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BRIEF COMMUNICATION

Anticandidal activity of medicinal plants and *Pseudomonas aeruginosa* strains of clinical specimens



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This study was designed to investigate the *in vitro* anticandidal activity of some medicinal plants and *Pseudomonas aeruginosa* strains against *Candida* species. The antifungal activity of methanolic extracts of five medicinal plants, namely, *Cinnamomum porrectum*, *Lippia nudiflora*, *Cestrum nocturnum*, *Trachyspermum ammi*, and *Sida carpinifolia* were studied. The medicinal characteristics of these plants were compared with commercially used antibiotics. The antimicrobial assay was done by agar well diffusion and the broth dilution method. Among the plants used, *T. ammi* and *C. nocturnum* were found to be more potent than the others. Twenty *P. aeruginosa* strains were isolated from various clinical specimens. The total inhibitions obtained were found to be 47%, 38%, and 36% in blood agar, whereas in Sabouraud dextrose agar (SDA) the inhibitions were 57%, 48%, and 37%, respectively. Copyright © 2014, Taiwan Society of Microbiology. Published by Elsevier Taiwan LLC. All rights reserved.

Introduction

Northeast India has been identified as one of the biodiversity hotspots among the 32 hotspots identified worldwide.¹ The medicinal plants of this area are still to be identified and studied properly. The objective of the present investigation is to screen medicinal plant species and

Pseudomonas aeruginosa strains isolated from patients at the local civil hospital for antifungal activities against the pathogenic microorganism *Candida* species, which inhabits the gastrointestinal tract, buccopharyngeal cavity, and vulvovaginal tract, is capable of causing serious systemic infections, and is frequently associated with indwelling catheters and immunosuppressive agents.² *Candida* species are also benign members of skin and mucosal flora. When host defenses falter, however, *Candida* species initiate invasive growth that can lead to severe diseases.

According to an National Nosocomial Infection Surveillance (NNIS) survey from 1980 to 1990, the most frequently

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isolated nosocomial fungal pathogen was *Candida albicans* (59.77%) followed by other *Candida* species (18.6%). From time immemorial, medicinal and aromatic plants have been used to get relief from various ailments. The widespread belief that green medicine is healthier than synthetic products has revived the interest in natural drugs.

P. aeruginosa is a part of the human microflora of healthy individuals. Because of the ingestion of the organism through the colonization respiratory tract, the bowel is the most likely colonized site. The likelihood of colonization increases with the longevity of hospitalization. Pseudomonads represent the major group of nondifferentiating microorganisms that produce antibiotics. The antibiotic substances are pyocyanin, pyrrolnitrin, and pseudomonic acid. It has been demonstrated that some secondary metabolites, production of phytotoxins, slime production, production of antifungal compounds, produced by pseudomonads, give an obvious selective advantage to these organisms in their natural environment.^{3,4}

This study investigated the *in vitro* efficacy of some medicinal plants and *P. aeruginosa* strains against *Candida* species.

Materials and methods

Plant material

Five medicinal plants were chosen for this study. The plant materials (leaves, fruits, and seeds) were collected from Tezpur (border of Northeastern India). The plant materials collected were brought to the laboratory in polythene bags, identified by experts of the institute, and processed. Fresh plant materials were washed under running water, shade dried, homogenized to a fine powder, and processed accordingly.

Microorganisms

Twenty *P. aeruginosa* isolates were isolated from the culture of patients of a local civil hospital. Each single isolate was collected from different patients. The strains were identified by standard bacterial identification methods.⁵ *P. aeruginosa* isolates were identified on the basis of their characteristic colony morphologies and the production of diffusible pigments. *P. aeruginosa* strains were identified based on the positive oxidase test, triple sugar ion reaction of alkaline over no change, no growth at 42°C. The strains of *Candida* including *Candida krusei* ATCC 6258, *C. albicans*, and *Candida tropicalis* were the clinical isolates obtained from the School of Tropical Medicine, Kolkata, India. To investigate the *in vitro* anticandidal activity of *P. aeruginosa* strains, the Kerrs method⁴ was taken into consideration.

Preparation of the plant extract and test extracts

A total of 5 g of the material was extracted with 150 mL methanol in Soxhlet apparatus. The solvent was evaporated at room temperature and the extracts thus obtained were stored in airtight bottles for further studies. Each extract

was dissolved in dimethyl sulfoxide at a concentration of 0.2 mg/mL and was used as working stock. Clotrimazole 1000 µg/mL was used as the standard for comparison.

Technique

The antifungal assay of the plant extracts was evaluated employing an agar well diffusion method.⁶ Sterilized Sabouraud dextrose agar (SDA) medium was poured into sterile Petri plates and allowed to solidify. After solidification of the medium, the broth was vortexed and 100 µL of the broth was spread evenly over the surface of the agar plates. A well of 8 mm diameter was made in each plate with a sterile cup borer. Into each well was introduced 100 µL of the test extract and the plates were incubated at 28°C for 24 hours. The experiment was replicated three times. The efficacy was determined by measuring the diameter of the inhibition zone exhibited by the extracts against the test pathogen. The minimal inhibitory concentrations (MICs) of the aqueous plant extract as well as reference drugs against *Candida* strains were determined by the broth dilution method.⁷

Activity index determination

The activity index of the extracts was determined by the following formula provided by Jain and Sharma:⁸

$$\text{Activity index} = \frac{\text{Inhibition zone of extracts}}{\text{Inhibition zone of standard}}$$

To investigate the *in vitro* anticandidal activity of *P. aeruginosa* strains, the Kerrs method was employed. The isolated *P. aeruginosa* strains were stored at +4°C on the nutrient agar slopes and *Candida* isolates were maintained on SDA plates. The inoculums were prepared by fresh 24 hour plate cultures of each *P. aeruginosa* strain of 10⁶ CFU/mL in 0.08% NaCl to test the anticandidal activity. Freshly prepared inoculum (20 µL) was streaked on SDA and blood agar with a width of 1 cm across. Plates were then incubated at 30°C for 24 hours. Further growth was removed using a glass slide. Filter paper disks of 5 cm in diameter were cut, soaked in chloroform, and laid on a metal tray in a safety cabinet. Each plate was then placed face down on the top of a chloroform-containing filter paper disk and was left for 20 minutes so that the microscopic remnants of the culture were killed. The plates were removed from the cabinet, and traces of chloroform were eliminated by exposure to air for few minutes. A fresh 24 hour plate culture of each fungal strain was used to prepare an inoculum of 10⁶ CFU/mL. This fungal suspension was streaked onto the chloroform treated medium at right angles to the line of the original medium: plates were then incubated for 24 hours at 30°C. Each of the 20 *P. aeruginosa* strains was tested against each of the three *Candida* strains. Plates were read as follows: total inhibition fungal growth was recorded as (a ± b) and no inhibition of fungal growth was recorded as (–).

Statistical analysis

The Chi-square test was used to compare *in vitro* anticandidal activity on blood agar and on SDA.

Results

In the present study, the extracts of five medicinal plants were investigated for their antifungal activities against *C. albicans*. The details of the results are shown in Table 1. The study shows that all of the plant extracts are active against the test pathogens, but the seeds extract of *Trachyspermum ammi* and the leaf extract of *Cestrum nocturnum* exhibited more potent antifungal efficacy, showing maximum inhibition zones of diameters 38.3 mm and 31.3 mm, respectively, against *C. albicans* (Table 1). The leaf extract of *Lippia nudiflora* revealed less activity than that of the standard. The activity index, as revealed in Table 1, is at maximum in the seed extract of *T. ammi* followed by *C. nocturnum*, while it is the least in the case of *L. nudiflora*. The MICs of the potent plant extract, nystatin, and clotrimazole are shown in Table 2. The effect of the unrefined nature of the plant extract as compared to the reference drugs is once more apparent. However, there is obvious evidence that the plant extract has a substantial level of anticandidal activity. Comparatively, the *Candida* strains isolates seemed to be more sensitive to the plant extract. It would appear that the strains were more resistant to the plant extracts and nystatin than to clotrimazole.

P. aeruginosa strains were isolated from tracheal aspiration materials, wound, and sputum. The anticandidal activity of *P. aeruginosa* strains was determined in eight of 10 tracheal, three of six wound, and two of four sputum sample isolates. All *P. aeruginosa* strains have pyocyanin pigment.

The total inhibition rates were obtained using SDA for *C. albicans*, *C. tropicalis*, and *C. krusei* and were 47%, 38%, and 36%, respectively. When SDA was used, the rates were detected as 57%, 48%, and 37%, respectively. There was no significant difference between blood agar and SDA results ($p > 0.06$) which demonstrates *in vitro* anticandidal activity on the blood agar and SDA (Table 3).

Discussion

In recent years, there has been a gradual revival of interest in the application of medicinal plants in developed, as well as in developing, countries and the medicinal properties of several plants have been explored. A successful search for medicinal property of any plant largely depends on the solvent utilized in the process of extraction. Most traditional practitioners use water as a solvent and are unable to get the desired results.

Despite the striking success of the pharmaceutical industries in creating new antibiotics, finding new broad-spectrum antimicrobial agents is still a priority because of resistant bacterial infections.⁸ Antifungal agents currently available in the market are limited due to their toxicity, low effectiveness, and cost for prolonged treatment. Therefore, there is a need to develop antifungal agents which can satisfy the present scenario. The efficacies of the extracts were also compared with the activity of the standard antibiotic clotrimazole, available in the market. The present results showed that, with the exception of one (*L. nudiflora*), all of the extracts were more promising than the standard antifungal. Significant differences are

Table 1 Antifungal activities of methanolic extracts of the medicinal plants and efficacy of the medicinal plants against *Candida* strains

Organisms	Plants											
	<i>Cinnamomum porrectum</i>		<i>Lippia nudiflora</i>		<i>Cestrum nocturnum</i>		<i>Trachyspermum ammi</i>		<i>Sida carpinifolia</i>		Clotrimazole	
	ZI	AI	ZI	AI	ZI	AI	ZI	AI	ZI	AI	ZI	AI
<i>Candida albicans</i>	17.5 ± 0.40	1.346 ± 0.55	12.8 ± 0.45	0.984 ± 0.45	31.3 ± 0.65	2.423 ± 0.35	38.3 ± 0.45	2.946 ± 0.35	23.5 ± 0.55	1.807 ± 0.40	13.0 ± 0.45	
<i>Candida tropicalis</i>	16.8 ± 0.05	1.12 ± 0.35	11.4 ± 0.30	0.76 ± 0.40	30.8 ± 0.75	2.053 ± 0.65	32.6 ± 0.45	2.173 ± 0.35	24.6 ± 0.75	1.640 ± 0.40	15.0 ± 0.45	
<i>Candida krusei</i>	17.0 ± 0.40	1.214 ± 0.65	12.2 ± 0.05	0.871 ± 0.45	28.9 ± 0.35	2.064 ± 0.45	34.6 ± 0.45	2.471 ± 0.45	22.8 ± 0.60	1.628 ± 0.40	14.0 ± 0.45	

AI = activity index; ZI = zone of inhibition.

Table 2 Minimum inhibitory concentrations (MIC) of potential plant extracts and standard antifungals against the test strains

Organisms	Mean MIC (\pm SD)			
	<i>Cestrum nocturnum</i> mg/mL	<i>Trachyspermum ammi</i> mg/mL	Nystatin IU/mL	Clotrimazole μ g/mL
<i>Candida albicans</i>	20.44 \pm 0.45	26.44 \pm 1.41	30.33 \pm 2.02	10.08 \pm 2.45
<i>Candida tropicalis</i>	42.40 \pm 10.08	40.42 \pm 1.08	40.08 \pm 3.32	11.08 \pm 3.56
<i>Candida krusei</i>	38.68 \pm 11.18	41.08 \pm 1.34	40.44 \pm 2.28	12.08 \pm 3.38

SD = standard deviation.

Table 3 Anticandidal activity of *Pseudomonas aeruginosa* strains on blood agar and on Sabouraud dextrose agar (SDA)

Organisms	Blood agar		SDA	
	Total inhibition n (%)	Partial inhibition n (%)	Total inhibition n (%)	Partial inhibition n (%)
<i>Candida albicans</i>	21 (47)	4 (8)	24 (57)	6 (14)
<i>Candida tropicalis</i>	18 (38)	9 (17)	21 (48)	8 (18)
<i>Candida krusei</i>	16 (36)	5 (9)	16 (37)	4 (10)

normally observed when crude (unpurified) plant extracts are compared with pure drugs.⁹ The agar-cup diffusion method is commonly employed for preliminary susceptibility testing, but it is not a dependable and accurate method for comparison, because of the high degree of interference due to drug diffusion problems.¹⁰ A more generally accurate method of assessment is the broth dilution technique. In this study, therefore, the broth dilution method was used in determining the activities measured as MIC. These observations point "to the fact that in order to make" an affirmative conclusion on the activity of a drug (especially a new product), strains of the organisms from a wide variety of sources should be employed at the evaluation stage.

The study was also designed to investigate the *in vitro* anticandidal activity of *P. aeruginosa* strains against three *Candida* species. The total inhibition rates obtained by using blood agar suggested that *C. albicans* and *C. tropicalis* were the most inhibitive strains. *C. krusei* was found to be the least inhibitive strain. There was no significant difference between the blood agar and SDA results. The total inhibition rates observed for *C. albicans* and *C. krusei* were 10% and 6%, respectively, using blood agar.¹¹ Our results are comparable with the results of Yucesoy et al¹¹ because *C. albicans* is the most inhibitive strain and no significant difference was detected on blood agar and SDA results. However, their anticandidal activity rates reported are less than ours. Different *Pseudomonas* species such as *P. aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas multivorans*, *Pseudomonas aureofaciens*, and *Burkholderia cepacia* were implicated for antibiotic production in medical literature. Total inhibition of *Candida* strains by 10 *P. aeruginosa* strains was observed by Kerr.³ The complete inhibition is due to the formation of pyocyanin, which is known to reduce cyclic adenosine monophosphate (cAMP) and adenosine triphosphate (ATP) in human epithelial cells and is a possible explanation of increased antifungal activity of pyocyanin against *C. albicans* in the well plate assay.

The growth of dermatophyte and non dermatophyte fungi was found to be poor in the presence of *P. aeruginosa* as observed by Foster et al⁵ and they also showed that large *P. aeruginosa* populations in infected nails resulted in a lower fungal population. It may interact with the endothelium derived relaxing factor or nitric oxide through formation of a complex, or it may act by nitric oxide synthase. An elaborate study on the interaction of *C. albicans* with *Pseudomonas* spp. *in vitro* showed that *Pseudomonas* spp. forms biofilm on *C. albicans* cells and kills them.¹⁰ It has been suggested that only the filamentous form of *C. albicans*, not the yeast form, is affected by *Pseudomonas* spp., because of the difference in their cell walls.

Several clinical reports have shown that the presence of *P. aeruginosa* appears to limit the growth of *C. albicans* and that attention of *P. aeruginosa* by treatment with antibiotics is often followed by the increase in the *C. albicans* population. The interactions between fungi and bacteria in natural settings are complicated by a number of factors including the host response, environmental parameters, and species composition of the community. The present study reveals the potential of medicinal plants to be used as antifungal agents and the potential of *P. aeruginosa* and its compounds in the treatment of candidiasis.

Conflicts of interest

There are no conflicts of interest.

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