ORGINAL RESEARCH article

Fungicidal action of endophytic fungi, obtained from *Justicia carnea*, against multidrug-resistant *Candida albicans*

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Abstract: *Candida albicans* causes high morbidity and mortality and is becoming a danger to public health. The problem created by its high occurrence and the treatment failures cannot be overstated. Endophytes derived from some medicinal plants serve as a new source of drug discovery. This study is aimed at evaluating the fungicidal potential of endophytic fungi obtained from *Justicia carnea* against multidrug-resistant *Candida albicans*. *Candida albicans* were obtained from clinical samples at Nnamdi Azikiwe Teaching Hospital in Anambra State, Nigeria. The susceptibility study to isolate multi-drug-resistant *Candida albicans* with antifungal agents was determined using the Kirby-Bauer technique. The isolation and extraction of fungal metabolites were carried out. The fungicidal activity of the metabolites against multi-drug-resistant *Candida albicans* was studied using *in vitro* method. Molecular characterization of endophytic was carried up to the species level. The findings have established that *Candida albicans* species are becoming resistant to fluconazole, followed by miconazole, within the environment. The leaves of *Justicia carnea* produced a high yield of secondary metabolites. These metabolites have significant antifungal effects against the isolates of multi-drug-resistant *Candida albicans* up to a concentration of 18.8 mg/ml. The DNA sequence of the endophytic fungi isolate is the same as *Sordariomycetes sp*. This study indicates that *Justicia carnea* harbors endophytic fungi with biosynthetic capacities for a new bioactive agent.

Introduction

Candida albicans is a versatile and opportunistic fungus that normally exists in the human microbiome without causing harm [1]. However, under specific conditions, it can lead to various infections, from minor skin issues to severe systemic infections [2-4]. It causes high morbidity and mortality globally [5-7] and is becoming a serious threat to public health [8]. *Candida albican* is becoming resistant to many antifungals [3, 9, 10], and the obtainability of antifungal drugs to treat people with candida infections is inadequate [11]. The problem created by the rising occurrence of *Candida albican* and failures in the treatment of its infections cannot be overestimated. As such, there is a need for the development of an alternative therapy that is easily accessible to support the antifungal agent. In a continuous search of new products, a study of endophytic fungi isolated from *Justicia carnea,* a flamingo plant, was carried out. This study is aimed at evaluating the fungicidal potential of endophytic fungi gotten from *Justicia carnea* against multidrug-resistant *candida albicans*.

Materials and methods

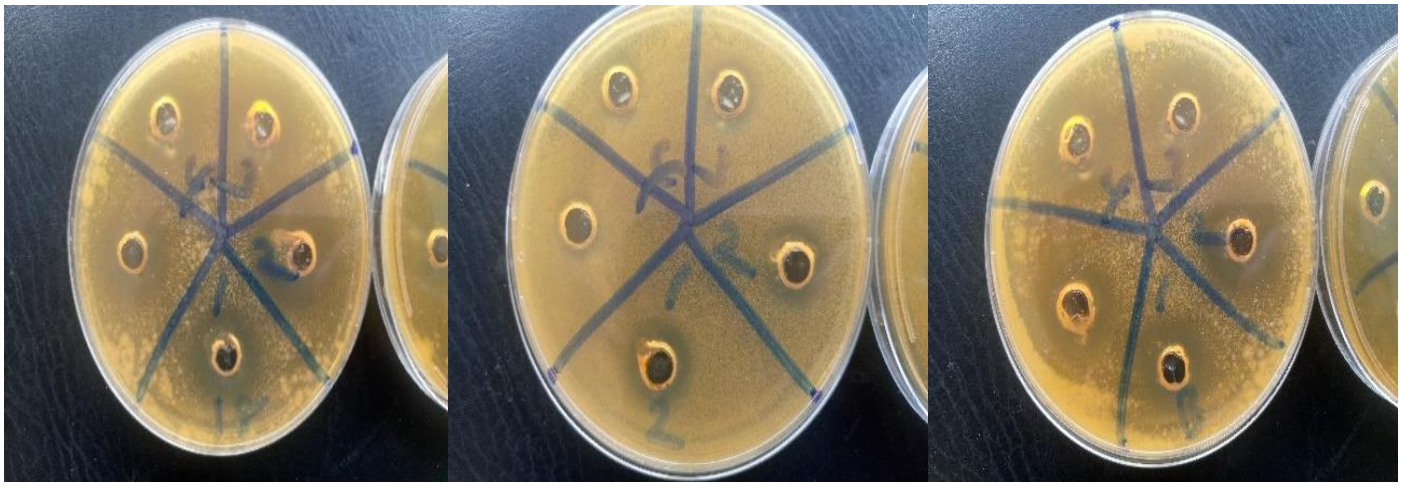
Plant material: Fresh and healthy leaves of *Justicia carnea* were harvested in February 2022 from the botanical garden of the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu campus, Anambra State, South-Eastern Nigeria. The leaves were identified and authenticated by a plant taxonomist at the Department of Pharmacognosy and Traditional Medicine in the same University.

Culture media and drugs: Sabouraud dextrose agar (SDA), potato dextrose agar and sabouraud dextrose broth (Titan Biotech. Limited, India) were the culture media used. These media were prepared according to the manufacturer's instructions. Miconazole (2.0 mg), fluconazole (2.0 mg) and chloramphenicol (500 mg/L) (Titan Biotech. Ltd; India) were the antifungal and antibacterial agents used.

Test organism: A total of 20 isolates of *Candida albicans* were obtained from clinical samples at Nnamdi Azikiwe Teaching Hospital in Anambra State. An approval for these samples was obtained from the hospital Committee. The isolates were obtained from high vaginal swabs. Each of these strains were reconfirmed by macroscopy, microscopy and biochemical test, such as sugar utilization test/Glucose test and Germ tube test [12-14] and stored in Sabouraud dextrose broth at 25ºC.

Isolation of multi-drug-resistant Candida albicans: The susceptibility study of the *Candida albicans* isolates with the antifungal agents was determined using the Kirby-Bauer technique as follows. 0.1 ml of standardized *Candida albicans* cultures was diluted with distilled water to get the turbidity match of 0.5 McFarland standards and dispersed evenly into SDA plates using a sterile swab stick to make a lawn. Inoculated plates were allowed to dry. A sterile cork borer was used to bore five wells of size 8.0 mm in the plates. Using a micropipette, 80 µl of each concentration was put into the wells and the plates were allowed for a period of 30 min and then incubated at 25°C for two days. The zone of inhibition was observed, and the diameter of the zone was measured and recorded **(Figure 1)**. The organisms that showed resistance to the antifungal agents were isolated as MDR *Candida albicans* [15].

Figure 1: Inhibition zones produced by *Sordariomycetes spp* crude extract against *Candida spp.*



A **B** **C**

Key: A: Isolate 1; B: Isolate 2; C: Isolate 3

Isolation of endophytic fungi: The leaves of *Justicia carnea* were washed with water, disinfected with 2.5% sodium hypochlorite and 70.0% ethanol. They were aseptically cut to 2.0 cm and inoculated onto sterile PDA plates containing chloramphenicol. These plates were incubated for five days at 25ºC while observing the development of mycelium. Isolation of pure cultures was attained by continuous sub-culturing of isolates on fresh PDA. Colonial/morphological characteristics of the fungal isolates were carried out by observing the colony texture, color and pigmentation [16, 17].

Extraction of metabolites: Each pure fungal isolate was grown in 1.0 L Erlenmeyer flasks with sterilized rice medium, previously autoclaved for one hour at 121ºC at 15 psi [13]. The fermentation flasks were sealed and incubated under static conditions for 21 days at 28°C. Extraction of the fungal metabolites was attained using ethyl acetate. The filtrates were concentrated by evaporating the solvent at 40ºC using a rotary evaporator.

Determination of fungicidal activity: The antifungal effects of the endophytic fungal extracts were tested *in vitro* against a test culture of multidrug-resistant *Candida albicans* as follows. The suspensions of test organisms were adjusted to 0.5 McFarland turbidity standards and inoculated onto previously sterile SDA plates using sterile cotton swabs. A sterile cork borer was used to make five wells (8.0 mm in diameter) on each of the SDA plates. Aliquots of 80 µl of each dilution of the extract reconstituted in DMSO at different concentrations were put in each of the wells. Fluconazole (100 mg/mL) served as the positive control. The plates were then incubated at 25ºC for 48 hrs. The antimicrobial potential for each extract was determined by measuring the zone of inhibition around each well. The assay was carried out in duplicates and the mean IZD was used [18].

Isolation of DNA and polymerase chain reaction (PCR): DNA isolation and amplification was done to characterize the endophytic fungi up to the species level. The genomic DNA of endophytic fungi was extracted and quantified using Quick-DNATM Fungal/Bacterial Miniprep Kit (Zymo Research), as laid-out in the endorsed protocols with minor modification. The PCR amplifications were undertaken in a 25.0 µl reaction volume (reaction mixture) comprising of 12.5 µl of One Taq Quick-Load 2X Master Mix with standard buffer, 0.5 µl each of forward and reverse primers, 9.0 µl of Nuclease free water and 3.0 µl es of DNA template. The reaction was gently mixed and transferred to a thermal cycler. The PCR cycling conditions were in the order: Initial denaturation at 94ºC lasted for 30 sec, followed by 35 cycles of denaturation at 94ºC for 20 sec, primer annealing at 54ºC for 45 sec and strand extension at 72ºC for one minute. Final extension at 72ºC for five min on an Eppendorf Nexus gradient Mastercycler. PCR products were separated on a 1.5% agarose gel and DNA bands were visualized with ethidium bromide [15].

Sequencing: PCR products were cleaned using EXOSAP protocol whereby EXOSAP mix was prepared by the addition of Exonuclease 1 (20.0 U/µl 50.0 µl) and shrimp alkaline phosphatase (1.0 U/µl 200 µl). Amplified PCR product 10.0 µl EXOSAP and 2.5 µl were mixed and incubated at 37ºC for 15 min. The reaction was stopped by heating the mixture at 80ºC for 15 min. Fragments were sequenced using Nimagen, Brilliant Dye Terminator cycle sequencing kit, according to the manufacturer's instructions.

Results

Susceptibility study of the isolates with antifungal agents: The sensitivity of *Candida albicans* to these two standard drugs (Fluconazole and Miconazole) revealed that the drugs have no activity on most of the isolates, indicating the resistance of the organisms to these drugs (**Tables 1** and **2**).

Table 1: Inhibition zone diameter of miconazole against isolates of *Candida albicans*

Concentration (µg/mL)	Isolates						
	1	2	3	4	5	6	7
300	8±0	4±0	0±0	0±0	0±0	0±0	0±0
150	5±0	0±0	0±0	0±0	0±0	0±0	0±0
75	0±0	0±0	0±0	0±0	0±0	0±0	0±0
37.5	0±0	0±0	0±0	0±0	0±0	0±0	0±0
18.8	0±0	0±0	0±0	0±0	0±0	0±0	0±0

Table 2: Inhibition zone diameter of fluconazole against isolates of *Candida albicans*

Concentration (µg/mL)	Isolates						
	1	2	3	4	5	6	7
300	0±0	0±0	0±0	0±0	0±0	0±0	0±0
150	0±0	0±0	0±0	0±0	0±0	0±0	0±0
75	0±0	0±0	0±0	0±0	0±0	0±0	0±0
37.5	0±0	0±0	0±0	0±0	0±0	0±0	0±0
18.8	0±0	0±0	0±0	0±0	0±0	0±0	0±0

Extraction of metabolites: The colonial features and yield of endophytic fungi isolated from the leaf blade of *Justicia carnea* is presented in **Table 3**.

Table 3: Colonial features and yield of fungal extracts

Isolate code	Colour	Texture	Pigment	Yield (g)
PLB	White and light green	Cottony	No pigment	4.0

Key: PLB - Plant leaf blade

Determination of fungicidal activity: The antifungal assay results obtained exhibited that the extract has activity on most of the isolates based on their concentrations (**Table 4** and **Figure 2**).

Table 4: Inhibition zone diameter of the extract against *Candida albicans*

Concentration (mg/mL)	Isolates					
	1	2	3	4	5	6
150	9±0	13±0.7	13±0	12±0	7±0	12±0.7
75	8±0	12.5±0	12±0.7	7±0	5.5±0.7	4.5±0.7
37.5	5.5±0	9±0.7	10±0	0±0	3.5±0.7	0±0
18.8	2.5±0	4.5±0.7	3.5±0.7	0±0	2.5±0	0±0
9.4	0±0	0±0	0±0	0±0	0±0	0±0
Fluconazole (100 mg/mL)	0±0	0±0	0±0	0±0	0±0	0±0

Sequencing: The result of the molecular characterization (**Table 5** and **Figure 2**) revealed that the endophytic fungus has a DNA sequence as same to *Sordariomycetes*.

Table 5: Molecular identification of endophytic fungus isolated from the leaf of *J. carnea*

DNA Sequence	Name of fungus	GenBank accession number
>UG1_ITS-1_C06_09 AACCCCATGTTGAACTTATCTCTTTGTTGCCTCGGCGCAAGCTACCC GGGACCTCGTGCCCCGGGCGGCCCGCCGGCGGACAAACCAAACTC TGTTATCTTCGTTGATTATCTGAGTGTCTTATTTAATAAGTCAAAACT TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCG AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT CTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGT TCGAGCGTCATTTCAACCCCTAAGCACAGCTTATTGTTGGGAATCCA CGCCTGTGGTTCCTCAAAGACATTGGCGGAGTGGCAGTAGTCCTCT GAGCGTAGTAATTCTTTATCTCGCTTTTGTTAGGTGCTGCCCCCCCG GCCGTAAAACCCCCAATTTTTTCTGGTTGACCTCGGATCAGGTAGG AATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA	*Sordariomycetes spp.*	KP306960.1

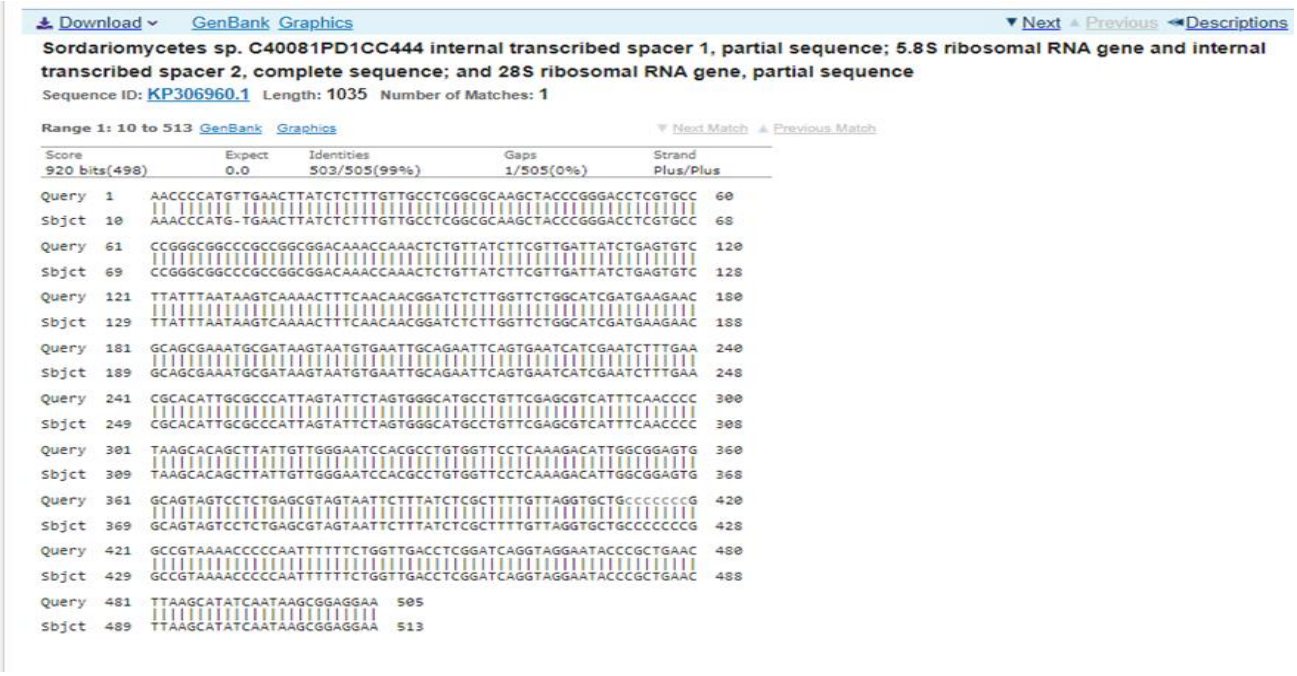


Figure 2: Molecular characterization of the endophyte as *Sordarimycetes sp*

Discussion

The findings of the current study provide compelling insights into the antifungal resistance patterns of *Candida albicans* and the potential of endophytic fungi from *Justicia carnea* as alternative therapeutic agents. The susceptibility profile revealed a concerning resistance of *Candida albicans* isolates to two commonly used antifungal agents-fluconazole and miconazole. This aligns with global reports of increasing antifungal resistance [19], particularly among immunocompromised patients and those with recurrent candidiasis. The lack of activity observed in most isolates suggests possible overuse or misuse of these agents in clinical settings, leading to selective pressure and resistance development. These findings underscore the urgent need for alternative antifungal strategies and routine susceptibility testing to guide effective treatment. The successful isolation of endophytic fungi from the leaf blade of *Justicia carnea*, as evidenced by distinct colonial features and metabolite yield, highlights the plant's potential as a reservoir of bioactive compounds. Endophytes are known to produce secondary metabolites that mimic or enhance the host plant's pharmacological properties. The antifungal assay demonstrated that the crude extract from the isolated endophyte exhibited significant fungicidal activity against most *Candida albicans* isolates, with efficacy dependent on concentration. This dose-dependent response suggests the presence of potent bioactive compounds within the extract. The ability of the extract to inhibit resistant strains further supports its therapeutic potential and warrants further purification and characterization of the active constituents. Molecular sequencing revealed that the isolated endophytic fungus shares a DNA sequence with members of the class *Sordariomycetes*. *Sordariomycetes* are one of the largest classes of ascomycota that comprises a highly diverse range of fungi. They include many important pathogens, as well as saprobes, endophytes, epiphytes, coprophilous and fungicolous, lichenized taxa. They are found in terrestrial, marine and freshwater habitats globally. They are commonly isolated as endophytes from various plants [20], and known for their rich biosynthetic capabilities, including the production of antimicrobial and antifungal compounds. The identification of *Sordariomycetes* as the source organism strengthens the hypothesis that the observed antifungal activity is linked to its metabolic profile.

Conclusion: This study highlights the resistance of *Candida albicans* to standard antifungals, the promising activity of *Justicia carnea*-derived endophytes, and suggests a valuable avenue for developing novel antifungal agents.

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