International Journal of Innovative Research and Advanced Studies (IJIRAS)
Volume 10 Issue 7, July 2023

ISSN: 2394-4404

Antifungal Activity Of Endophytic Fungi Isolated From *Justicia Carnea* On *Candida Albicans*

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Abstract: ***Candida infections remain an important cause of morbidity and mortality worldwide and pose a grave threat to public health. The trouble created by the rising incidence of failures in the treatment of this pathogen and the high cost cannot be overrated. Endophytes derived from some of these medicinal plants serve as excellent source of novel drug discovery. This work is aimed at assessing the antifungal potentials of endophytic fungi isolated from J. carnea against Candida albicans.***

Candida albicans were obtained from Dei Salvatorum Lab at Ekwulobia, Anambra State and cultured for isolation and purification. The isolation and colonial examination of fungal endophytes gotten from the leaf blade (LB) parts of the leaves was carried out. DNA isolation and amplification to characterize the endophytic fungi up to species level were carried out. The antimicrobial potentials of the fungal extracts were tested in vitro against test culture of C. albicans.

Our study has demonstrated that the leaves of Justicia carnea produced a high yield of secondary metabolites. The endophytic fungi isolated from this plant exhibited antifungal activity up to a concentration of 18.8 mg/ml against the isolates of C. albicans. Molecular identification of the endophytic fungi isolate showed its DNA sequence to bear high similarity to the Sordariomycetes spp.

This result strongly suggests that Justicia carnea harbors endophytic fungi with biosynthetic capacities for a new bioactive compound.

Keywords: Candida albicans, Endophytic fungi, J. carnea, Sordariomycetes.

I. INTRODUCTION

Candida albicans is an endogenous opportunistic fungal organism that grows in moist, warm, dark environments like the gastrointestinal and genitourinary tracts and has an obligate association with mammalian host (Gow and Yadav, 2017). It causes a range of superficial infections like urinary tract infection (UTI), vaginitis, oral and cutaneous candidiasis (Wachtler *et al*., 2012, Mayer *et al*., 2013), that cause a little discomfort, to a more severe and life-threatening systemic infections (Anejionu *et al*., 2012) like candidemia, endocarditis, endophthalmitis, pneumonia, septicemia,

meningitis, osteomyelitis and fungal arthritis. Candida infections remain an important cause of morbidity and mortality worldwide and pose a grave threat to public health (Pfaller *et al*., 2014; Matthaiou *et al*., 2015; Pappas *et al*., 2016). Up to 75% of women may be infected with this organism once in their lifetime minimum (Kabir *et al*., 2012). Although, it is most often seen in women during the childbearing ages, men, children, babies and even pets can suffer from candida infection (Pappers *et al*., 2000).

The availability of antifungal agents to treat patients with candida infections are limited (Andrade and Ribeiro, 2020) and the development of new antifungal drug is slow and costly (Terry and Damian, 2014). Candida strains are also becoming resistance to many antifungals (Prasad *et al*., 2019; Kabir *et al*., 2012; Bhattacharya *et al*., 2020). The trouble created by the rising incidence of failures in the treatment of this pathogen and the high cost cannot be overrated. As such, there is a need for the development of alternative therapy that is easily available and cheaper to support the antifungals.

Plants that have healing values have been considered for their endophytic fungal variety and for the production of distinct secondary metabolites with remarkable medicinal values (Talukdar *et al*., 2020, Okezie *et al*., 2023). In constant search for innovative products targeted against *C. albicans*, our study of endophytic fungi isolated from Flamingo plant (*Justicia carnea*) was conceptualized. This was based on evidence that has shown that *J. carnea* is used to treat cancer, epilepsy, malaria, HIV, sickle cell disease, inflammation, anemia, arthritis, tumor, whooping cough, liver, disease, typhoid, diabetes, hepatitis and bronchitis cold (Anaradol et al., 2021; Nduche et al., 2019; Oladele et al., 2016; Orjiakor et al., 2019). These study results thus strongly suggest that *Justicia carnea* harbors endophytic fungi with biosynthetic capacities for a new bioactive compound. Other therapeutic properties like its hypocholesterolemic, antioxidant, antiallergic, analgesic and antimicrobial potentials have also been reported (Orjiakor et al., 2019; Ukpabi-Ugo et al., 2019). The antimicrobial activity of *J. carnea* was expressed in the methanol, n-hexane, and ethylacetate extracts of the plant. The results showed concentration-dependent inhibition against *S.aureus*, Ba*cillus spp, Aspergillus spp. K. pneumonia, S. typhi* and *Candida albicans* were resistant in all concentrations of the extracts (Anaradol et al., 2021). However, there are no reports on the antifungal potentials of endophytic fungi isolated from *J. carnea* against *Candida albicans*.

Thus our study aims to identify the antifungal activity of the endophytic fungi isolated from *J. carnea* against *Candida albicans*.

II. MATERIALS AND METHOD

A. MATERIALS

a. TEST ORGANISM

Candida albicans previously isolated from 25 clinical samples at "Dei Salvatorum laboratory" located in Anambra, Southeast of Nigeria were used for the study. The clinical sample was vaginal discharge.

b. PLANT MATERIAL

Healthy leaves of *Justicia carnea* (Figure 1) were collected and taken to the laboratory. The leaves were identified and authenticated by a plant taxonomist at the Department of Pharmacognosy and Traditional Medicine, Nnamdi Azikiwe University, Awka, Nigeria.

Figure 1: Flamingo plant (Justicia carnea)

c. CULTURE MEDIA

The culture media used were Sabouraud Dextrose Agar (SDA), Sabouraud Dextrose Broth and Potato Dextrose Agar (Titan Biotech. Limited, India). Manufacturer's instructions were strictly followed during each of the media preparation.

d. DRUGS AND REAGENTS

Chloramphenicol (an antibacterial) was also used to prevent bacterial infection. Lactophenol cotton blue stain, Absolute ethanol (Sigma-Aldrich, Germany), Ethyl Acetate (JHD,China), Dimethyl sulphoxide (Sigma-Aldrich, Germany), Hypochlorite solution**,** Alcohol 70%**,** were the reagents used**.**

B. METHOD

a. ISOLATION AND CHARACTERIZATION OF TEST MICRO-ORGANISM

The isolates were inoculated onto SDA plates containing 0.03% w/v chloramphenicol and incubated at 25 ^{o}C for 48 h. Each of these isolates was reconfirmed by standard method (Abiroo *et al*., 2018) and stored in sabouraud dextrose broth at 25 ^{o}C. Before use, an aliquot of the test isolates were activated by sub culturing into sabouraud dextrose agar with chloramphenicol and then incubated for 48 h at 25 ^{o}C. For standardization of reactivated cultures, an overnight (18 hrs) subcultures in sabouraud's dextrose broth were adjusted to 90% transmittance at 530nm using distilled water (Anejionu, *et al*., 2012).

b. ISOLATION AND COLONIAL EXAMINATION OF ENDOPHYTIC FUNGI

The Leaves of *Justicia carnea* were thoroughly washed with running tap water for about 10 minutes, disinfected with 2.5 % Sodium hypochlorite, 70 % ethanol, and then distilled water. They were aseptically cut to about 2 cm and inoculated onto sterile Potato Dextrose Agar (PDA) plates containing 500 mg/L Chloramphenicol. These plates were incubated for 5 days at 25^{o}C while observing the development of mycelium. By continuous sub-culturing of isolates on fresh PDA, isolation of pure cultures was achieved. Examination of the colonial/morphological characteristics of the fungal isolates was carried out by observing the colony texture, color and pigmentation (Okezie *et al*., 2023; Senanayake *et al*., 2020).

c. FERMENTATION AND METABOLITES EXTRACTION

Each pure fungal isolate was grown in 1 L Erlenmeyer flasks containing sterilized rice medium, previously autoclaved at 121ºC at 15 psi for 1 h (Okezie *et al*., 2017). The fermentation flasks were properly sealed and incubated under static conditions at 28°C for 21 days. Extraction of biosynthesized fungal metabolites was achieved using 500 mL of ethyl acetate. The filtrates were concentrated by evaporating the solvent at 40ºC using a rotary evaporator.

d. DNA ISOLATION AND POLYMERASE CHAIN REACTION (PCR)

Genomic DNA was extracted using Quick-DNATM Fungal/ Bacterial Miniprep Kit; (Zymo Research), according to recommended protocols with slight modification.

Using a micropipette, 12.5 microlitres of One Taq Quick-Load 2X Master Mix with standard buffer (New England BiolabsInc); 0.5 microlitre each of forward and reverse primers; 8.5 microlitres of Nuclease free water and 3 microlitres of DNA template were used to prepare 25 microlitres reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to a thermal cycler. Amplification conditions for the PCR include Initial denaturation for 30secs at 94 degree Celsius, followed by 35 cycles of denaturation at 94^{o}**C** for 20secs, primer annealing at 54^{o}**C** for 45secs and strand extension at 72 ^{o}**C** for 1min.

Final extension at 72 ^{o}**C** for 5 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.

e. SEQUENCING

PCR products were cleaned using EXOSAP protocol whereby EXOSAP mix was prepared by addition of Exonuclease 1 (20U/ul 50ul) and Shrimp Alkaline Phosphatase (1U/ul 200ul).

Amplified PCR product 10 ul EXOSAP Mix 2.5 ul were mixed and incubated at 37 ^{o}**C** for 15mins. The reaction was stopped by heating the mixture at 80^{o}**C** for 15mins. Fragments were sequenced using Nimagen, Brilliant Dye Terminator cycle sequencing kit, according to manufacturer's instructions.

f. EVALUATION OF ENDOPHYTIC FUNGAL EXTRACT

The antimicrobial effects of the fungal extracts were tested *in vitro* against test culture of *C. albicans* (Okezie *et al*., 2017). Sterile Sabouraud dextrose agar plates were inoculated with 0.5 McFarland suspension of test organism using sterile cotton swabs. After inoculation, a volume of 80 µl of each dilutions of the extract reconstituted in DMSO at concentrations of 150, 75, 37.5, 18.8 and 9.4mg/mL were transferred into the wells made in the agar using 8mm sterilized cork borer and incubated at 25 ^{o}C for 48 h. DMSO served as the negative control. The assay was carried out in duplicates (Okezie *et al*., 2017).

III. RESULTS AND DISCUSSION

A. RESULTS

Figure 2 shows the isolates of *Candida albican* which appeared as white, creamy, convex and smooth colonies. The photomicroscopic images of the organism in Figure 3 showed that they are opaque cells that are spherical to oval in shape.

Figure 2: Picture showing the cultures of Candida albicans.

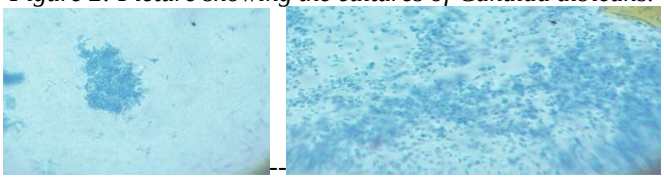

Figure 3: Photomicroscopic Images of Candida albicans

The endophytic fungi *FP-LB* (Flamingo plant-Leaf Blade) were isolated from the leaf blade of *Justicia carnea* leaf on PDA. Their colonial features and yield are presented in Figure 4 and Table 1 respectively.

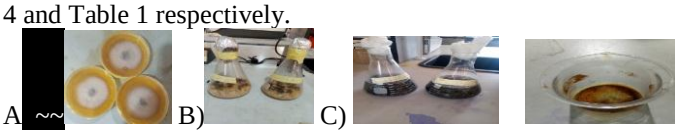

Figure 4: Colonial features and concentrated crude extracts of the isolated fungal endophytes
4A-Purification, 4B-Fermentation, and 4C-Extraction

Isolate code	COLOUR	TEXTURE	PIGMENT	Yield of fungal extract (g)
FP-LB	White and light green	Cottony	No pigment	4.20

Key: FP-LB = Flamingo plant leaf blade.

Table 1: Colonial features and Yield of fungal extracts

The result of the molecular characterization (Table 2) indicated that the endophytic fungi isolate has the same DNA sequence with *Sordariomycetes*.

DNA Sequence	Name of fungus	GenBank Accession number
>UG1_ITS-1_C06_09 AACCCCATGTTGAACTTATCTCTTTG TTGCCTCGGCGCAAGCTACCCGGGA CCTCGTGCCCCGGGCGGCCCGCCGG CGGACAAACCAAACTCTGTTATCTTC GTTGATTATCTGAGTGTCTTATTTAA TAAGTCAAAACTTTCAACAACGGAT CTCTTGGTTCTGGCATCGATGAAGA ACGCAGCGAAATGCGATAAGTAATG TGAATTGCAGAATTCAGTGAATCAT CGAATCTTTGAACGCACATTGCGCC CATTAGTATTCTAGTGGGCATGCCTG TTCGAGCGTCATTTCAACCCCTAAGC ACAGCTTATTGTTGGGAATCCACGC CTGTGGTTCCTCAAAGACATTGGCG GAGTGGCAGTAGTCCTCTGAGCGTA GTAATTCTTTATCTCGCTTTTGTTAG GTGCTGCCCCCCCGGCCGTAAAACC CCCAATTTTTTCTGGTTGACCTCGGA TCAGGTAGGAATACCCGCTGAACTT AAGCATATCAATAAGCGGAGGAA	*Sordariomycetes spp.*	KP306960.1

Table 2: Molecular Identification of Endophytic Fungus Isolated from the Leaf of J. carnea.

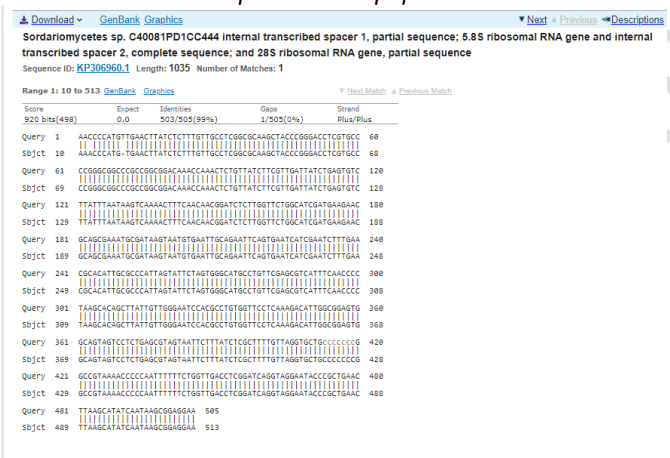

Figure 5: Image showing BLAST results of the sequenced endophytic fungi DNA

The antimicrobial effects of the fungal extracts results showed that the extracts have activity on majority of the isolates based on their concentrations (Table 3).

Concentration (mg/mL)	Isolates					
	1	2	3	4	5	6
150	9±0	13±1.4	13±1.4	4±0	5.5±0.7	4.5±0.7
75	7±0	12±0	12.5±0.7	3±0	4.5±0.7	3.5±0.7
37.5	5±0	9.5±0.7	10±1.4	0±0	2.5±0.7	0±0
18.8	0±0	6.5±0.7	4.5±0.7	0±0	0±0	0±0
9.4	0±0	0±0	0±0	0±0	0±0	0±0
DMSO	0±0	0±0	0±0	0±0	0±0	0±0

Table 3: Inhibition Zone Diameter of the Extract against Candida albicans

B. DISCUSSION

Isolates of *C. albicans* growing on SDA appear as white colonies that are creamy, convex, and have a smooth surface. This is consistent with the result of (Jabri *et al*., 2022; Lamichhane *et al*., 2015). Our finding suggests the strain as the most populous organisms in the clinical samples investigated. This is in agreement with the findings of Masri *et al* (2015) and Tseng *et al* (2005), which revealed that isolates of *C. albicans* were the highest *Candida* species isolated in vaginal candidiasis. The emergence and superiority of *C. albicans* over the rest of other species of candida is due to its aggressiveness, polymorphism and the ability to adhere to epithelial cell membranes in a high degree likened to other types (Mayer *et al*., 2013; Abirami *et al*., 2020).

Sordariomycetes is the second largest class of fungi in the subdivision Ascomycota. They are found in different niches of freshwater, marine and terrestrial habitats worldwide (Lee *et al*., 2019; Maharachchikumbura *et al*., 2016). Some of the species are endophytes and pathogens of various plants, while some cause diseases in mammals and arthropods (Lee *et al*., 2019; Maharachchikumbura *et al*., 2015; Hyde KD *et al*., 2016).

The antimicrobial effects showed by these fungal extracts against the test organism were observed to be concentration-dependent with the least and maximum inhibition zones observed to be 2 and 4.9 mm respectively. Hence, the extracts might be used to treat infections caused by *candida albicans.* This contradicts the work of Anarado *et al*., 2021 which stated that C. albicans and some bacteria such as *S. typhi*, *P. aerogenosa* and *K. pneumonia* were resistant to the extracts. The antifungal effect shown by these extracts is an indication that this species harbor endophytic fungi with biosynthetic capacities for a new bioactive compound.

IV. CONCLUSIONS

Our study has demonstrated that the leaves of *Justicia carnea* produced a high yield of secondary metabolites. The endophytic fungi isolated from *J. carnea* contain interesting and remarkable antifungal activities against the isolates of *C. albicans*. The endophytic fungi isolate has almost the same DNA sequence with *Sordariomycetes* as indicated in the molecular characterization.

ACKNOWLEDGMENTS

The authors are thankful to the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka, Anambra State, Nigeria.

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