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Original Article

Pharmacology Section

Antifungal Activity of *Calliandra haematocephala* against Selected Pathogenic Fungi: An In-vitro Study

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ABSTRACT

Introduction: Pathogenic fungi cause a wide range of infections which include life-threatening diseases particularly in immunocompromised individuals. Plant derived secondary metabolites have significant therapeutic role in pathogenic fungal infection because of their fungicidal, immunity enhancing and least toxic properties. *Calliandra haematocephala* also called 'Red Powder puff' possess greater antimicrobial and immunoadjuvant activities.

Aim: To evaluate the antifungal activity of *Calliandra haematocephala* against selective pathogenic fungal species.

Materials and Methods: The present in-vitro study was conducted in central laboratory in association with Department of Pharmacology at Sree Balaji Medical College, Chennai, Tamil Nadu, India, during six months from June 2018 to December 2018. Antifungal activity of the *Calliandra haematocephala* (leaves extract) were examined against six pathogenic fungal species *Candida albicans, Aspergillus niger, Trichoderma viride, Rhizopus microsporus, Penicillium chrysogenum* and *Trichophyton rubrum* using agar disc diffusion method by measuring the zone of inhibition.

Results: The ethanolic leaf extract of *Calliandra haematocephala* showed strong inhibitory activity against all six fungal species. However, it showed significant inhibitory activity against *Aspergillus niger* (12 mm) and *Penicillium chrysogenum* (11 mm) when compared with standard drug Amphotericin B.

Conclusion: The antifungal activity of *Calliandra haematocephala* suggested its potential role in opportunistic fungal infections with immunocompromised conditions.

Keywords: Agar disc diffusion method, Fungal infection, Opportunistic fungal infections, Zone of inhibition

INTRODUCTION

Infectious diseases caused by pathogenic fungi possess significant threat to the public health care system. The rise in incidence of serious fungal infections and annual death rate of 1.5 million globally necessitates the further development in antifungal therapy [1]. Contraction of opportunistic fungal infections in most of the patients with immunosuppressive drugs, chemotherapy and Human Immunodeficiency Virus (HIV) infection plays a major role for increasing the morbidity and mortality. Particularly in immunocompromised patients, these infections invade their immune system in multiple ways. The antifungal immune based treatment consists of host-targeting agents (immunomodulators) and pathogen targeting agents (immunotherapeutics) which manipulate the host immune system help in fighting with the invasive fungal pathogen [2]. These protective immunological components build the innate and acquired antifungal immunity and augment the efficacy of existing antifungal drugs. However, the hyper activation of proinflammatory cytokines makes the eradication of severe fungal infection tougher. So, the potent fungicide with immunomodulatory properties could be an effective measure in the management of serious life-threatening mycosis.

Plant kingdom has been an abundant source for mankind in providing the compounds with therapeutic properties. These natural bioactive compounds with novel structure could provide the lead antifungal compounds with least toxicities. For the past five decades, only few antifungal drug classes like Polyene, Azoles, Allylamines and Echinocandins have been developed which particularly focus on fungal cell wall ergosterol synthesis [3]. But the cost, resistance and toxicities of these synthetic antifungal drugs insist the search of potential plant based drugs [4]. Medicinal plants have been used as potent antifungal drugs traditionally and their secondary metabolites considered as effective human and animal fungicide [5]. Plants own defense mechanisms against fungal organisms proved that it could be an ideal alternative in treating fungal infections [6]. These naturally occurring bioactive compounds are environment safe, biodegradable, and responsible for crop protection by inhibiting the pathogenic microorganisms.

Calliandra haematocephala (Hassk) is an evergreen shrub with branched petiole or axis, silky leaves and powder-puff-like balls of conspicuous dark crimson stamens. It belongs to the family Fabaseae and native to tropical and subtropical regions of America. The plant genus found to have the anti-inflammatory, immunomodulatory, antiulcerogenic, anticonvulsant, anthelmintic and anti-rotaviral properties [7]. The plant has been used traditionally as antioxidant and blood purifier [8]. Gas Chromatography/Mass Spectroscopy (GC/MS) analysis revealed the presence of sterols, triterpene (Lupeol) and fatty acid methyl esters. The high content phenolic compounds and flavonoids attribute for its hepatic protective activity. The acetylated quercetin rhamnosides, flavonoids and condensed tannins isolated from the leaves of *Calliandra haematocepahala* are found to have moderate-severe radical scavenging properties and exhibited antioxidant activities. [9,10]. The secondary metabolites tannins and flavonoids showed strong antifungal activity as there is strong relationship with their antioxidant properties [11].

In the recent years, the increasing frequency of pathogenic fungal infections and fatal disseminated diseases necessitates the discovery of potent plant fungicide with immunomodulatory properties. So, this study aims to postulate the antifungal activity of *Calliandra haematocephala* against the selected fungal species using agar disc diffusion method.

MATERIALS AND METHODS

The present in-vitro study was conducted in central laboratory in association with Department of Pharmacology at Sree Balaji Medical College, Chennai, Tamil Nadu, India, during the period of six months from June 2018 to December 2018. Antifungal activity of the *Calliandra haematocephala* (leaves extract) were examined against

six pathogenic fungal species *Candida albicans, Aspergillus niger, Trichoderma viride, Rhizopus microsporus, Penicillium chrysogenum* and *Trichophyton rubrum*.

Study Procedure

Plant identification: The fresh leaves of *calliandra haematocephala* were collected from different parts of the Madipakkam area in Chennai, India. The leaves washed several times with distilled water and used for extraction. The plant materials were identified and authenticated by the Director, Plant Anatomy Research Centre, West Tambaram-Chennai, Tamil Nadu, India.

Extract preparation: The leaves of *calliandra haematocephala*, cut into pieces, air dried and powdered. A 25 grams of powdered sample were taken and extracted with 300 mL of ethanol in Soxhlet apparatus for 12 hours of time. The crude extract was filtered, and the solvents were further condensed using rotary evaporator. The crude extract was stored at room temperature in airtight container for further analysis. A portion of the extract was used for antifungal evaluation.

Phytochemical analysis: The Phytochemical analysis showed the presence of alkaloids, tannins, flavonoids, saponins and glycosides [12].

Test Organisms: The test organisms used: *Candida albicans* (MTCC 4748), *Aspergillus niger* (MTCC 8652), *Trichoderma viride* (MTCC 1763), *Rhizopus microsporus* (MTCC 3934), *Penicillium chrysogenum* (MTCC 6494), *Trichophyton rubrum* (MTCC 3272) were purchased from Microbial Type Culture Collection and Gene Bank (MTCC) at Chandigarh, India.

Standard: Amphotericin B (20 µL/disc)

Inoculum preparation: Stock cultures were maintained at 4°C on sabouraud dextrose agar Slant. Active cultures for experiments were prepared by transferring the stock cultures into the test tubes containing sabouraud dextrose broth that were incubated at 48 hours at room temperature. The culture media and Amphotericin B discs were purchased from Hi media labs, Mumbai, Maharashtra, India. Dimethylsulfoxide (DMSO) was purchased from sigma labs, Chennai, Tamil Nadu, India. The assay was performed by agar disc diffusion method [13,14].

Antifungal susceptibility testing: Antifungal activity of the extract was determined by disc diffusion method on Sabouraud Dextrose Agar (SDA) medium. The medium was poured into the petri plate. After the medium was solidified, the inoculums were spread on the solid plates with sterile swab moistened with the fungal suspension. Amphotericin B (20 µL/disc) was taken as positive control and DMSO as negative control. Samples and positive control of 20 µL each were added in sterile discs (Concentration: 1000 µg, 750 µg and 500 µg) and placed in SDA plates. The plates were incubated at 37ºC for 24 hours. Then antifungal activity was determined by measuring the diameter of zone of inhibition. The diameter of less than 5 mm was considered as insignificant. The diameter of zones of inhibition produced by various concentrations of plant extracts and Amphotericin B were compared.

STATISTICAL ANALYSIS

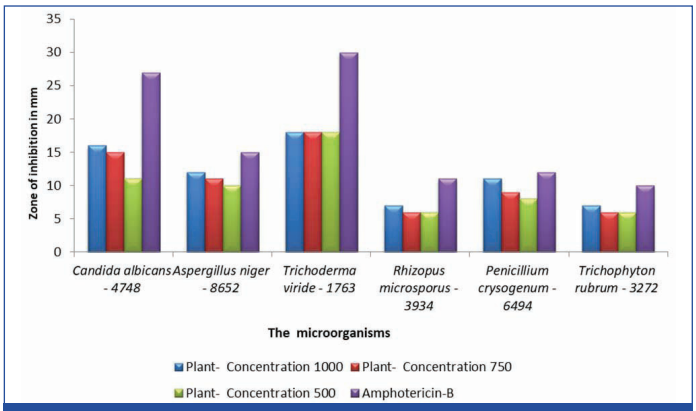
All the tests have been repeated three times. Statistical Package for the Social Sciences (SPSS) version 26.0 was used for statistical analysis. Unpaired t-test was performed and the p-value <0.001 considered significant. The values were represented as mean±SD.

RESULTS

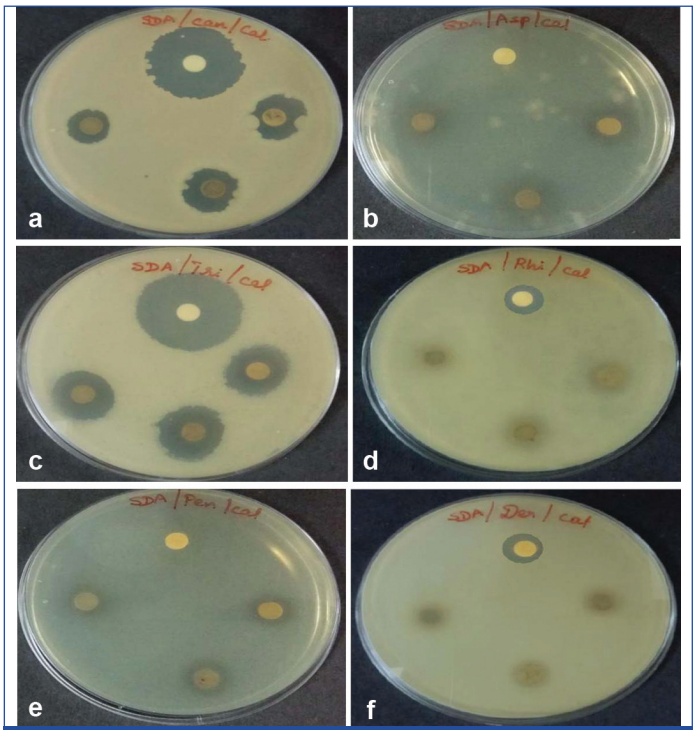
The antifungal activity of *calliandra haematocephala* leaves were assessed by comparing the zone of inhibition against six fungal species in the three different dilutions (1000, 750, 500 µg/mL) of plant extracts with Amphotericin B (20 µL/disc) [Table/Fig-1]. The maximum zone of inhibition measured as *Trichoderma viride* (18mm), *Candida albicans* (16 mm), *Aspergillus niger* (12 mm), *Penicillium chrysogenum* (11 mm), *Rizopus microporus* (7 mm), and *Trichophytum rubrum* (7 mm) for 1000 µg /mL dilutions of *Calliandra haematocephala* leaf extract [Table/Fig-2,3a-f]. In that the zone of

Organisms	Zone of inhibition (mm)			
	Plant concentration 1000 µg/mL Mean±SD	Plant concentration 750 µg/mL Mean±SD	Plant concentration 500 µg/mL Mean±SD	Amphotericin B 20 µL/disc Mean±SD
Candida albicans- 4748	16.2±0.28	15.25±0.57	11.4±0.50	27.3±1.2
Aspergillus niger- 8652	12.3±0.24*	11.8±0.31	10.3±0.42	15.7±0.13
Trichoderma viride- 1763	18.2±0.31	18.5±0.54	18.6±0.65	30.3±1.23
Rhizopus microsporus- 3934	7.5±0.32	6.54±0.12	6.8±0.36	11.1±0.95
Penicillium chrysogenum- 6494	11.6±0.25*	9.65±0.52	8.4±0.36	12.8±1.21
Trichophyton rubrum- 3272	7.6±0.56	6.7±0.45	6.1±0.52	10.4±0.85

[Table/Fig-1]: Antifungal activity of ethanolic extract of *Calliandra haematocephala* determined by zone of inhibition using agar diffusion method in various concentrations compared with Amphotericin B as control.
*p-value <0.001, SD: Standard deviation. Data reported are the mean diameters expressed in mm. Values are presented as mean±SD



[Table/Fig-2]: Antifungal activity of ethanolic extract of *Calliandra haematocephala* in various concentration compared with Amphotericin B as control against fungal species.



[Table/Fig-3]: Antifungal activity of *Calliandra haematpocephala* extract (1000 µg/mL) in comparison with the control Amphotericin B (20 µL/disc) measuring zone of inhibition using agar disc diffusion method. a) *Candida albicans* (16 mm); b) *Aspergillus niger* (12 mm); c) *Trichoderma viride* (18 mm); d) *Rhizopus microsporus* (7 mm); e) *Penicillium crysogenum* (11 mm); f) *Trichophyton rubrum* (7 mm).

inhibition showed of 18 mm for *Trichoderma viridae* in all three dilutions. However, the plant extract showed significant inhibitory activity against *Aspergillus niger* and *Penicillium crysogenum* in 1000 µg/mL dilution (p-value <0.001).

DISCUSSION

The leaves of *Calliandra haematocephala* exhibited marked antifungal activity against all the six fungal species. However, it showed significant activity against *Aspergillus niger* and *Penicillum chrysogenum* in highest dilution (1000 µg/mL) by producing maximum zone of inhibition when compared with standard drug Amphotericin B. It was similar with the previous study which also showed the fungitoxic effect of nonprotein imino acids isolated from leaf extract of *Calliandra haematocephala* against saprophytic fungi [15]. Several studies revealed that medicinal plant extracts showed significant reduction in growth of *Aspergillus niger* and *Penicillum chrysogenum*. They showed significant difference in efficacy in various concentrations [16,17]. When compared with these studies, our study proved the significant fungicidal effect in highest dilution.

Among the six hundred infective fungal species, *Trichoderma viride, Candida albicans, Aspergillus niger, Penicillum chrysogenum, Rizopus microporus,* and *Trichophytum rubrum* are the main pathogens that attribute localised to severe systemic infections. The fatal disseminated infections caused by pathogenic yeast *Candida albicans* (mucocutaneous infections, meningitis, endocarditis, osteomyelitis) and other fungal species *Aspergillus niger* (Bronchopulmonary aspergillosis), *Penicillum chrysogenum* (Necrotizing oesophagitis), *Rizopus microporus* (Nosocomial infections), *Trichophytum rubrum* (Athlete's foot, ringworm) in immunocompromised patients has increased recently. The availability of few antifungal drugs and multidrug resistant strains increased the incidence and prevalence of mild to serious life-threatening infections like Candidiasis, Cryptococcosis, Aspergillosis and Mucormycosis. The complex biological nature of acquired drug resistance mechanisms restricts the treatment option which eventually affects the patient management [18]. The important antifungal drugs include polyene antibiotics, azoles and terbinafine mainly target the ergosterol while the newer potent drug echinocandins inhibits the β-1.3-glycan the unique component of fungal cell wall. However, side effects are more with these selective antifungal drugs [3]. So, it is necessary to discover natural sources which could be a potential least toxic fungicide for such infections.

Plants produce secondary metabolites either in constitutive or inducible form to protect it from human and animal pathogen. The constitutive first-class metabolites of phenols (Flavonoids, tannins and alkaloids) have greater biological activities against microorganisms. It has been shown that bioactive phenolic compounds derived from plants possess both antifungal and antioxidant activities [19,20]. However, tannins and saponins are also considered to have potent fungicidal properties and toxic to dangerous fungal species [21]. Several plants have been tested and exhibited the relationship between its secondary metabolites and antifungal activity.

In immunocompromised patients, the weaken immune system led to the contraction of severe fungal infections. In chronic dermatophytosis infection, reactivation of defective cell mediated immunity is vital to overcome drug resistance [22]. So, potent fungicidal with immunomodulatory drugs will be effective in managing such patients. Studies explored that the immunomodulatory therapies (cytokine agonists, cellular therapies, and monoclonal antibodies) improve the immune system and augment the existing antifungal therapy in invasive fungal disease [23]. But in fungal sepsis the Interleukin-17 (IL-17) family of cytokines acts a regulatory mechanism in promoting pro-inflammatory cytokine expression to amplify inflammatory responses. So, focusing on the hyper inflammation and targeting the Damage Associated Molecular Pattern (DAMP) could be a novel immunomodulatory strategy in systemic fungal infections [24].

Previous data proved the radical scavenging and immunomodulatory properties of *Calliandra haematocephala* and the phytochemical constituents like flavonoids and tannins must be responsible for its antioxidant, anti-inflammatory and antimicrobial activity [25]. The immunoadjuvant activity of aerial parts of *Calliandra haematocephala* was evaluated against ovalbumin antigen with measuring the delayed type of hypersensitivity reaction as invivo assay of cellular immune response [26]. It has been observed that saponins exhibit immunoadjuvant activity by releasing the cytokines and antibodies through humoral and cellular responses [27]. So, immunomodulatory effect of *Calliandra haematocephala* might augment the fungicidal property and potentiate the therapeutic efficacy in invasive mycosis. Considering these facts, it is proved that *Calliandra haematocephala* could be a potent fungicide and enhances the efficacy of existing synthetic antifungal drugs.

Limitation(s)

The major limitation of the study was that authors did not investigate the minimum inhibitory concentration of plant extract which will be the next step present research work. Authors did not conduct in-vivo study to co-relate with in-vitro test findings.

CONCLUSION(S)

The complex immune responses of pathogenic fungi necessitate the understanding of its mechanism to optimise the treatment strategies. The leaves of *Calliandra haematocephala* showed significant antifungal activity against the infective life-threatening pathogens. So, it could exhibit synergistic effect with synthetic antifungal drugs in combating mild to severe life-threatening fungal infections.

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Authors contribution: GJI- Concept, planned and conducted the experiments, analyzed the data and graph, Manuscript writing. PK-Analyzed the data and graph, Manuscript editing.

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