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In vitro antifungal activity of bioactive compound of *Centella asiatica* (L.) urban

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Abstract
The present study deals with *in vitro* antifungal activity of bioactive compounds present in leaves of *Centella asiatica*. Extracted with hexane, chloroform, ethyl acetate, and ethanol were tested against human pathogens, such as *Apergillus flavus*, *Apergillus fumigates*, *Aspergillus niger*, *Candida albicans Candida glabrata*, *Candida tropicalis*, *Microsporium canis*, *Microsporium gypseum* and *Trichophyton rubrum* by disc diffusion method. The phytochemical tests were revealed the presence of alkaloids, total phenols, glycosides, terpenoids, steroids, flavonoids, tannins, saponins and reducing sugars. The ethanolic extract showed maximum yield of bioactive compounds than the other solvent extracts. In the present investigation all the extracts were found to be effective against nine human fungal pathogenic species. The maximum inhibition was observed on *Candida trophicalis* (25.3 mm) with ethanol extract of leaves followed by *Aspergillus flavus* (24.4 mm), *Candida glabrata* (24.0 mm), *Apergillus fumigates* (23.5 mm), *Aspergillus niger* (22.2 mm), *Candida albicans* (21.5 mm), *Microsprium canis* (20.7 mm) and *Microsporium gypseum* (19.8 mm). The minimum inhibition was observed on *Trichophyton rubrum* (12.6 mm). The hexane extract of leaves also showed the promising antifungal activity against to all tested fungal cultures. The results were validating the traditional uses of this plant in treatment of various diseases. This kind of study could generate more such ideas for re-inventing and using herbs in combination to treat many more diseases.

Keywords: *Centella asiatica*, bioactive compounds, pathogenic fungi, antifungal activity

1. Introduction
Many of the plant materials used in traditional medicine are readily available in rural areas at relatively cheaper than modern medicine. Medicinal plants have also been reported in traditional systems of medicine for the treatment of both human and animal mycoses, and are considered to be a valuable source for the discovery of new antifungal drugs [1]. There are 2600 plant species of which more than 700 are noted for their uses as medicinal herbs [2]. Higher plants produce hundreds to thousands of chemical compounds with different biological activities [3]. Thus, they have been used in the treatment of various human diseases for thousands of years all over the world. Most of the plants used for medicinal purpose have been identified, and their uses are well documented and described by different authors [4] but the efficiency of many of these plants is yet to be verified. There are several reports on the antimicrobial activity of different herbal extract in different region of the world [5]. Medicinal plants represent a rich source of antimicrobial agents [6].

In folk medicine, medicinal herbs and plant products were used in treating a wide spectrum of infections and other diseases. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the matched less availability of chemical diversity [7]. Plants generally produce many secondary metabolites which constitute an important source of micro biocides, pesticides and many pharmaceutical drugs. Plant products still remain the principal source of pharmaceutical agents used in traditional medicine [8]. A survey of literature reveals that there are many essential oils which possesses antifungal activity [9-15]. Hence, there is a great demand for novel antifungal belonging to a wide range of structural classes, selectively acting on new targets with fewer side effects. One approach might be the testing of plants traditionally used for their antifungal activities as potential sources for drug development. Plants are traditionally used in the treatment of bacterial and fungal infections for its wide range of bioactive molecules. Phytochemicals are applied as natural anti pathogenic, which can be derived from leaves, stems, barks, flowers and other parts [16]. According to WHO reports, over 80% of the world population depends on traditional medicine for their primary healthcare needs [17]. There is an alarming increase in the incidence of new and re-emerging infectious diseases [18]. Hence, there is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms, especially due to development of resistance to the antibiotics in current clinical use [19]. There are many synthetic and natural product-based drugs available for treating fungal infections, but they are not consistently effective [20].

Furthermore, the development of resistance in fungi against most of the drugs has now been reported for several years [21]. In addition, the low efficacy, and side-effects and resistance associated with the existing drugs, highlight the advent of safe, novel, and effective antifungal drugs. Based on the knowledge that plants develop their own defense against fungal pathogens [22], they appear as an interesting source for antifungal compounds. There are alarming reports of opportunistic fungal infections [23]. Aspergillosis is caused due to inhalation of *Aspergillus fumigatus* spores. *Aspergillus*

fumigatus is an opportunistic pathogen which usually affects cavities that have formed in the lungs from preexisting lung diseases. *Candida* is a genus of yeasts and is the most common cause of fungal infections worldwide. Many species are harmless commensals or endosymbionts of hosts including humans. *Candida albicans* is the most commonly isolated species, and can cause infections (candidiasis or thrush) in humans and other animals. Fungal infections or mycoses are a common public health problem ranging from superficial to deep infections. Superficial mycoses sometimes reach high endemic levels, especially in tropical areas [24] and dermatophyte fungi are usually the principal cause [25]. Microorganisms have developed resistance to many antibiotics and as a result, immense clinical problem in the treatment of infectious diseases has been created [26]. The resistance of the organisms increased due to indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious disease [27]. Several plants used traditionally have potential antimicrobial and antiviral properties and this has raised the optimism of scientists about the future of phyto-antimicrobial agents [28]. With this background, the present study was carried out to evaluate the antifungal activity of hexane, chloroform, ethyl acetate and ethanol extracts from leaves of *Centella asiatica*.

Centella asiatica (L.) Urban belongs to the family Apiaceae (Umbelliferare) is a significant medicinal herb employed based on the familiarity, which is very popular in most tropical and subtropical countries [29]. It is commonly known as Asiatic pennywort containing 20 species grows fastly in most parts wet-rocky and higher elevations [30]. The whole plant parts are used as medicinal values. The leaves are extensively utilized as a blood purifier, memory enhancement and for treating elevated blood pressure and prevent ageing [31]. The herb contains many types of active compounds, Terpenes or Terpenoids [32]. This plant is used as brain tonic, and to treat chronic diseases and mental disorders. The plant contains several valuable bioactive compounds viz., centellasaponin, asiaticoside, madecassoside and sceffoleoside, pectin, castilliferol 1 and castillicetin 2 [33]. In Asiatic countries, *C. asiatica* is used as an ingredient in traditional systems of medicine such as Ayurveda, Siddha and Unani. Biological effects of *C. asiatica* have been attributed to the existence of major triterpene derivatives including asiatic acid, madecassic acid, asiaticoside, madecassoside, and brahmic acid [34]. The fatty oil isolated from the plant consists of glycerides of oleic, linolic, centoic, linolenic, lignoceric, palmitic, and steric acids; the leaves contain triterpene madasiatic acid as well as 3-glycosyl quercetin, 3-glycosyl kaempferol and 7-glycosyl kaempferol [35]. Asiaticoside is one of the prime triterpene saponin found in leaves in large amount is utilized commercially as a wound healing agent due to its potent anti-inflammatory effect and showed the potential use as anti-gastric ulcers drugs [36]. Many plants were found to contain compounds, which are used as natural medicines to treat common bacterial infections. Medicinal plants are regularly used in various system of medicine because of minimal side effect and cost effectiveness. *Centella asiatica* is one of the important plant shows antibacterial activity against wide variety of bacteria [37]. Therefore, the present study has been carried out to evaluate the phytochemicals present in the leaves and it's *in vitro* antifungal activity present in leaves of *C. asiatica*.

2. Materials and Methods

2.1 Collection of Plant Materials

The leaf samples of *Centella asiatica* were collected from Dharmapuri district in Tamilnadu, India. These plants were then identified, confirmed and have been deposited in the herbarium of PG and Research Department of Botany, Government Arts College, Dharmapuri for the future reference. Fresh leaves were washed thoroughly under running tap water followed by sterile distilled water and dried under shade. The dried plant parts were crushed to fine powder and stored in airtight bottles which were later used for solvent extraction.

2.2 Preparation of plant extract

The presence of different chemical constituents in crude drugs can be detected by subjecting them to successive extraction using solvents in the order of increasing polarity. Fresh leaves were washed thoroughly under running tap water followed by sterile distilled water and dried under shade. They were ground into coarse powder by using mechanical pulveriser. The leaf powder, about 100 g were weighed and extracted repeatedly with hexane, chloroform, ethyl acetate and ethanol in a 500 mL round bottom flask containing 250 mL solvent individually. The reflux time for each solvent was varying with 25 to 40 cycles for complete extraction in soxhlet apparatus [38, 39]. The filtrate was collected and concentrated by using rotary evaporator under controlled condition of temperature and pressure. The extracts were concentrated to dryness to yield crude residue. The dry leaf powder of *C. asiatica* also was extracted by cold percolation method [40, 41] and using different organic solvents like Hexane, Chloroform, Ethyl acetate, and Ethanol. Twenty five gram of dried powder was taken in 250 of Hexane in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 120 rpm for 24 h. After 24 h, the extract was filtered with eight layers of muslin cloth; centrifuged at 5000 rpm for 10 min. The supernatant was collected and the solvent was evaporated. The extract was weighed and the extractive yield was calculated. These residues were stored at -20°C, used for preliminary phytochemical screening of secondary metabolites and *in vitro* antifungal activity screening study.

2.3 Phytochemical Screening study

Preliminary phytochemical screening of the *C. asiatica* leaf extract was carried out for the detection of the various plant constituents. Shaded dried and powdered of aerial part of plant samples were successively extracted with hexane, chloroform, ethyl acetate and ethanol. The extracts were filtered and concentrated using vacuum distillation. The different extracts were subjected to qualitative tests for the identification of various phytochemical constituents as per standard procedure [42].

2.4 Tested Microorganisms

The clinical pathogenic fungal strains were aseptically collected form Dharmapuri Government Medical College and Government General Hospital, Dharmapuri. The fungal

cultures such as *Apergillus flavus*, *Apergillus fumigates*, *Aspergillus niger*, *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Microsporium canis*, *Microsporium gypseum* and *Trichophyton rubrum* were maintained in potato dextrose broth (PDB) at the laboratory of PG and Research department of Botany, Government Arts College, Dharmapuri, Tamil nadu, India. The stock cultures of fungi were maintained on potato dextrose agar slants at 4ºC. Antifungal activity of crude extracts was tested against all these fungi.

2.5 *In vitro* Antifungal Assay by Disc Diffusion Technique

The screening of the extracts for antifungal effect was carried out by determining the zone of inhibition using disc diffusion method. Sterile potato dextrose agar plates were prepared. Then 0.1 ml of spore's suspension of test organism was taken from the stock (broth) and swabbed on the agar medium in aseptic condition. The filter paper disc of 2 mm diameter (Whatman's No.1 Filter paper) were prepared and sterilized. The plant extracts to be tested were prepared with various concentrations *viz*., 25%, 50%, 75% and 100% and were added to each disc of holding capacity of 10 µl [43]. The sterile impregnated disc with plant extracts were placed on the agar surface with framed forceps and gently pressed down to ensure complete contact of the disc with the agar surface. Positive control disc of Fluconazole (10 µl) were prepared and placed on the agar surface. The cultured plates were incubated at 37°C for 3 to 5 days. After incubation, the antifungal activity area was measured the zone of inhibition by two directions at right angles to each other against test organisms. Experiments were carried out with three replicates per treatment and each treatment was repeated at least three times [44].

2.6 Statistical Analysis

The resultant of clear zones around the discs were measured in mm. Data of all experiments were statistically analysed and expressed as Mean ± Standard Deviation.

3. Results

The present study revealed the presence of bioactive compounds in leaf extracts of *Centella asiatica* was evaluated by hexane, chloroform, ethyl acetate and ethanol extracts. This investigation carried out through cold percolation as well as Soxhlet extraction methods have showed the presence of bioactive compounds in varying concentrations. The ethanolic extract showed maximum yield of bioactive compounds when compare to other solvent extracts. (Data were not shown). The results were clearly demonstrated that the ethanol and ethyl aceate extracts yielded maximum nine bioactive compounds such as carbohydrates, glycosides, alkaloids, terpenoids, phenols, anthraquinones, amino acid, reducing sugars and proteins, these compounds were yielded minimum in hexane and chloroform extracts. Similarly, other six compounds were yielded high in hexane and chloroform extracts such as tannins, flavonoides, steroids, saponins and terpenoids respectively.

All the extracts were showed antifungal activity against all the selected fungal isolates. The maximum inhibitory activity was observed in ethanol extracts. The plant extracts were prepared with various concentration *viz*., 25%, 50%, 75% and 100% were tested for their antifungal effect against *Aspergillus flavus*, *Aspergillus fumigates*, *Aspergillus niger*, *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Microsporium canis*, *Microsporium gypseum* and *Trichophyton rubrum*. The results were presented in (Table 1 & 2). The extracts were tested against nine different human pathogenic fungal organisms at different concentrations (25%, 50%, 75% and 100%). From the extracts 100% of the ethanol extracts showed the maximum activity against all the fungal isolates followed by 75%, 50% and 25% respectively. The ethanol extract exhibited pronounced inhibition against all tested fungal organisms followed by ethyl acetate, chloroform and hexane. The maximum *in vitro* growth inhibition was observed on *Candida tropicalis* (25.3 mm) leaf ethanol extract 100% (10 µl) (Table-3). The positive control of fluconazole recorded the inhibition of 17.9 mm, 16.3 mm, 15.6 mm, 15.2 mm, 15.1 mm, 14.5 mm, 14.2 mm, 12.3 mm and 10.2 mm in *Aspergillus niger*, *Aspergillus flavus*, *Microsporium gypseum*, *Candida glabrata*, *Aspergillus fumigates*, *Candida tropicalis*, *Microsporium canis*, *Trichophyton rubrum* and *Candida albicans*, respectively.

The ethanol extract of leaf exhibited maximum antifungal activity against 100% (10µl) in *Candida trophicalis* (25.3 mm) followed by *Aspergillus flavus* (24.4 mm), *Candida glabrata* (24.0 mm), *Apergillus fumigates* (23.5 mm), *Aspergillus niger* (22.2 mm), *Candida albicans* (21.5 mm), *Microsporium canis* (20.7 mm) and *Microsporium gypseum* (19.8 mm). The minimum inhibition was observed on *Trichophyton rubrum* (12.6 mm). The hexane extract of leaves also showed the promising antifungal activity against to all tested fungal cultures (Table-2). From the extracts hexane extract showed the least inhibition against 100% (10µl) in *Candida trophicalis* (16.4 mm) followed by *Microsporium canis* (14.4 mm), *Aspergillus flavus* (14.2 mm), *Aspergillus fumigates* (13.3 mm), *Candida albicans* (13.2 mm), *Aspergillus niger* (12.7 mm), *Microsporium gypseum* (11.3 mm), *Candida glabrata* (10.5 mm) and *Trichophyton rubrum* (10.0 mm), respectively (Table-1). The chloroform and ethyl acetate extracts 100% (10µl) were showed the moderate antifungal activity to the other extracts. The inhibition values were *Candida trophicalis* (18.6 mm, 20.2 mm) followed by *Microsporium canis* (16.2 mm, 19.6 mm), *Candida albicans* (15.00 mm, 18.4 mm), *Aspergillus flavus* (14.8 mm, 18.0 mm), *Microsporium gypseum* (13.5 mm, 17.3 mm), *Aspergillus niger* (13.1 mm, 16.6 mm), *Aspergillus fumigates* (12.1 mm, 16.3 mm), *Candida glabrata* (11.2 mm, 13.6 mm) and *Trichophyton rubrum* (10.4 mm, 11.4 mm), respectively (Table-1 & 2).

Table 1: *In vitro* Antifungal activities of hexane and chloroform extracts of *Centella asiatica* leaves.

Sample tested	Diameter zone of inhibition in mm									
	Hexane extract					Chloroform extract				
	C	25%	50%	75%	100%	C	25%	50%	75%	100%
Aspergillus flavus	16.3	-	10.4	11.7	14.2	16.3	-	10.7	12.2	14.8
Aspergillus fumigates	15.1	-	-	10.2	13.3	15.1	-	-	10.4	12.1
Aspergillus niger	17.9	-	-	10.1	12.7	17.9	-	-	11.0	13.1
Candida albicans	10.2	-	-	10.6	13.2	10.2	10.7	12.4	14.2	15.0
Candida glabrata	15.2	-	-	-	10.5	15.2	-	-	10.3	11.2
Candida tropicalis	14.5	-	11.8	14.2	16.4	14.5	12.2	14.1	16.0	18.6
Microsporium canis	14.2	-	10.2	12.7	14.4	14.2	-	11.2	13.8	16.2
Microsporium gypseum	15.6	-	-	10.1	11.3	15.6	-	10.1	11.5	13.5
Trichophyton rubrum	12.3	-	-	-	10.0	12.3	-	-	-	10.4

Table 2. *In vitro* Antifungal activities of ethyl acetate and ethanol extracts of *Centella asiatica* leaves.

Sample tested	Diameter zone of inhibition in mm									
	Ethyl acetate extract					Ethanol extract				
	C	25%	50%	75%	100%	C	25%	50%	75%	100%
Aspergillus flavus	16.3	10.1	13.7	15.4	18.0	16.3	12.6	15.3	19.4	24.4
Aspergillus fumigates	15.1	-	10.6	15.2	16.3	15.1	11.4	14.2	18.8	23.5
Aspergillus niger	17.9	-	10.2	14.3	16.6	17.9	10.7	13.5	18.0	22.2
Candida albicans	10.2	-	10.7	13.4	18.4	10.2	11.4	14.6	17.5	21.5
Candida glabrata	15.2	-	-	10.2	13.6	15.2	12.2	15.4	18.7	24.0
Candida tropicalis	14.5	10.2	13.2	17.4	20.2	14.5	13.2	16.4	19.2	25.3
Microsporium canis	14.2	-	12.0	15.3	19.6	14.2	10.7	13.4	16.5	20.7
Microsporium gypseum	15.6	-	10.1	13.6	17.3	15.6	10.5	12.7	15.8	19.8
Trichophyton rubrum	12.3	-	-	10.1	11.4	12.3	-	-	10.5	12.6

4. Discussions

In the present study, successive soxhlet extraction was carried with hexane, chloroform, ethyl acetate and Ethanol have revealed the presence of bioactive compounds in phytochemical screening and have *in vitro* antifungal activity in different solvent extracts. The studies of antifungal activity in leaves were extracted with different solvents. From the ethanol extracts showed the maximum inhibitory activity. Similar observations were recorded in methanol extracts of *Gloriosa superba* showed high antifungal activity against *Aspergillus flavus* and *Candida glabrata* [45]. Ethanolic and ethyl acetate extracts of *Centella asiatica* plant shows significantly higher rate of antifungal activity against various fungal strains like *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans* when compare to hexane extracts [46]. Hexane, carbon tetrachloride, chloroform and aqueous soluble fractions of methanolic extract showed antimicrobial activity against various yeast and mold strains like *Aspergillus niger*, *Saccharomyces cerevisiae* and *Candida albicans*. Methanolic extract of *Centella asiatica* showed significant inhibitory effect on spore germination against various fungal strains like *Alternaria*, *Cercospora*, *Curvularia*, *Drechslera* and *Fusarium*. Bobbarala *et al* [47]. examined the antifungal activity of forty nine plants including *Centella* against *Aspergillus niger* using agar well diffusion method. Among the 49 plants studied the methanolic extracts of 43 plants including *Centella* exhibited varying degrees of inhibition activity against the above fungi. Methanol, chloroform and acetone extracts of *Centella asiatica* showed significant inhibitory effect on growth and sporulation of *Colletotrichum gloeosporioides* [48]. Similar to the work of Samy and Ignacimuthu [49]. Dash *et al*. [50] studied antibacterial and antifungal activities of several extracts of *C. asiatica* against some human pathogenic microbes. The results showed that ethanol extracts gave highest antibacterial activity against *Candida tropicalis* followed by ethyl aceate, chloroform, and hexane, respectively.

5. Conclusions

In the present study clearly demonstrated that of using natural products. *Centella asiatica* has been mentioned in Ayuravedic record. It can be used to treat leprosy, insanity, asthma, ulcers, eczema, skin tuberculosis, wounds, stomach aches, arthritis, varicose veins and high blood pressure. The extracts even at low concentrations they showed antifungal activity nearly equal to that of the commercial fungicide used as a positive control. Further studies are needed to determine the chemical identity of the bioactive compounds responsible for the observed antifungal activity. Extensive screening programs of plants used mainly in traditional medicine have resulted in the discovery of thousands of phytochemicals with inhibitory effects on different types of microorganisms in vitro. Studies from all over the world have indicated that several plant extracts and their phytocompounds have been identified in an effort to supplement the relatively sparse portfolio of antifungal drugs. There is a need to exploit these bioactive compounds in disease caused by pathogenic fungi.

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