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EVALUATION OF ANTIFUNGAL ACTIVITY OF *CALOTROPIS GIGANTEA* LATEX EXTRACT: AN *IN VITRO* STUDY

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ABSTRACT

Medicinal plants are important source of potentially useful structures for the development of novel chemotherapeutic agents. The present study was aimed to evaluate the antifungal effect of partially purified *C. gigantea* latex extract on some human pathogenic fungi. Ethanolic extract of the latex were tested *in vitro* against fungi strains. The inhibitory effect was assessed by disc diffusion method. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were also determined by serial dilution method. The ethanolic extract was subjected to qualitative phytochemical screening for the presence of bioactive ingredients. The ethanolic extract shows the presence of many biologically active molecules such as flavonoids, alkaloids, triterpenoids, steroids, saponins, phenols and glycosides. The latex extract shows significant zone of inhibition in dose dependent manner. The MIC and MFC values of latex extract against fungal strains varies from 1 mg to 8 mg and the results are comparable with Amphotericin B. From this study, we conclude that the latex extract possesses potent fungicidal activity which may be due to the presence of biologically active ingredients with antimicrobial activity in the ethanolic extract of *C. gigantea* latex.

INTRODUCTION: The prevalence of invasive, opportunistic fungal infections has increased at an alarming rate especially in immune-compromised individuals [1, 2]. This trend has also been attributed to the increasing use of cytotoxic and immunosuppressive drugs to treat both malignant and nonmalignant diseases. Mycologists estimate that there were about one lac validly characterized species of fungi, with at least as many species waiting to be discovered. Of those identified, nearly 150 species are recognized as pathogens of humans and animals. They cause a broad spectrum of infections ranging from systemic and potentially fatal diseases to localized cutaneous, subcutaneous or mucosal infections [3]. Although it appears to be a great array of antifungal drugs, there is at present a quest for new generations of antifungal compounds due to the low efficacy, side effects or resistance associated to the existing drugs. Based on the knowledge that plants develop their own defense against fungal pathogens [4] they appear as an interesting source for antifungal compounds.

Medicinal plants are the 'back bone' of traditional remedy [5]. In addition, the traditional medicine related to treatment of both human and animal mycoses with plant-derived preparations is considered a valuable knowledge for the discovery of new antifungal drugs [6]. Plants contain many biologically active molecules with different medicinal properties [7, 8]. India is very rich in natural resources and the knowledge of traditional medicine and the use of plants as source of new drugs is an innate and very important component of healthcare system. However, very little information is available about many useful herbs as experimental data. *Calotropis gigantea* R. Br (Asclepiadaceae) is a xerophytic, erect shrub, growing widely throughout the tropical and subtropical regions of Asia and Africa. This plant is popularly known because it produces large quantity of latex. Latexes are source of various biologically active compounds, including glycosides, tannins and many proteins, among others [9, 10]. Previous work, using different parts of the plant has advocated its use for a variety of disease conditions [11] in addition to the application as an antidote for snake poisoning. The plant has potential pharmacological properties [12]. Fractionation of the latex into its rubber and rubber-free fractions affords better insight into its potentials and limitations [13]. Based on folklore claims, the present study was aimed to evaluate the antifungal activities of *C. gigantea* latex extract against prominent human pathogenic fungi by disc diffusion method. Besides, the biological activity of the extract in terms of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were also determined. The latex extract has also been qualitatively analyzed for the presence of different phytochemicals. Recently we have evaluated the wound healing and antibacterial activity of *C. gigantea* latex [14, 15].

MATERIALS AND METHODS:

Plant material and latex collection: The fresh latex of *C. gigantea* was aseptically collected from the aerial parts of the healthy plants as described by Aworh *et al.,* [16] in clean glass tubes containing distilled water to yield a dilution rate of 1:1 (v/v). The plant exsiccate was deposited at the herbarium of the Centre for Advance Studies in Botany, University of Madras, where the plant was identified by a plant taxonomist a voucher specimen is preserved (Voucher No. 197/09). The latex mixture was gently handled to maintain homogeneity during transport to the laboratory where it was kept overnight at 4°C.

Preparation of *C. gigantea* latex extract: The supernatant was selectively decanted and

centrifuged at 5000xg for 20 min at 25°C. The precipitated material showing rubber aspect (poly-isoprene) was pooled apart and the supernatant was decanted carefully and subjected to exhaustive dialysis using a membrane of 8000 MW cut off against distilled water at 25°C. Finally the samples were centrifuged as previously described and the clear soluble supernatant was collected and lyophilized. The lyophilized fraction was subjected to ethanolic extraction (70% (v/v)) in Soxhlet extractor at room temperature and the extraction process was performed repeating 4 cycles [17].

The extract was filtered through Whatman No.1 filter paper and filtrate was concentrated with a rotary evaporator under reduced pressure at 60°C to afford crude ethanolic extract. The dry-crude extracts were irradiated with ultraviolet light for 24 h for sterilization; sterility was confirmed by plating the sample suspension on Sabouraud Dextrose Agar and stored in labeled sterile brown glass containers at 4°C until used for the screening of antifungal activity. At that time, the extracts were freshly reconstituted to yield desired concentration.

Phytochemical screening of *C. gigantea* latex extract: The phytochemical screening of the ethanolic extract of *C. gigantea* latex was performed qualitatively for the presence of alkaloids, glycosides, flavonoids, phenols, tannins, saponins, sterols and triterpenes according to the method of Harborne [18].

Fungal strains and growth medium: Fungal cultures of *Candida albicans* (MTCC 227), *Saccharomyces cerevisiae* (MTCC 463), *Trichophyton mentagrophytes* (MTCC 183), *Trichophyton rubrum* (MTCC 296), *Aspergillus fumigatus* (MTCC 343), *Aspergillus niger* (MTCC 277), *Aspergillus flavus* (MTCC 418), *Penicillium chrysogenum* (MTCC 886) were used in the present study. The fungal strains were all standard laboratory strains obtained from the stock cultures of the Division of Microbiology, CAS in Botany, University of Madras, Chennai and maintained on slopes of Sabouraud Dextrose Agar (SDA) at 28°C.

Preparation of inoculums: The fungal strains were subcultured on slants of SDA at 28°C for 7 days and the colonies were suspended in 1 ml of sterile normal saline. The resulting mixture of conidia and hyphal fragments was vortexed and the turbidity of each homogenous suspension was adjusted to match that of a 0.5 McFarland standard, as read at 530 nm. At this turbidity, the fungi density was 3×10^6 - 5×10^6 CFU/ml.

Preparation of the McFarland standard: Add 0.5 ml of 0.048M $BaCl_2$ to 99.5 ml of 0.18M H_2SO_4 with constant stirring. Distribute the standard into screw cap tubes of the same size and with the same volume as those used in growing the broth culture. Seal the tubes tightly to prevent loss by evaporation. Store protected from light at room temperature. Vigorously agitate the turbidity standard on a vortex mixture before use. Standards may be stored for up to 6 months, after which time they should be discarded.

Determination of antifungal activity: Antifungal activity of the ethanolic extract of *C. gigantea* latex was evaluated by disc diffusion method [19]. The inoculate with respective fungi were homogenously seeded onto the 90mm Petri dishes containing 20 ml cooled molten SDA medium using sterile swab in such a way as to ensure thorough coverage of the plates and a uniform lawn of growth following incubation. These inoculated plates were left to dry for at least 15 min. The lyophilized latex extract was dissolved in distilled water to obtain the different

concentrations of 300, 150, 75, 37.5 and 18.75 mg/ml. Amphotericin B at concentration 10 µg/disc was used as positive control and was dissolved in dimethyl sulphoxide (DMSO). Sterile filter paper disc (6mm in diameter) were impregnated with 10 µl of each different concentration of latex extract. The discs were allowed to dry and then placed on the agar surface of each Petri dish. DMSO was used as negative control. Zone inhibitions (in mm) were measured after 48-72 h at 28^{o}C. The complete antifungal analysis was carried out under strict aseptic conditions. Each assay was repeated three times.

Minimum inhibitory concentration (MIC): The minimum inhibitory concentration (MIC) of *C. gigantea* latex extract was determined by using broth microdilution method as described by the National Committee for clinical laboratory standards for fungi (M27-A2) [20]. The stock solutions of *C. gigantea* latex extract was diluted suitably as required from stock solution. The ranges should be prepared one step higher than the final dilution range required that if a final dilution range of 0.5, 1, 2, 4, 8, and 16 mg/ml is required then a range of 1, 2, 4, 8, 16 and 32 mg/ml should be prepared to compensate for the addition of an equal volume of inoculum.

Two rows of 12 capped test tubes were arranged in the rack. In a sterile 30 ml (universal) screw capped bottle, 8 ml of SD broth containing the required concentration of *C. gigantea* latex extract for the first tube in each row was prepared from the appropriate stock solution already made. The contents of the universal bottle were mixed using a sterile pipette and transferred 2 ml to the first tube in each row. Using a fresh sterile pipette, 4 ml of broth was added to the remaining 4 ml in the universal bottle, mixed well and transferred 2 ml to the second tube in each row. Dilutions were continued in this way to as many as 10 tubes. 2 ml of broth free from *C. gigantea* latex extract was added to the last tube in each row. The density of the suspension was adjusted (3×10^6 to 5×10^6 CFU/ml) to equal that of the 0.5 McFarland standard by adding sterile distilled water as detailed above. Amphotericin B was included in the assays as positive control 10 µg/disc. After incubation at 28^{o}C for 42-78 h, turbidity of the tubes was assessed visually by comparison to uninoculated control. MIC was determined as the lowest concentration of *C. gigantea* latex extract with no visible growth after incubation. All assays were carried out in triplicates.

Minimum fungicidal concentration (MFC): The MFC was determined by plating a 100 µl volume on SDA from the tubes showing no visible growth. The plates were incubated as described above in MIC. The MFC was defined as the lowest concentration of substance that did not allows any visible growth on the agar plate.

RESULTS AND DISCUSSION: In the present study, the ethanolic extract of *C. gigantea* latex yield was 15.5%. **Table 1** shows the antifungal activity of ethanolic extract of *C. gigantea* latex against eight different fungal species. The antifungal potency of *C. gigantea* latex extract was evaluated by the presence or absence of inhibition zones and zone diameters (mm). From the results, it is evident that the ethanolic extract of *C. gigantea* latex showed a maximum inhibitory zone in a dose dependant manner. However, there was no significant difference between the levels of zone of inhibition at the concentration of 150 mg and 300 mg. The antifungal potency of *C. gigantea* latex extract on the *C. albicans* showed a larger diameter of clearance than that of other strains. Moreover, the zone of clearance achieved by *C. gigantea* latex extract is comparable to that of standard drug, Amphotericin B.

TABLE 1: ANTIFUNGAL ACTIVITY OF *CALOTROPIS GIGANTEA* LATEX EXTRACT AGAINST FUNGAL SPECIES TESTED BY DISC DIFFUSION ASSAY

Strains	Control	0.175 mg/disc	0.375 mg/disc	0.75 mg/disc	1.5 mg/disc	3 mg/disc	Amphotericin B
Candida albicans	-	10.0	13.6	14	24	25.2	26
Saccharomyces cerevisiae	-	-	8.5	10	15.2	17	19.4
Trichophyton mentagrophytes	-	9.5	14.3	15.2	20	21.5	23
Trichophyton rubrum	-	9	12.5	18	23	24	25
Aspergillus fumigatus	-	12	15.5	17.2	22	23.5	24
Aspergillus flavus	-	11	14	16.5	20	22	23.5
Aspergillus niger	-	7	10.2	14	21	21.5	23
Penicillium chrysogenum	-	8.5	12	15	19.5	21	22

The increasing incidence of invasive fungal infections is the result of many factors, including increasing number of patients with severe immunosuppressant. Although new drugs have been introduced to combat this problem, the development of resistance to antifungal drugs has become increasingly apparent, especially in patients who require long-term treatment or who are receiving antifungal prophylaxis, and there is growing awareness of shifts of flora to more-resistant species [21].

Plants are important source of potentially useful structures for the development of novel chemotherapeutic agents. Plant extract has been used traditionally to treat a number of infectious diseases including those caused by bacteria, fungi, protozoa and viruses [22]. Only the alcoholic extract was used in the present study, as ethanol was reported to be a better solvent for extracting the antimicrobial active substances from Calotropis compared to other solvents [23, 24]. The fungal strains used in the present study were selected on the basis of their clinical importance. For example, *Saccharomyces cerevisiae*, commonly known as brewer's yeast, has been reported as the cause of both superficial and invasive infections [25]. *Candida albicans*, while naturally occurring in the intestinal flora, can cause oral thrush and systemic infection [26]. Aspergillus and penicillium species are known to produce many secondary metabolites commonly known as mycotoxins. The secondary metabolites are potent carcinogens, hepatotoxins, teratogens and immunosuppressive compounds [27].

According to Mock *et al.* [28] agar-based methods are not recommendable for testing azoles because these drugs can diffuse poorly in the agar. However, the disk diffusion and E-test methods have shown a good correlation with the broth-based reference methods for testing yeasts. Hence, agar disc diffusion method was performed in the present study to investigate the antifungal activity of *C. gigantea* latex extract. The highest activity (diameter of zone of inhibition 26 mm) was demonstrated by the ethanolic extract of *C. gigantea* against *C.*

albicans while the lowest activity was observed against *S. cervisiae*. The results of the *in vitro* antifungal assay revealed that the growths of fungal strains were affected by the latex extract by forming clear inhibition zones. The minimum inhibitory concentration and minimum fungicidal concentration of *C. gigantea* latex extract and the standard antifungal drug, Amphotericin B was depicted in **Table 2**. The MIC value of *C. gigantea* latex extract against fungal strains varies from 1 mg to 8 mg and the results are comparable with the standard antifungal agent, Amphotericin B.

Minimum inhibitory concentrations are considered the "gold standard" for determining the susceptibility of microorganisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. MICs are used in diagnostic laboratories to confirm unusual resistance, to give a definite answer when a borderline result is obtained by other methods of testing, or when well diffusion methods are not appropriate. Results of MICs and MFCs showed that *S. cerevisiae* had the highest MIC (8 mg/ml) and MFC (8 mg/ml) while the lowest MIC of 1 mg/ml was demonstrated by *C. albicans*. The fungistatic or fungicidal effect of natural products and the mechanisms involved are cytoplasm granulation, cytoplasmic membrane rupture and inactivation and/or inhibition of intracellular and extracellular enzymes. These biological events could take place separately or concomitantly culminating with mycelium germination inhibition and it is also reported that plant lytic enzyme act in the fungal cell wall causing breakage of β-1,3 glycan, β-1, 6, glycan and chitin polymer [29]. Studies on the antifungal activity of *C. gigantea* latex are scanty in the literature.

However, few reports are available on the antimicrobial activity of *C. procera*. Recently Kareem *et al.*[24] have reported that the latex of *C. procera* possess significant inhibitory effect on fungal strains. Similarly Hassan *et al.*[30] reported that the aqueous extracts of *C. procera* stem, root and leaves have demonstrated strong inhibitory effect on the test microorganisms. The mechanism of action of the constituents of *C. procera* could be by inhibition of fungal cell wall, protein and amino acid, sphingolipid biosynthesis and electron transport chain [31].

TABLE 2: ANTIFUNGAL ACTIVITY OF *CALOTROPIS GIGANTEA* LATEX EXTRACT AGAINST FUNGAL SPECIES TESTED BY MIC AND MFC

Fungal species	MIC		MFC	
	***C.gigantea* (mg/ml)**	**Amphotericin B (mg/ml)**	***C.gigantea* (mg/ml)**	**Amphotericin B (mg/ml)**
Candida albicans	1	0.5	2	2
Saccharomyces cerevisiae	8	4	8	4
Trichophyton mentagrophytes	4	2.5	4	2
Trichophyton rubrum	2.5	4	5	8
Aspergillus fumigatus	2	1	4	2
Aspergillus flavus	2	1	2	2
Aspergillus niger	2.5	2	5	5
Penicillium chrysogenum	2	4	4	2

Table 3 shows the phytochemical screening of ethanolic extracts of *C. gigantea* latex. Bioactive ingredient of ethanolic extracts of latex indicates the presence of alkaloids, flavonoids, glycosides, saponins, tannins, steroids, triterpenoids, and phenols.

TABLE 3: QUALITATIVE ANALYSIS OF THE PHYTOCHEMICALS OF *CALOTROPIS GIGANTEA* LATEX EXTRACT

Phytoconstituents	Inference
Alkaloids	+
Tannins	+
Flavonoids	+
Terpenoids	+
Amino acids	+
Saponins	+
Steroids	+
Cardiac glycosides	+

The aqueous and methanolic extracts of leaves of *C. procera* were found to exhibit both antifungal and antioxidant properties. However, the aqueous extract was reported to have mild antioxidant activity. The presence of pharmacologically active phytochemicals in the ethanolic extract of *C. gigantea* may provide a justification for the observed antifungal activity. Phytochemical constituents such as flavonoids, alkaloids, tannins and other aromatic compounds are secondary metabolites of plants that serve as defense mechanisms against predation by many microorganisms, insects and herbivores [32]. Tannins have been found to form irreversible complexes with proline rich protein [33] resulting in the inhibition of cell protein synthesis. Parekh and Chanda [34] reported that tannins are known to react with proteins to provide the typical tanning effect which is important for the treatment of inflamed or ulcerated tissues. These observations therefore support the use of *C. gigantea* latex in herbal cure remedies. Alkaloids which are one of the largest groups of phytochemicals in plants have amazing effects on humans and this has led to the development of powerful pain killer medications [35]. Flavonoids, another constituent exhibits a wide range of biological activities like antimicrobial, anti-inflammatory, analgesic, anti-allergic, cytostatic and antioxidant properties [36]. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency [37, 38]. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. Plant extracts have great potential as antimicrobial compounds against microorganisms.

Thus, they can be used in the treatment of infectious diseases caused by resistant microbes. The demonstration of antifungal activity of *C. gigantea* latex extract against fungal species may be an indicative of the presence of broad spectrum antibiotic compounds [39]. To our knowledge, the present study is the first one which systematically reports the antifungal activities of *C. gigantea* latex extract. The results of our preliminary screening assays justify the use of *C. gigantea* latex extract in the Indian ethnomedicine. However, it is important to note that the crude extract of *C. gigantea* latex need to be further purified through bio activity guided fractionation to isolate and identify the compounds responsible for antifungal activity.

CONCLUSION: The remarkable fungicidal effects of *C. gigantea* latex extract suggest that the latex may be a useful source for the development of novel antifungal agent against pathogenic fungi.

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