials and Methods

Plant identification and sample collection

Centella asiatica (L.) was collected from herbal garden, Dravidian University, Kuppam, A.P. India. The taxonomic identification of the plant was carried out at the herbarium of Botany department and voucher specimens were properly authenticated.

Preparation of plant extracts

Leaf, stem and root of the plant allowed to air dried in the shade for two weeks and packed in paper bags and stored. Samples were kept under constant observation to avoid any growth of contaminants. Dried leaves stem and roots were pulverized in an electric grinder to obtain fine powder sample [10]. By using this powder different samples were prepared by using organic solvents such as ethanol, methanol, petroleum ether, chloroform and aqueous preparation by using distilled water. The samples were

stored in a shaker for 72 hours at room temperature. The samples were reduced to 10% of its original volume and filtered using whatmann filter paper. The filtrate was allowed to concentrate in a vacuum by using rotatrory evaporator leaving behind crude extracts.

Phytochemical screening

Phytochemical analysis of all the evaporated solvent extracts was conducted following the procedure of Indian pharmacopoeia [11].

Test for alkaloids

200 mg of plant material was dissolved in 10 ml methanol and filtered. For 2ml filtrate and 1% Hcl+steam, 1ml filterate+6 drops Mayer's reagent/Wagner's reagent/Dragendorff's reagent was added. Creamish precipitate/brownish red precipitate/orange precipitate indicated the presence of respective alkaloids.

Test for tannins

200 mg of plant material was dissolved in 10ml of distilled water and filtered. For 2 ml filtrate+ 2ml $FeCl_3$ was added. Blue/ black precipitate indicated the presence of tannins.

Test for flavonoids

200 mg of plant material was dissolved in 10ml of ethanol and filtered. For 2ml filtrate +conc. Hcl + magnesium was added. Ribbon pink/tomato red color indicated the presence of flavonoids.

Test for steroids

(Liebermann-Burchard reaction)
200 mg of plant material was dissolved 10ml of chloroform and filtered. For 2 ml of filterate+2 ml of acetic anhydride+ conc. H_2SO_4 was added. Blue/green ring indicated the presence of steroids.

Test for terpenoids

200 mg of plant material was dissolved in 10ml of distilled water and filtered. For 2 ml of filterate, 2ml of chloroform and then 3ml of conc. H_2SO_4 was added. A reddish brown coloration of the line was indicated the presence of terpenoids

Test for saponins

About 2ml of solvent extract was introduced into a tube containing 2 ml of distilled water, and the mixture was shaken for 3-5 min results in formation of foam indicated the presence of saponins

Test for phenols

1 ml of each solvent extracts dissolved in alcohol/water was separately treated with 1ml of neutral $Fecl_3$. The change in color indicated the presence of phenols.

Bacterial test strains used and growth conditions

Gram-positive bacterial strains such as *Bacillus subtilis*, *Staphylococcus aurius* and gram -negative bacterial strains such as *Pseudomonas aurigenosa*, *Escherichia coli*, *Klebsiella pneumonia* and *Salmonella typhi* were used for screening antibacterial activity respectively. The bacteria used for the study were obtained from Department of Microbiology, Sri Venkaterwara University, Tirupati. All the cultures were maintained at 4^0 C in nutrient agar slants. All glass wares and other materials needed for test were thoroughly sterilized.

Preparation of inoculums

A loop full of the test culture was taken from respective strains of agar slants and sub cultured in to fresh tubes containing nutrient broth and incubated for overnight at 37^0C. The obtained cultures was centrifuged at 5000 rpm for 15 min. Bacterial suspension was added to fresh media which gives final concentration of 10^7 cfu /ml [12].

Determination of antibacterial assay

Agar well diffusion method with some minor modification was used in the present study [13]. Approximately 100 µl of inoculums of each microbial culture was cultured on nutrient agar medium which was poured into petridishes to occupy the deepness of 3-4 mm. By using a sterile cork borer about 6 mm in diameter wells were punched in the medium. The punched agar disc was removed by vacuum device. Then five different solvent extracts of ethanol, methanol, petroleum ether, chloroform and aqueous extracts were placed appropriate position on the plate with the quadrants marked at the backside of each plate. Standard antibiotics such as penicillin, kanamycin, tetracycline and cefotaxime were used at a final concentration at 100 µg/ml. wells at the distance of 4cm were made in the agar plate. 50 µl for each dilution (50 µg/ml), (100 µg/ml) plant extracts were filled in the wells. Wells for standard antibiotic (50 µg/ml), (100 µg/ml) were also run parallel. The plates were incubated at 37^0 C for overnight. In addition to that quantitative analysis of phenols and tannins of both in conventional plants and in *in vitro* propagated plants were also traced.

Statistical evaluation

The diameter of the zones of inhibition (mm) was measured by calculating the difference between cork borer (5 mm) and the diameters of inhibition. The antimicrobial activity was determined by calculating the mean of triplicates ± SD of three replicates.

Results

Phytochemical analysis

Qualitative phytochemical analysis of selected crude organic solvent extracts and an aqueous extracts revealed the presence of various compounds such as alkaloids, flavonoids, glycosides, tannins, terpenoids, saponins, aminoacids (Table 1).

Table 1- Qualitative phytochemical analysis of secondary metabolites such as alkaloids, flavonoids, terpenoids, glycosides, phenols, and tannins in different plant parts of *Centella asiatica*

Type of extract	Leaf					Stem				
Phytochemicals	E	M	C	PE	AQ	E	M	C	PE	AQ
Alkaloids	_	+	+	+	-	_	+	+	+	-
Flavonoids	+	+	+	+	+	+	+	+	+	+
Terpenoids	+	-	+	+	+	+	-	+	+	+
Glycosides	+	+	-	+	+	+	+	-	+	+
Phenols	+	+	+	-	+	+	+	+	-	+
Tannins	+	+	-	-	+	-	+	-	+	-
Steroids	-	+	+	+	-	-	+	+	+	-

Positive = (+), Negative = (-)
M = Methanol, E = Ethanol, C= Chloroform, PE= Petroleum ether,
AQ = Aqueous Extract.

The main active principle compounds are triterpenoids, glycosides such as asiatic acids. Diterpenes, triterpenes as madecassic acid,or brahmic acid were proved as highly potential against various diseases.. Alkaloids, flavonoids detected in the extracts are compounds that have been documented to possess medicinal properties and health promoting effects. Phenolics are the largest group of phytochemicals and have been said to account for most of the antioxidant activity of plant extract.

Antimicrobial activity

Antimicrobial activity of *Centella asiatica* extracts was determined by agar well diffusion method. All the four extracts that were employed were showed maximum activity against all tested microbial cultures. The zone of inhibition obtained was compared with standard antibiotics were represented in Table 2 and Figure 1 respectively. Methanolic extract of leaves showed maximum activity against all microorganisms (8.6-14.8 mm).

All the four leaf extracts such as ethanol, methanol, petroleum ether, chloroform were showed maximum activity (8.0-15.0 mm) except aqueous extracts. Stem extracts were showed moderate such as (6.4-9.2 mm). Aqueous extracts which showed less activity or else poor activity (4.2 mm). The root extracts of petroleum ether and chloroform were does not show any activity against tested cultures. Methanol and ethanol showed moderate activity (6.1 mm) against some gram-positive bacterial strains such as *Bacillus subtilis*, *Staphylococcus aureus.* Chloroform root extract was showed least activity (4.0 mm). The results were compared with standard antibiotics such as penicillin, kanamycin, tetracycline and cefotaxime. All the tested strains showed the maximum activity against methanolic extract of leaves (8.6-14.8 mm) which is near to the activity of standard antibiotic kanamycin (8.5-15.5 mm).

Quantitative analysis of phenols and tannins revealed that maximum concentration of phenols (3.3 mg/gm) and tannins (4.8 mg/gm) were observed in tissue cultured plants followed by the conventional field grown plants (Table 3).

Table 2- Susceptibility of test microbial strains to leaf, stem and root extracts of Centella asiatica and standard antibiotics.

Type of extract / antibiotic used	Zone of inhibition (mm)											
	Staph. aureus		Bacillus subtilis		Escherichia coli		Salmonella typhi		Klebsiella pneumonia		Pseudomonas aureginosa	
	50µl	100µl	50µl	100µl	50µl	100 µl	50µl	100µl	50µl	100µl	50µl	100 µl
Leaf												
Ethanol	3.2	5.8	3.6	7.4	5.4	11.2	6.4	10.8	3.6	5.8	4.1	8.4
Methanol	4.8	8.6	4.2	8.5	6.8	13.2	7.2	14.8	4.8	9.4	5.7	12.1
P. ether	3.0	5.6	3.7	6.6	5.3	10.8	6.2	9.3	3.4	6.1	3.9	8.3
Chloroform	3.2	5.4	3.6	7.1	4.3	9.2	5.0	8.3	2.9	4.7	5.5	7.8
Aqueous	2.8	4.0	3.1	5.2	5.0	7.8	6.0	7.8	2.0	4.4	3.9	4.2
Stem												
Ethanol	3.5	5.6	4.1	7.9	3.9	6.8	4.5	8.6	6.4	9.3	5.2	8.9
Methanol	6.4	11.8	5.9	11.4	6.4	10.7	7.3	13.2	7.1	12.4	6.1	11.4
P. ether	4.5	8.4	3.6	8.2	5.8	9.4	5.2	9.4	4.9	7.4	5.9	10.1
Chloroform	3.9	7.0	4.2	7.6	4.1	8.2	4.6	8.1	5.1	7.9	4.1	7.6
Aqueous	3.2	5.9	4.2	6.3	4.2	6.3	4.1	6.0	4.0	6.4	3.2	4.8
Root												
Ethanol	4.1	6.1	-	-	3.0	5.0	2.1	4.2	-	-	-	-
Methanol	5.1	8.1	2.2	3.4	-	-	2.5	3.6	3.4	4.1	-	-
P. ether	4.0	6.2	-	-	-	-	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-	2.4	4.0	-	-
Aqueous	-	-	-	-	-	-	-	-	-	-	-	-
Standard antibiotics												
Pencillin	11.4	22.5	10.8	22.0	6.5	14.9	5.5	12.1	9.3	10.5	10.2	21.0
Kanamycin	10.5	20.8	9.5	19.8	5.5	13.1	6.2	11.5	6.2	9.5	7.5	13.0
Cefotaxime	7.5	14.8	8.3	16.0	5.4	10.7	9.3	16.5	6.2	12.8	5.8	10.9
Tetracycline	5.0	10.5	8.5	15.8	8.0	15.5	4.5	10.2	5.5	9.8	6.0	11.6

Table 3- Estimation of total phenols and tanins present in field grown conventional plants and in vitro regenerated plants of *Centella asiatica*

Name of the plant material	Phenols (mg/g)	Tannins (mg/g)
Field grown conventional plants	2.5	3.6
in vitro regenerated plants	3.3	4.8

Discussion

In India, the Ayurvedic system has described a large number of medicines based on plants or plant products. Phytochemical assay of the plant showed the presence of various compounds such as alkaloids, flavonoids, glycosides, tannins, terpenoids, saponins, amino acids are major compounds of effective medicinal property with increased potential which exhibit the antiproliferative, antioxidant [14, 15]. Alkaioids rank among the most proficient and pharmaco-therapeutically significant plant substances and the largest single class of secondary plant metabolites. Phenolics are the largest group of phytochemicals and have been said to account for maximum degree of antioxidant activity of plant extracts of *Baccopa monnieri* and *Centella asiatica* have been reported [16]. Saponins are function as potent antimicrobial agents. The antimicrobial activity of saponins extract of was reported in *Sorghum bicolor* (L.) [17]. Tannins are complex phenolic polymers, which will bind to the proteins and carbohydrate molecules resulting in reduction and inhibition of microbial growth [18].

An active principle compounds such as flavonoids have been reported in *Combretum* genera and in *Terminalia* Sps. [19,20] which indicates the medicinal potential of plant. Our reports were accordance with previous investigations of same plant species *Centella asiatica* [21]. Triterpenoids of *Centella* was reported to exhibit antitumor activity. This plant was proved to be efficient in

the treatment of rheumatic disorders. Asiaticoside is a major triterpene and posses anti-inflammatory and gastric ulcer drugs [22].

In the present study our results showed that leaf extracts posses bioactive molecules with maximum antibacterial activity against all tested strains. The observed potential could be due to the presence of tannins and cyanogenic glycosides. This will suggest the presence of bioactive compounds or groups in the extracts with similar mechanism of action (MoA) to that of kanamycin to that of higher concentration. The extract exhibited some appraisal level of activity. Gram positive species such as *Staphylococcus aureus* and *Streptococcus* species are causative pathogen in community acquired pneumonia. *Klebsiella pneumoniae* is also causative pathogen of *Pneumonia* and respiratory tract infections [23, 24].

Our study suggests that crude extracts of *Centella asiatica* was effective against many infection causing microbial agents. This study was accordance with previous reports of *Ficus thonningsii* [25], *Amaranthus tristis* [26] and *Osimum sanctum* [27]. It is also states that the bioactive compounds present in aerial parts than that of stems and roots *Spermanthus indicus* [28]. These results are in concord with the findings in leaf extracts of *Mentha pipertia* which was also showed the maximum antimicrobial activity [29]. The lack of antimicrobial activity of root extracts may due to the absence of antimicrobial compounds or else present in low levels [30]. The present study reveals that more phenols and tannins were present in the tissue cultured plants followed by conventional plants. Our results are in line with *Bacopa monnieri* [31]. The results obtained were optimistic as the methanolic extract of leaves has showed significant antibacterial activity against the tested microbial cultures.

Conclusion

In conclusion the methanolic extracts of *Centella asiatica* showed varying inhibitory activities against all tested organisms. The results were encouraging enough to pursue fractionation of this extracts and to find out the functional properties of phytochemical compounds in view to discover useful potential chemotherapeutic agents.

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