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Original Article

ANTIFUNGAL ACTIVITY OF *CYANOTIS AXILLARIS* (L.) D. DON EX SWEET AGAINST OPPORTUNISTIC FUNGAL STRAINS

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ABSTRACT

Objective: The main focus of the study was to compare and determine the antifungal activities of different solvent extracts of *Cyanotis axillaris*.

Methods: The dried whole plant of *C. axillaris* was extracted by sequential extraction method using solvents hexane, ethyl acetate and methanol based on their polarity. The antifungal activity of the extracts was tested against 12 opportunistic fungal strains by disc diffusion method. Minimum inhibitory concentration (MIC) was determined using microtiter plate method.

Results: The hexane, ethyl acetate and methanol extracts showed significant antifungal activities. The highest antifungal activity was recorded for ethyl acetate extract of *C. axillaris*. In disc diffusion method at high concentration (5 mg/ml), the ethyl acetate extract exhibited the zone of inhibition>30 mm against *C. krusei*, *mentagrophytes*, *Scopulariopsis* sp. and *B. cinerea*. In MIC the ethyl acetate extract inhibited the growth of *T. mentagrophytes*, *Scopulariopsis* sp., *B. cinerea* in its low dose (0.031 mg/ml). The hexane, ethyl acetate and methanol extracts of *C. axillaris* did not show activity against *M. gypseum, T. rubrum* and *E. floccosum*.

Conclusion: This is the first report for the antifungal efficacy of *C. axillaris*. The results proved that the extracts of *C. axillaris* have high potential antifungal principles which could fight against the opportunistic and multidrug resistant fungal strains.

Keywords: Antifungal, *Cyanotis axillaris,* Disc Diffusion, MIC

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INTRODUCTION

Fungi are widespread in the environment. Some seen in the normal commensal of animals and humans. During infection, they alter the host immune system and turn as pathogenic, opportunistic and life-threatening [1]. It is estimated that nearly 300 million people are affected worldwide with serious fungal infections and 25 million are at high risk of dying or losing their sight. The severity of fungal infection in susceptible population ranges from acute, severe to chronic [2-7]. Azoles are the most widely used drugs to treat fungal infections due to their bioavailability and safety profile. Even though it shows a positive response to patients, due to its fungi static effect rather than fungicidal effect frequent relapses occur [8]. Continuous and prolonged treatment with these drugs to an immunocompromised person resulted in the development of multidrug resistant fungal strains [9]. Moreover, prolonged antifungal therapy in heavily immunocompromised people can lead infection-related toxicities, nephrotoxicity, hepatotoxicity, recurrent drug infections, organ dysfunction, cutaneous reactions and malignancies [10and11]. For example, oral itraconazole causes nausea, hypertension, hypokalemia, edema in old age people. Therefore it is restricted to patients with heart failure [12]. Voriconazole treatment in ambulatory patients cause phototoxic reactions and this can lead to the development of squamous cell carcinoma and melanoma [13and14]. Increase in the multidrug resistant fungal strains, lack of drugs with new antifungal targets and the adverse effects of antifungal drugs for a long term use urge the need for the new antifungal agents which have new target site or mechanism to fight against multidrug-resistant (MDR) fungal strains as well as to overcome the disadvantage of commercially used antifungal drugs.

Cyanotis axillaris is an herbal weed belongs to the family of Commelinaceae. Traditionally C. axillaris is used to treat tympanitis, ascites, abscesses, fever, worms and ear drum infection [15]. There is no data for its antifungal activity in traditional use. Moreover, there is no scientific evidence for its antifungal activity. Since Products from plants are safe, easily available, less expensive, efficient over the synthetic drugs used in the market and having less side effect, we chose C. axillaris to examine its antifungal activity to explore new antifungal bioactive principle to overcome the existing old antifungal drugs to treat opportunistic fungal diseases. This study is the first to report the in vitro antifungal efficacy of C. axillaris.

MATERIALS AND METHODS

Collection and processing

Fresh whole plants of *C. axillaris* were harvested from the banana field in Pongumoodu, Thiruvananthapuram, Kerala, in the month of May and authenticated by the taxonomist Dr. D. Narasimhan, Centre for Floristic Research, Department of plant biology and biotechnology, Madras Christian College, Chennai. The herbarium specimen has been deposited in Loyola College, Chennai, India, with the voucher number of LCH 403. Plants were washed thoroughly and dried completely at room temperature under shade. Dried plants were ground into coarse powder using an electric blender and filtered through sieves. The powdered drug was stored in airtight container at ambient temperature for further use.

Chemicals and reagents

All solvents used for the extraction purpose were purchased from Merk, Germany. Chemicals and standard discs (fluconazole and clotrimazole) were procured from Himedia, Mumbai, India.

Preparation of plant extracts

Plant extracts were prepared by serial extraction method which involves successive extraction with solvents of increasing polarity from a low polar (Hexane) to high polar solvents (Ethyl acetate and Methanol) to ensure that a wide polarity range of compounds could be extracted. About 50 g of dried powder was soaked in 800 ml hexane for 72 h with intermittent shaking at 120 rpm in a shaker. The extract was filtered through Whatman No. 1 filter paper. The filtrate was dried completely to get a constant dry weight of the extract. The remaining plant residue from hexane extract was dried completely and soaked in 800 ml of ethyl acetate and then methanol

successively as above mentioned and the extracts were collected. The concentrated extracts were stored at 4 °C for further use.

Fungal strains

All the microbial cultures were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and Department of Microbiology, Christian Medical College, Vellore, Tamilnadu, India and National Chemical Laboratory (NCL), Pune, India. The fungal strains were *Candida albicans* (MTCC 227), *Candida krusei, Candida tropicalis* (MTCC 230), *Microsporum gypsum, Malassezia pachydermatis*, *Trichophyton rubrum* 57/01, *Trichophyton mentagrophytes* 66/01, *Epidermophyton floccosum* 73/01, *Scopulariopsis* sp. 101/01, *Aspergillus flavus* (MTCC-277), *Botrytis cinerea* and *Curvularia lunata* 46/0. Fungal strains were stored at 4 °C in Sabouraud Dextrose slants.

Inoculum preparation

Fungal strains were subcultured in sabouraud dextrose agar (SDA) and incubated overnight at 37 °C for yeast, 3-7 d at 37 °C for *Aspergillus* species or 25 °C for dermatophytes. Fungal spores were collected from the subculture and suspended into 5 ml of sterile distilled water. The concentration of the spore suspension was adjusted to 10^4cfu/ml-using 0.5% McFarland as standard. The spore suspension was further used to determine the antifungal assays such as disc diffusion and MIC.

Antifungal activity by disc diffusion method

Antifungal disc diffusion was performed according to standard procedure for yeast [16] and filamentous fungi [17]. The extract was dissolved in dimethyl sulfoxide (DMSO). 20 µl of extract with 3 different concentrations (1.25 mg, 2.5 mg and 5 mg) were loaded in the disc and allowed to dry. The sterile Petri plates with SDA medium were swapped with fungal spore suspension. After drying the disc with extracts were placed on the Petri plates. The plates were kept inverted and incubated overnight at 37 °C for yeast; 3-7 d at 37 °C for *Aspergillus* species or 25 °C for dermatophytes and zones formed were measured and tabulated. The experiment was performed in triplicates and mean values taken as the zone of inhibition which was expressed in millimetres (mm). Fluconazole (10 µg) and clotrimazole (10 µg) were used as positive control. DMSO was used as negative control.

Determination of antifungal activity by MIC

The MIC was performed according to the standard procedure for yeast [18] and filamentous fungi [19]. The extracts were dissolved in DMSO. The microtitre plates were dispensed with 100 µl of sabouraud dextrose broth (SDB). The initial concentration of extract was 1 mg/ml and it was serially diluted two-fold. 20 µl of fungal spore suspensions were added to each well. Broth with fungal inoculum and DMSO was kept as negative control. Fluconazole and clotrimazole were used as positive control. The microtitre plates were sealed and incubated overnight at 37 °C for yeast, 3-7 d at 37 °C for *Aspergillus* species or 25 °C for dermatophytes. After incubation, 40 µl of 0.2 mg/ml P-iodonitrotetrazolium (INT) violet was added to all the wells and incubated for half an hour to one hour to note the colour change. When there is any fungal growth, an electron from the living cell is transferred from NADH to INT violet and converted to formazan which gives rise to red colour. The lowest concentration in which red colour is not seen is taken as the MIC of the extract. The MIC values were expressed as mg/ml.

Table 1: Antifungal disc diffusion method of *C. axillaris* extracts

Fungi	Zone of inhibition in mm										
	Hexane			Ethyl acetate			Methanol			Flu	Clot
	1.25 mg/ disc	2.5 mg/ disc	5 mg/ disc	1.25 mg/disc	2.5 mg/ disc	5 mg/ disc	1.25 mg/ disc	2.5 mg/ disc	5 mg/ Disc		
F_1	15	20	21	21	23	25	15	16	19	-	14
F_2	14	18	31	14	20	32	11	13	22	-	10
F_3	8	10	12	11	14	18	9	12	14	-	11
F_4	-	-	-	7	8	13	7	8	9	-	9
F_5	10	17	25	17	23	35	10	15	20	-	14
F_6	-	-	8	8	8	11	-	-	-	-	12
F_7	11	19	28	23	30	35	13	21	24	-	15
F_8	-	-	-	10	11	13	-	-	-	-	14
F_9	9	15	26	24	29	34	14	20	27	-	-
F_{10}	11	18	24	9	12	21	24	29	35	-	11
F_{11}	18	22	27	24	31	35	23	31	36	-	13
F_{12}	12	12	17	19	21	27	9	12	24	-	13

Note: F_1-*C. albicans*; F_2-*C. krusei*; F_3-*C. tropicalis*; F_4-*M. gypseum*; F_5-*M. pachydermatis*; F_6-*T. rubrum*; F_7-*T. mentagrophytes*; F_8-*E. floccosum*; F_9-*Scopulariopsis* sp.; F_{10}-*A. flavus*; F_{11}-*B. cinerea*; F_{12}-*C. lunata*; nt: not tested; (–) no activity; Flu-Fluconazole; Clot-Clotrimazole.

Table 2: MIC values of *C. axillaris* extracts

Fungi	Hexane (µg/ml)	Ethyl acetate (µg/ml)	Methanol (µg/ml)	Fluconazole (µg/ml)	Clotrimazole (µg/ml)
F_1	125	62.5	125	125	<31.625
F_2	62.5	62.5	125	125	31.625
F_3	250	125	250	125	31.625
F_4	nt	Nt	nt	125	62.5
F_5	125	62.5	250	125	<31.625
F_6	nt	Nt	nt	125	<31.625
F_7	62.5	31.25	125	125	<31.625
F_8	nt	Nt	nt	125	<31.625
F_9	125	31.625	125	125	125
F_{10}	125	125	62.5	62.5	31.625
F_{11}	62.5	31.625	31.625	125	<31.625
F_{12}	125	62.5	125	125	<31.625

Note: F_1-*C. albicans*; F_2-*C. krusei*; F_3-*C. tropicalis*; F_4-*M. gypseum*; F_5-*M. pachydermatis*; F_6-*T. rubrum*; F_7-*T. mentagrophytes*; F_8-*E. floccosum*; F_9-*Scopulariopsis* sp.; F_{10}-*A. flavus*; F_{11}-*B. cinerea*; F_{12}-*C. lunata*; nt: not tested.

RESULTS

Disc diffusion method

In antifungal disc diffusion method, *C. axillaris* showed high antifungal activity by forming a zone of inhibition up to 36 mm at high concentration (5 mg/ml). The antifungal efficiency of the hexane, ethyl acetate and methanol extracts of *C. axillaris* by disc diffusion method was summarised in table 1.

Determination of MIC

After the incubation with INT, the appearance of pink colour was observed in wells which had living fungal cells and well which appeared colourless was indicated the inhibition of fungal growth by the extract of *C. axillaris*. The MIC values of *C. axillaris* tabulated in table 2. It showed a wide range of MIC values from 0.25–0.031 mg/ml. Ethyl acetate extract showed high MIC values for many tested fungi.

DISCUSSION

Worldwide fungal diseases are one of the main causes of morbidity and mortality [20]. Recent years the fungal infections in human have dramatically increased from superficial to disseminate or deeply invasive due to the increasing immunocompromised population with cancer, organ transplantation, HIV infection, and individuals receiving immunosuppressive treatments. The prolonged immunosuppression with more intensive regimens causes invasive fungal infections in patients undergoing transplants or treatment for malignancies [21]. In the present study, we have investigated 5 yeasts and 7 filamentous fungi which cause various infections in immunocompromised people. In immunocompromised patients, Candida sp. cause invasive candidiasis and attribute 77% death [22]. *T. rubrum*, *T. mentagrophytes*, *E. floccosum*, *M. gypseum* cause dermatophytosis in immunocompromised patients [23]. In immunocompromised patients, *M. pachydermatis* cause invasive infections and Scopulariopsis sp. causes invasive sinusitis [24 and 25]. *A. flavus* cause aspergillosis, otitis, keratiti, pulmonary and systemic infections in immunocompromised patients [26]. *C. lunata* is most common in immunocompromised people and causes keratitis [27].

In our present study for the first time, the antifungal activity of *C. axillaris* was investigated. Even though plants from Commelinaceae family were investigated for its antimicrobial activity, there are very fewer investigations have been made to prove their antifungal efficacy. The ethanol extracts of *C. benghalensis* from Commelinaceae family exhibited high antifungal activity than the standard drug at the minimum concentration [28]. *C. axillaris* showed high antifungal activity against the tested fungal strains. The highest antifungal activity was recorded for ethyl acetate extract against *T. mentagrophytes*, *Scopulariopsis* sp., *B. cinerea* and methanol extract against *B. cinerea*. It inhibited the growth in its low dose (0.031 mg/ml). The hexane extract of *C. axillaris* showed high antifungal activity against *C. krusei, T. mentagrophytes, B. cinerea* and moderate activity against C. *albicans*, *C. tropicalis*, M. *pachydermatis*, *Scopulariopsis* sp*., A. flavus* and *C. lunata*. The hexane, ethyl acetate and methanol extracts of *C. axillaris* were inactive against *M. gypseum, T. rubrum* and *E. floccosum*. In this current study, we used two commercial azoles such as fluconazole and clotrimazole which are used to treat infections caused by fungal strains in immunocompromised patients. Fluconazole is a triazole and clotrimazole is an imidazole, are used to treat antifungal infections such as thrush, candidiasis, funguria, ear infections, skin infections, urinary infections and various fungal infections in AIDS patients, bone marrow transplant recipients and leukemia patients [29-36]. Extracts of *C. axillaris* showed superior activity than fluconazole. Clotrimazole showed sensitivity towards the tested fungal strains. Among these 3 extracts (hexane, ethyl acetate and methanol) of *C. axillaris,* the ethyl acetate extract showed the highest antifungal activity against *C. albicans*, *C. krusei, M. pachydermatis*, *T. mentagrophytes, Scopulariopsis* sp., *B. cinerea* and *C. lunata*. It exhibited the strongest and most potential antifungal activity than hexane and methanol extracts. Tamokou *et al.* studied the antifungal activity of ethyl acetate extract of *Albizia adianthifolia* and isolated compounds with antifungal activity [37]. The ethyl acetate fraction of Garcinia mangostana-Linn pericarp extract showed antifungal activity against *candida albicans* [38]. Bisht *et al.* proved the antifungal activity of banana leaf ethyl acetate extract against *P. oxalicum* [39]. These works support our study, that the ethyl acetate extract of plants can have antifungal principals. Hence this study proved that the ethyl acetate extract of *C. axillaris* poses more active and potential antifungal compounds that can fight against opportunistic fungal diseases.

CONCLUSION

From the present study, it is clear that the ethyl acetate extract of *C. axillaris* has great potential antifungal principles. Since ethyl acetate extract showed better activity than the commercially used drug (fluconazole) for fungal infections, it is clear that they should poses active principles with high antifungal activity as well as with better efficacy to replace the older drugs to fight against updating MDR fungal strains. The antifungal activity may be due to the single compound or a synergetic effect of multiple compounds. Phytochemical evaluation and isolation of compounds responsible for its antifungal activity are needed to establish a high potential antifungal drug that can fight against the opportunistic and multidrug resistance fungal strains.

AUTHOR CONTRIBUTION

Conception and design of this study, acquisition of data, analysis, interpretation of data and drafting the manuscript was done by the first author (Anto Suganya Regis). Conception of the study, revising the manuscript critically for important intellectual content was performed by the corresponding author (Jeya Jothi Gabriel).

CONFLICT OF INTERESTS

Declared none

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