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Hypersaline fungi as a source of potentially active metabolites against pathogenic *Candida* species

SHWETA PANCHAL[1]*, THOKUR SREEPATHY MURALI[2], TRICHUR SUBRAMANIAN SURYANARAYANAN[3]*, KAUSTUV SANYAL[1]

[1] Molecular Mycology Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, IN-560064, India
[2] Department of Biotechnology, Manipal School of Life Sciences, Manipal Academy of Higher Education, Manipal, IN-576104, India
[3] Vivekananda Institute of Tropical Mycology (VINSTROM), Ramakrishna Mission Vidyapith, Chennai, IN-600004, India
*corresponding authors: shwetapanchal@jncasr.ac.in; t_sury2002@yahoo.com

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Opportunistic and nosocomial human infections by various *Candida* species are of serious global health concern especially due to the spread of drug resistant strains and lack of treatment options. One of the main problems of bioprospecting for novel antimycotics is the rediscovery of already known molecules. To reduce the chances of such rediscoveries, one option is to search for unique metabolites from microbes of less studied and extreme habitats.

Thirty-five fungal strains were isolated from solar salterns and the methanolic extracts of their culture supernatants were tested for the inhibition of human-pathogenic *Candida albicans*, *C. dubliniensis*, *C. glabrata*, *C. lusitaniae*, *C. tropicalis*, and two clinical isolates of *C. auris*. Of the fungi screened, two, viz. *Curvularia nodosa* and *Fusarium* cf. *foetens*, showed significant growth inhibition of all the *Candida* species. Although the effective molecules were not identified in this preliminary screening, it highlights the importance of bioprospecting fungi from extreme environments which have been neglected in the search for novel antibiotics.

Key words: antifungal, bioprospecting, filamentous fungi, extremotolerant, hypersaline environment.

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Oportunní a nosokomiální infekce, působené různými druhy rodu *Candida*, představují celosvětový zdravotní problém, zejména kvůli šíření rezistentních kmenů a nedostatečným možnostem léčby. Jedním z hlavních problémů bioprospekce nových antimykotik je opakované objevování již známých molekul. Coby možnost, jak toto eliminovat, se jeví pátrání po jedinečných metabolitech, jež mohou produkovat mikroorganismy z méně prozkoumaných a extrémních stanovišť.

Metanolické extrakty supernatantů 35 kmenů hub, izolovaných ze solných polí, byly testovány na inhibici lidských patogenů *Candida albicans*, *C. dubliniensis*, *C. glabrata*, *C. lusitaniae*, *C. tropicalis* a dvou klinických izolátů *C. auris*. Dva druhy, konkrétně *Curvularia nodosa* a *Fusarium* cf. *foetens*, vykázaly významnou inhibici růstu všech druhů rodu *Candida*. Ačkoli jde o předběžný screening, při kterém nebyly identifikovány účinné molekuly, ukazuje se význam bioprospekce hub z extrémních prostředí, dosud opomíjené při hledání nových antibiotik.

INTRODUCTION

Of the approximately 140,000 known fungal species (Lücking et al. 2020), only a few are obligate human pathogens; most other human-associated fungi are opportunistic pathogens and cause disease only when immunity is compromised (Hall et Noverr 2017). Under weakened host immunity caused by surgical interventions, occurrence of pre-existing infections, or administration of immunosuppressive drugs, some of the commensals may turn pathogenic and cause invasive infections (mycoses). Such opportunistic fungal pathogens are adapted to surviving at human-body temperature, evade or suppress immunity, and break down human tissues to absorb nutrients (Köhler et al. 2017), causing over 1.6 million deaths annually (Almeida et al. 2019). The substantial increase in immunocompromised patients due to organ transplants, diseases like AIDS and the emergence of new strains of pathogenic fungi have projected mycoses as a major health issue (Rodrigues et Nosanchuk 2020).

Among the fungal pathogens, species of *Candida* are the most common fungi causing mycoses in immunocompromised patients probably because of their widespread association with humans as commensals (Köhler et al. 2017). Systemic candidiasis caused by *Candida albicans* is life-threatening with a high mortality rate of 35–70%; infections caused by non-albicans *Candida* species like *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. auris* are also significant in hospital settings, with *C. tropicalis* prevalent as the leading fungal pathogen in intensive care units (ICUs) across hospitals in India (Chakrabarti et al. 2015). The limited choice of drugs for treating candidiasis and the rapid evolution of multidrug resistance among *Candida* owing to chromosomal aneuploidy, karyotype diversity (Legrand et al. 2019, Narayanan et al. 2021) and differential gene expression (Ji et al. 2020) has resulted in these pathogens affecting more than a quarter of a million patients every year (Kullberg et Arendrup 2015). Thus, the need for novel antibiotics to manage drug-resistant species of *Candida* cannot be overstressed.

In the past decade, the rapid evolution of multidrug resistance among *Candida auris* isolates across the globe (Clancy et Nguyen 2017, Lone et Ahmad 2019) has underscored the need for alternatives to existing antifungal drugs. Currently, only three primary antifungal drug types are used in treating invasive

fungal infections, viz. polyenes, azoles and echinocandins. Resistance to even one of these drugs reduces the therapeutic options by at least 33% (Berman et Krysan 2020). The evolution and spread of drug resistance to currently available antifungals combined with the slow pace of synthetic drug discovery emphasises the urgent need for new natural product-based antifungals. Furthermore, most drug discovery pipelines are dedicated to modifications of existing drugs. Resistance to these drugs is inevitable in the foreseeable future and hence bioprospecting the natural environment for novel antifungal drugs presents an excellent option.

However, since the search for such antibiotics among natural products has resulted in recurrent discovery of the same molecules (Cox et al. 2017), it is prudent to screen microbes from extreme habitats for novel antibiotics. Such environments would support only microbes adapted to the harsh conditions prevailing in them, thus possibly establishing novelty in the chemical interactions between them when compared to 'normal' environments. Extreme environments influence the metabolism of the microbes they harbour, possibly leading to the synthesis of unique metabolites not met within microbes of regular environments (Gunde-Cimerman et Zalar 2014, Chávez et al. 2015, Charlesworth et Burns 2016, Giddings et Newman 2022). Recent discoveries of several antimicrobial metabolites from extremophilic microbes including fungi (Rogozhin et al. 2018, Zhang et al. 2020, Hui et al. 2021, Igarashi et al. 2021) add credence to this argument.

While bacteria have been subjected to intense scrutiny in search of antibiotics, the fungal kingdom remains poorly explored in this aspect (Suryanarayanan et Hawksworth 2018). Fungi produce an extraordinary number of secondary metabolites which provide them with a competitive edge to survive in their varied environmental niches. Since a few studies show that fungi from extreme habitats, like sites with hypersaline conditions, produce novel secondary metabolites (reviewed by Chung et al. 2019, Corral et al. 2019), we screened crude extracts of the culture supernatants of fungi isolated from solar salterns in southern India for inhibition of several species of human pathogenic *Candida* species.

MATERIAL AND METHODS

The soil samples were collected from 4 cm depth from salterns at Thoothukudi (8°48' N, 78°11' E, salinity 9.9%) and Villupuram (11°57' N, 79°32' E, salinity 9.1%), Tamil Nadu State, India. We used both soil dilution plating (Waksman 1922) and the Warcup method (Warcup 1957) for isolating filamentous fungi, since the former generally selects fungi existing as spores in the soil while the latter reveals fungi present in relatively low numbers. By using potato dextrose agar (PDA) medium amended with chloramphenicol (150 mg/l) (Thirunavukkarasu et al. 2017), thirty-five filamentous fungi were isolated from hypersaline soil samples.

Each fungus was cultured in 100 ml liquid potato dextrose medium (static culture, 21 days at 26 °C) with XAD™ 16 amberlite™ resin (Rohm and Hass, Philadelphia, PA, USA). XAD polystyrene resin adsorbs small molecular weight metabolites secreted by the fungus facilitating their easy extraction

(Suryanarayanan et al. 2010). After incubation, the mycelium was filtered, the XAD was air dried and shaken with 10 ml methanol. The methanol was then evaporated in a Rotavapor (Büchi Labortechnik, Flawil, Switzerland), and the residue resuspended in 20% dimethyl sulfoxide (DMSO) and the crude extract was tested for its activity against *Candida* species by checking growth inhibition using an antifungal assay in a 96-well plate (Hoque et al. 2015). The *Candida* species screened were *C. albicans* SC5314, *C. dubliniensis* Cd36 (clinical isolate), *C. glabrata* CBS138, *C. lusitaniae* (clinical isolate), *C. tropicalis* MYA-3404, and two clinical isolates of *C. auris* clade 1 (strains 45 and 46; Narayanan et al. 2021). Of these, *C. auris* strain 46 is resistant to two antifungal antibiotics, viz. fluconazole and caspofungin.

Each *Candida* species was cultivated for 12 hours in 10 ml YPD (1% yeast extract, 2% peptone, 2% dextrose) medium at 30 °C. The growth was estimated by measuring the absorbance at 600 nm, after which the culture was diluted to a concentration of ~10^5 cells/ml with sterile YPD broth. From this cell suspension, 200 µl was incubated with 25 µl of each of the 35 crude extracts (prepared in 20% DMSO) at 30 °C for 24 h in static condition, after which the optical density at 600 nm was measured. Untreated cells (in YPD + 25 µl of 20% DMSO) incubated in the absence of any crude extract were used as positive control. Student's *t*-test was used to compare the differences between the treatments.

To identify the two fungi which produced antifungal compounds (VIG 994 from Thoothukudi and VIG 1108 from Villupuram), genomic DNA was extracted following the protocol by Paranetharan et al. (2018), and the internal transcribed spacer (ITS) region was amplified using fungal-specific primers ITS1 and ITS4 (White et al. 1990). The resulting amplicons were sequenced using ITS1 primer in an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were edited manually and searched for the closest match using NCBI BLAST (megablast) limiting sequences from type material. Based on sequence similarity, the species were assigned when the similarity was above 99%. However, since the use of the ITS region alone for identifying species in the *Fusarium oxysporum* complex is unreliable, the assigned species name was prefixed with 'cf.'. The sequences were also submitted to GenBank and accession numbers were obtained (VIG 994 – MN153743 and VIG 1108 – MN153744).

RESULTS

Of the 35 crude extracts screened, those of VIG 994 and VIG 1108 inhibited significantly all *Candida* species tested and both extracts appeared to have similar potency with respect to growth inhibition for most *Candida* species tested (Fig. 1). Based on morphological and molecular analyses, we identified VIG 994 and VIG 1108 as *Curvularia nodosa* and *Fusarium oxysporum* complex, respectively. ITS sequence analysis showed that VIG 994 had high sequence similarity with *C. nodosa* (accession number NR154865; 99.61% similarity) and VIG 1108 had high similarity with *F. foetens* (accession number NR159865; 99.59% similarity), hence this species is further referred to as *Fusarium* cf. *foetens*. The culture characteristics also confirmed identification of the fungi as *C. nodosa* (Marin-Felix et al. 2017) and *F. foetens* (Schroers et al. 2004). The cultures were deposited in Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India under the accession numbers MTCC 13175 (*C. nodosa*) and MTCC 13176 (*F.* cf. *foetens*).

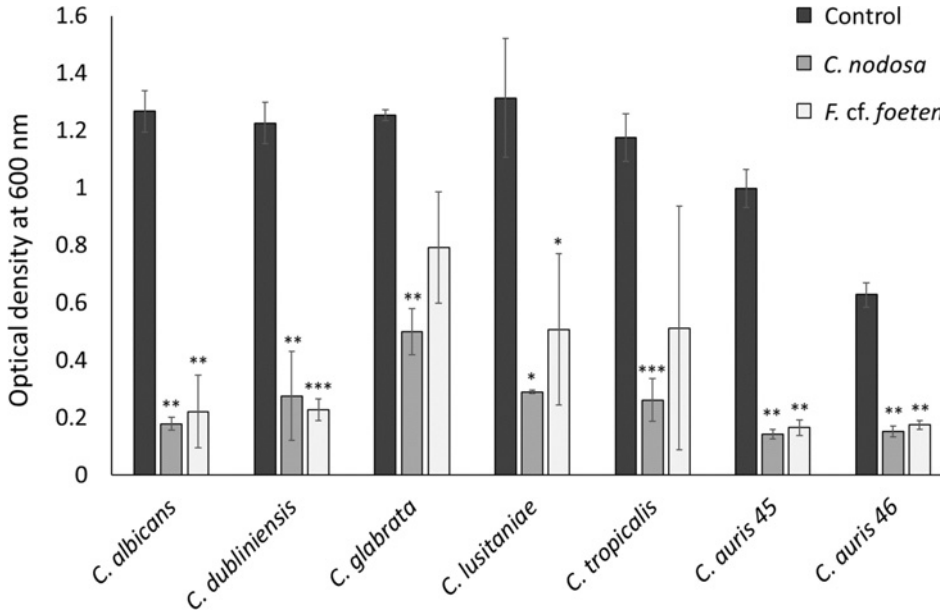

Fig. 1. Effects of crude extracts on the growth of various *Candida* species. Reduction in growth as compared to control as observed by significantly low optical density at a wavelength of 600 nm when the organisms are grown in the presence of crude extracts of hypersaline fungi VIG 994 (*Curvularia nodosa*) and VIG 1108 (*Fusarium* cf. *foetens*) for 24 h (N = 2; Student's *t*-test: *** $P < 0.01$, ** $P < 0.05$, * $P < 0.1$).

As seen in Fig. 1, the inhibition of *Candida* species growth was higher than 70% with the crude extract of *C. nodosa* and higher than 50% with the crude extract of *F.* cf. *foetens* for all *Candida* species tested, except for *C. glabrata*. For *C. glabrata*, 40% of the cells were found to be viable in the presence of the *C. nodosa* extract and 64% in the presence of the *F.* cf. *foetens* crude extract after an incubation time of 24 hours. The possibility of change in the growth of *C. glabrata* during longer incubation with the crude extract cannot be ruled out here. However, to avoid the ambiguity which may arise by using stationary phase cultures, a constant time point was chosen for reporting the results. In addition, *C. glabrata*, being a drug-resistant species, could be less susceptible to the active principles in the crude extracts. Strikingly, the two clinical isolates of *C. auris*, strains 45 and 46, were equally susceptible to both the crude extracts. Since strain 46 is a drug-resistant isolate with IC_{50} for fluconazole >64 µg/ml, it is possible that the crude extracts target a common conserved molecular pathway not related to drug resistance.

DISCUSSION

The chances of finding novel antibiotics from natural products are higher compared to the synthetic modification approach mainly due to their property of not strictly adhering to Lipinski's Rule of Five (Miethke et al. 2021). However, the major snag with this approach is the repeated discovery of the same molecules (Cox et al. 2017). One method to increase the possibility of finding novel antibiotics among natural products is to screen microbes including fungi of extreme habitats (Schneider 2021, Suryanarayanan et Sasse 2021). Antibiosis may not be a major competing method for resources among microbes of extreme environments due to decreased species richness. However, extremophilic fungi occupying hypersaline, acidic or alkaline, high- or low-temperature environments produce several novel metabolites including those with potential antibiotic properties (Chávez et al. 2015, Daletos et al. 2018, Jin et al. 2018, Baranova et al. 2020). In their review on extremophilic fungi, Zhang et al. (2018) state that of the 314 novel metabolites recorded to be produced by piezophilic, psychrophilic, thermophilic, halophilic, xerophilic, acidophilic, and alkaliphilic fungi, 161 are bioactive compounds.

Our results that *Curvularia nodosa* and *Fusarium* cf. *foetens* from solar salterns produce metabolites which inhibit the growth of several *Candida* species underscore the importance of screening fungi of extreme habitats for novel antibiotics. Hypersaline environments including solar salterns are known to harbour halophilic as well as halotolerant fungi (Azpiazu-Muniozguren et al. 2021). The fungi which we screened were halotolerant and not halophilic as they could grow on media with and without NaCl. Thus, our study suggests that, apart from halophilic fungi, halotolerant fungi should also be screened for novel chemicals; this is in accordance with findings by Zheng et al. (2013). Furthermore, halotolerant fungi should be screened for active compounds by culturing them in different salt concentrations since isozyme production in these fungi is controlled by external salt concentration (Paranetharan et al. 2018). Machine-learning analysis of the data set shows that the production of bioactive secondary metabolites in halophilic fungi could be influenced by the external NaCl concentration (Jančič et al. 2016).

It is possible that the antibiotic effect of *C. nodosa* and *F.* cf. *foetens* observed in the present study are due to already known compounds, since species of *Curvularia* (Zhang et al. 2011, Yin et al. 2018, Kaaniche et al. 2019, Polli et al. 2021) and the *F. oxysporum* complex (Pusztahelyi et al. 2015, Ibrahim et al. 2021) produce many novel secondary metabolites including antifungal compounds. Although our study is preliminary and needs further intensive investigations to identify the active molecules, it shows that bioprospecting not only extremophilic fungi but also extremotolerant ones for potentially novel antibiotics is important.

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