Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN 2320-3862
JMPS 2016; 4(5): 181-185

Received: 15-07-2016
Accepted: 16-08-2016

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Studies on the bioactive compounds and antimicrobial activities of medicinal plant *Centella asiatica* (Linn)

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Abstract
The leaves of the medicinal plant *Centella asiatica* is screened for phytochemical analysis and microbial activity using petroleum ether, acetone and methanol. The antimicrobial activity was performed by using disc diffusion method. Acetone and methanol crude extracts showed highest activity against fungal and bacterial strains.

Keywords: *Centella asiatica*, antimicrobial, disc diffusion, phytochemical

Introduction
Centella asiatica (Linn) Urban belonging to family *Apiaceae* (Umbelliferae) popularly known as 'Brahmi' is a very useful medicinal plant described by Charaka as an anti-ageing plant. The plant extract used to microbial infection are reported in our ancient Ayurvedic compendium Charaka Samhitha and Sushrat Samhitha [1] microbicides, pesticides and many pharmaceutical drugs. *C. asiatica* is very rich in terpenoides, a compound which plays a very active role in wound healing [2] and helps to reduce diastolic blood pressure and lowers the sugar level in blood [3]. Traditionally, *C. asiatica* is consumed as an herbal tea or the leaves are eaten fresh.
Phytochemical is a natural bioactive compound found in plants, such as vegetables, fruits medicinal plants, flowers leaves and roots that work with nutrients and fibers to act as defense system against disease or more accurately, to protect against diseases. Phytochemicals are divided into two groups, which are primary and secondary constituents; according to their functions in plant metabolism. Primary constituents comprise common sugars, amino acids, proteins and chlorophyll while secondary constituents consist of alkaloids, terpenoids and phenolic compounds [4]. Human infections particularly those involving microorganisms i.e., bacteria, fungi, viruses, nematodes cause serious damages in tropical and subtropical countries of the world. In recent years, multiple drug resistance in human pathogenic microorganisms has been developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of such diseases. Over the last centuries, intensive efforts have been made to discover clinically useful antimicrobial drugs [5-7].
Many plant families represent reservoir of effective chemotherapeutics and can provide valuable sources of natural antimicrobials [8, 9]. These for many thousands of years, plant extracts have been used for a wide variety of purposes [10]. Plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. Scientific experiments on the antimicrobial properties of plant components were first documented in the late 19th century [11].

Materials and Methods
The leaves of the medicinal plant *Centella asiatica* L., belongs to the family Apiaceae was selected for the present investigation.
C. asiatica is a prostrate herb, rooting at the nodes. Leaves orbicular, crenate, palmately nerved, deeply cordate with an angular sinus, long-petioled; stipules scarious. Flowers reddish, small, sessile, in simple axillary few-flowered umbels; involucral bracts two, small. Calyx truncate. Petals minute, ovate, acute, imbricate. Fruit laterally compressed, the mericarps with about 7-9 subsimilar ridges, the secondary ridges as prominent as the primary, reticulate between them, vittae absent; pericarp thickened.

Centella asiatica L.

Preparation of the extracts
The plant leaves were collected and shade dried for about two weeks and ground into coarse powder. About 50g powder of plant was extracted with petroleum ether, acetone and methanol using soxhlet apparatus. The extracts were concentrated to dryness to yield crude residues. These residues were used for preliminary phytochemical screening of secondary metabolites. In the present study, all preliminary phytochemical screening was carried out following the methodology of Harborne [12].

Alkaloids
Meyer's reagent (potassium mercuric iodide) 1.36gm of mercuric chloride was dissolved in 60ml of distilled water and 5 gm of potassium iodide was dissolved in 10 ml of water. These two solutions were mixed and diluted to 100 ml with distilled water. To 1 ml of the extract, a few drops of reagent were added. Formation of white or pale precipitate showed the presence of alkaloids.

Dragendorff's test
To a few ml of filtrate, 1 or 2 ml of Dragendorff's reagent are added. A prominent yellow precipitate indicates the test as positive.

Cardiac glycoside (Killer Killiani test)
Total 100 mg of extract was dissolved in 1 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under layered with 1 ml of concentrated sulphuric acid (H_2SO_4). A brown ring obtained at the interface indicates the presence of de-oxysugar characteristics of cardolides.

Flavonoids
In the test tube containing 0.5 ml of extract, 5 to 10 drops diluted HCL and small piece of ZnCl or magnesium were added and the solution was boiled for few minutes. The appearance of reddish pink or dirty brown colour indicates the presence of flavonoids.

Saponins
In a test tube containing about 5 ml of the extract, few drops of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 minutes. A honeycomb like froth was formed and it showed the presence of saponins.

Glycosides
A small amount of extract was dissolved in 1 ml of water and aqueous sodium hydroxide solution was added. Formation of yellow colour indicates the presence of glycosides.

Steroids
To 2 ml of extract, 1 ml of concentrated sulphuric acid was added carefully along the sides of the test tube. A red colour produced in the chloroform layer shows the presence of steroids.

Resins
To 2 ml of extract 5 ml of acetic anhydride was added, dissolved by gentle heating, cooling and then 0.5 ml of sulphuric acid was added. Bright purple colour indicates the presence of resins.

Phenols
Ferric chloride test
To 1 ml of the extract 3 ml of distilled water followed by few drops of 10% aqueous ferric chloride solution was added. Formation of blue or green colour indicates the presence of phenols.

Tannins
Ferric chloride test
(200 mg plant material in 10 ml distilled water, filtered); 2 ml filtrate + 2 ml ferric chloride gives blue-black precipitate which indicates the presence of tannins.

Lead acetate test
In a test tube containing about 5 ml of the extract, a few drops of 1% solution of lead acetate was added. A yellow or red precipitate indicates the presence of tannins.

Antimicrobial Study
The bacterial strains and the fungal strains were maintained in nutrient agar slants and potato dextrose agar slants respectively. Antibacterial and antifungal studies were carried out by Disc Diffusion method.
Test organisms

Bacterial strains	
Gram positive	Gram negative
Bacillus subtilis	*Escherichia coli*
Bacillus thuringiensis	*Klebsiella pneumonia*
Enterococcus faecalis	*Proteus mirabilis*
Staphylococcus aureus	*Proteus vulgaris*
Streptococcus faecalis	*Pseudomonas aeruginosa*
Streptococcus pyogenes	*Salmonella paratyphi*
	Salmonella paratyphi. A.
	Salmonella paratyphi. B.
	Serratia marcescens

Fungal strains
Fusarium oxysporum
Alternaria alternata
Curvularia lunata

Composition of Nutrient Agar medium for Bacteria

Peptone	10g
Beef extract	15g
Sodium chloride	3g
Distilled water	1000ml
Agar agar	20g

Composition of Potato Dextrose Agar (PDA) medium for Fungi

Potato tubers	200g
Dextrose	20g
Distilled water	1000ml
Agar agar	20g

Preparation of Culture Medium and Inoculation

The petriplates and the nutrient agar medium as well as potato dextrose medium were sterilized for 20 minutes at 120 ^{0}C. The rest of the procedure was carried out in laminar air flow. Approximately 20 ml of the media was poured into the sterile petriplates and allowed to get solidify for 15-20 minutes. After the media gets solidified, the bacterial and fungal organisms were swabbed in respective medium using cotton swabs.

Disc Diffusion Method

Antimicrobial activity of the plant extracts were tested using the Disc Diffusion method [13]. Sterile nutrient agar plates and potato dextrose plates were prepared for bacterial and fungal strains respectively and inoculated by a spread plate method under aseptic conditions. The filter paper discs of 6mm diameter (Whatman's No. 1 filter paper) were prepared and sterilized. The plant extracts to be tested were prepared with various concentrations of 25mg/ml, 50mg/ml, 75mg/ml and 100mg/ml and were added to each disc of holding capacity of 10 microlitres. The sterile impregnated disc with plant extracts were placed on the agar surface with flamed forceps and gently pressed down to ensure complete contact of the disc with the agar surface. Control discs were also prepared using the positive control amphicillin, a bactericide but it was not used for fungi. They were placed using respective solvents used for the extraction. All the plates including control plates were incubated at 37 ^{0}C for 24 hours. After incubation, the size (diameter) of the inhibition zones was measured.

Statistical analysis

The values were calculated in triplicate and were given as Mean.

Results

The present work deals with the evaluation of three different extracts (petroleum ether, methanol and acetone) of plant *C. asiatica* for preliminary phytochemical and antimicrobial activity.

The present study carried out in *C. asiatica* revealed the presence of medicinally active constituents. Alkaloids and flavonoids are present in all the three solvent extracts such as petroleum ether, acetone and methanol. Saponins, Phenols, Steroids, glycosides, tannins, terpenoids and triterpenoids are absent in petroleum ether solvent extracts and present in acetone and methanol solvent extracts. Cardiac glycosides and resins are absent in all the three solvent extracts. Comparing overall, among all the three extracts, the methanol extracts showed the higher rate of presence of alkaloids. Very poor response was showed only in the petroleum ether extract.

Antibacterial activity

Petroleum ether, acetone and methanol crude extracts of *C. asiatica* leaves were evaluated for antimicrobial activity against the clinical bacterial strains. The acetone and methanol leaf extracts were highly active compared to petroleum ether extract. At the same time the positive control (Ampicillin) was also maintained.

The gram negative bacterium *Proteus mirabilis* showed highest zone of inhibition to crude leaf extracts of *C. asiatica* (13.67 mm in methanol extract and 13.33 mm in acetone extract) as compared to other bacterial strains. The gram positive bacterium *Bacillus subtilis* showed least zone of inhibition to the crude leaf extract (8.33 mm)

Acetone and methanol extract of leaves of *C. asiatica* showed maximum activity against all the bacterial strains whereas petroleum ether showed no activity against all tested bacterial strains. Positive control Ampicillin showed similar activity to methanol.

Antifungal activity

Antifungal activity of crude leaf extracts of petroleum ether, acetone and methanol of *C. asiatica* was assessed using fungal strains. *Fusarium oxysporum* showed maximum inhibition zone (13 mm) when compared to other two fungal strains. *Alternaria alternata* showed minimum inhibition zone (11.67 mm) to the crude leaf extracts of *C. asiatica*.

Acetone and methanol crude extract of *C. asiatica* leaves showed highest activity against all the fungal strains. Petroleum ether showed no activity. The positive control Kanamycin highly inhibited the growth of *Alternaria alternata* (19 mm) and *Curvularia lunata* (19 mm).

Discussion

The preliminary phytochemical screening of the plant revealed the presence of alkaloids, flavanoids, saponins, phenols, steroids, glycosides, tannins, triterpenoids and terpenoids. Bioactive secondary metabolites have been utilized as natural medicines and plants containing those compounds have been used as medicinal plants and are prescribed in many recepies as forms of crude drugs [14].

Reports indicate that naturally occurring alkaloids and their synthetic derivatives have analgesic, antispasmodic and bactericidal activities [15]. Some alkaloids are known to be useful in correcting renal disorders [16]. Many alkaloids have pharmacological effects. It has been used to treat diseases like malaria, pain killers and managing heart diseases [17]. Flavanoids are known for substantial antimicrobial activity [18]. Saponins are known for their medicinal properties as a natural blood cleanser, expectorant and antibiotics [19]. Phenolics are ubiquitous secondary metabolites in plants and posses a wide range of therapeutic uses such as antioxidant, antimutagenic, anticarcinogenic, free radical scavenging activities and also decrease cardiovascular complications [20].

It should be noted that steroidal compounds are of importance and interest in pharmacy due to their relationship with such compounds as sex hormones [21]. Steroids have been reported to stimulate menstrual discharge and diminish secretion of milk [22]. The steroids and saponins are responsible for central nervous system activities [23]. Tannins are known for their astringent property, antimicrobial activity [24], anti-inflammatory [25] and anti-diarrhoeal properties [26]. The presence of tannins also aids in wound healing [27]. Many naturally occurring triterpenoids exhibited a good anti-inflammatory activity have been isolated from various plants [28, 29]. There is growing interest in natural triterpenoids caused as much by the scientific aspects extraction and structural analysis of these compounds, as by the fact of their wide spectrum of biological activities, they are bactericidal, fungicidal, antiviral, cytotoxic, analgesic, anti-inflammatory, anti-cancer and antiallergic [30].

C. asiatica is very rich in terpenoids, a compound which plays a very active role in wound healing [31]. From clinical studies, it is shown that terpenoids strengthen the skin, increase the concentration of antioxidants in wounds and restore inflamed tissue by increasing blood supply. Because of these properties, *C. asiatica* has been used widely in herbal medicine for burns,

psoriasis, prevention of scar formation following surgery, recovery from an episiotomy following vaginal delivery of a newborn and treatment of external fistulas [32].

The methanol extract of all plants material studied against *Staphylococcus aureus* showed average zone of inhibition ranging from (6 to 9.8 mm) whereas *C. asiatica* showed no zone of inhibition [33]. In contrast, the present study observed the methanol extract of *C. asiatica* showed 11mm zone of inhibition against *Staphylococcus aureus*. In the present study the petroleum ether extract of *C. asiatica* showed no inhibition zone against the bacterial strains *Staphylococcus aureus*, *Escherichia coli* and *Proteus vulgaris*. The same result was obtained by Jagtap [34].

C. asiatica whole plant methanol crude extract showed no zone of inhibition against the bacterial strains *Streptococcus sp*. and *Escherichia coli* [35]. But in the present study the methanol crude extract of *C. asiatica* showed the zone of inhibition to *Streptococcus species* (*Streptococcus faecalis* and *Streptococcus pyogenes* 12.67 and 10mm) and *Escherichia coli* (11.33mm). Panthi and Chaudhary [36] found that the methanolic extract of *C. asiatica* showed encouraging results against various Gram positive and negative bacteria. Similar results were also obtained in the present study.

Conclusion

The present study clearly indicates the methanol crude extracts of *Centella asiatica* showed highest zone of inhibition then compared to other extracts petroleum ether and acetone.

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