In Vitro Assessment of Conventional and Plant-derived Antifungal Agents against *Candida* Species Prevalence

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ABSTRACT

We investigated the *in vitro* assessment of conventional and plant-derived antifungal agents against *Candida* species prevalence among pregnant women in Southeastern Nigeria. A total of 300 non-duplicate clinical samples from pregnant women were processed using the standard microbiological protocol for isolation and characterization of *Candida* species on CHROM agar *Candida*. The antifungal susceptibility testing (AST) profile was performed using the Vitek 2 system. The plant-derived antifungal susceptibility testing with *Cocos nucifera* oil was performed using the agar-well diffusion method. The result of isolation shows that the most prevalent *Candida* species was *C. albicans* (41.0%), followed by *C. glabrata* (23.0%), *C. krusei* (14.0%), *C. tropicalis* (12.0%), and *C. dubliniensis* (8.0%). The antifungal susceptibility profile revealed that the *Candida* species were highly susceptible to voriconazole within the range of 82.6-100% but were extremely resistant to Nystatin 100%, micafungin 100%, and fluconazole 75.0-100%. The plant-derived antifungal susceptibility patterns of *Cocos nuficera* oil revealed that all the *Candida* species were 100% susceptible to *Cocos nucifera* oil at 100 mg/ml, 50 mg/ml, and 25 mg/ml (1:0, 1:2, and 1:4 dilutions) concentrations. Our findings have indicated that *Cocos nuficera* oil can serve as an alternative to contemporary antifungal agents if properly harnessed for *in vivo* utilization. Also, an agent such as voriconazole appeared as a better option for the management of *Candida* infection, *in vitro* susceptibility assay of another conventional antifungal agent for empirical antifungal treatment should rely on the outcomes of this study and antifungal susceptibility testing.

Key Words: *Candida species, Conventional, Plant-derived, Antifungal agents, Cocos nucifera oil*

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INTRODUCTION

Candida species are currently the most prevalent etiologic agent of fungal infections. Depending on the immunocompetence of the host, the infection may range from trivial or mild infection or systemic to mucocutaneous infection such as vulvovaginal, oropharyngeal, and genitourinary candidiasis [1]. Mucocutaneous infections are one of the clear indications of cell-mediated immunodeficiency, which may predictively enhance the occurrence of more than 90% of invasive candidiasis [1]. Approximately 20–50% of the vaginal microflora constitute *Candida* species [2] while 90% of infections [3] are caused by a few *Candida* species namely; *C. albicans*, *C. glabrata*, *C. auris*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis* [4, 5]. The pathogenesis of fungal infection by *Candida* species is associated with virulence factors such as yeast-to-hyphal transformation, hydrolytic enzyme production, biofilm development, expression of adhesion, and invasion proteins [2]. However, hemolytic enzymes and hydrolytic enzymes namely lipases, phospholipases produced by the *Candida* species contribute to its virulence while the secreted aspartyl proteinases play a role in the attachment, penetration, and invasion of host tissues, inducing tissue damage, thereby aiding the establishment of infection [2, 6]. As this opportunistic pathogen inhabits the genital tract, it has been reported that there's a higher incidence of *Candida* infection in pregnant women than in non-pregnant women [2, 7, 8]. As pregnancy is often a risk factor that disturbs the urogenital microflora, it enables the occurrence of vaginal infection due to an imbalance of hormonal secretion [9]. Also, the use of antibiotics, diabetes mellitus, and host behavioral-associated factors such as poor personal hygiene, consumption of oral contraceptives, and sexual intercourse enhance the establishment of *Candida* infection [10]. *Candida* species represents the most frequent yeast infections affecting pregnant women. Due to persistent, recurrent, and complicated incidences of *Candida* infection, the efficacy of most antifungal agents has been eroded. Thus, despite the availability of three main effective conventional antifungal agents such as azoles, polyenes, and echinocandins, *Candida* species infection remains a complicated infection to manage because of the emerging drug resistance and the recurrent character of the disease [11]. However, evidence of the increasing prevalence of *Candida* species resistance is also reported in antifungal surveillance studies globally [12-14]. As the looming threat of clinical antifungal tolerance or resistance persists, susceptibility tests remain essential for the screening and selection of conventional antifungal agents [3, 6, 15], it is important to look into plant-derived antifungal agents such as *Cocos nucifera* oil to curtail the scourge of antifungal resistant. *Cocos nucifera* oil also known as Coconut oil is a type of tropical oil that has been used for centuries in traditional diets and remedies. This plant-derived agent has been reported as an immune booster and also possesses anti-microbial, antioxidant, and anti-inflammatory properties. Presently, *Cocos nucifera* oil has been reported to possess antifungal, antibacterial, and antiviral properties [16] owing to its unique phytochemical component. In recent times, numerous plant-derived compounds have been on the increase for their promising antimicrobial activities linked to the existence of natural products in plants with medicinal properties [17, 18]. The hunt for plant-derived antifungal agents as alternative therapeutic against antifungal-resistant isolates that cause illnesses is critical, especially given the rise in *Candida* species' reduced susceptibility to routinely used conventional antifungal agents. Thus, there is a need for assessment of empirical conventional and plant-derived antifungal agent sensitivity data in Enugu Southeastern Nigeria, which may differ for every geographic region but significantly plays a crucial role in appropriate and effective management strategies.

MATERIALS AND METHODS

Clinical sample collection, isolation, and conventional antifungal susceptibility testing of candida species

Before the study, the approval for the study was obtained from the medical research and ethical committee of the University of Nigeria Teaching Hospital (UNTH), Ituku Ozalla, Enugu, Nigeria located at 6°18'05.0" N latitude and 7°27'34.9" E longitude. The study was done according to principles guiding human research or data. A total of 300 non-duplicate mid-stream urine {100}, endo-cervical swab {100}, and high virginal swab samples {100} were collected within the period of 9 months (August 2022-April 2023) from pregnant women attending UNTH, Ituku Ozalla, Enugu, Southeastern, Nigeria. All samples were processed following the standard microbiological protocol for the isolation of *Candida* species on CHROM agar *Candida* (BioMerieux, France). A typical colonial appearance of creamy, leaf green, pale pink, deep green, and metallic blue pigmentation on CHROM agar *Candida* (BioMerieux, France) phenotypically infers the presence of *C. glabrata*, *C. albicans*, *C. krusei*, *C. dubliniensis*, and *C. tropicalis*, respectively. *Candida* species were further confirmed using the Vitek 2 system (BioMerieux, France). The Antifungal Susceptibility Testing (AST) profile was carried out using the Vitek 2 system (BioMerieux, France). The antifungal susceptibility testing panel comprises five conventional antifungal agents namely; voriconazole, amphotericin B, micafungin, fluconazole, and nystatin.

The results were recorded as recommended by the Clinical and Laboratory Standards Institute (CLSI) performance guideline as resistant (R) and susceptible (S) [14, 19].

Processing of plant-derived antifungal agent

The processing of plant-derived antifungal agents; *Cocos nucifera oil* was performed according to Orji *et al.* [16]. Briefly, a fresh *Cocos nucifera* meat peel from the fibrous husk was sliced, blended, and pressed using a sterile sieve to obtain the *Cocos nucifera* milk. The milk was kept at room temperature to undergo fermentation for 48 hours. Thereafter, the oil was separated from the solids and the water content. The moisture content was removed by heating the oil at 50 °C and was then filtered with a filter (Savin, Nigeria Limited) to obtain a pure extract of *Cocos nucifera* oil [16]. The extract of *Cocos nucifera* oil was preserved in a sterile vial at 4 °C until further use [16]. A sterility test was performed by pouring 1ml of the extracted oil on brain-heart Infusion agar (bioMérieux, France) plates and incubating overnight at 37 °C. The absence of microbial growth was phenotypically confirmed after overnight incubation.

Plant-derived antifungal susceptibility testing

The plant-derived antifungal susceptibility testing was performed using the agar-well diffusion method as outlined by Peter *et al.* [17]. In brief, overnight culture of the test *Candida* species suspension equivalent to $1x10^6$ colony forming unit per milliliter (cfu/ml) were adjusted to 0.5 MacFarland turbidity standard and were spread over the entire surface of solidified Mueller-Hinton agar (Merck Co., Germany) plates using a sterile cotton-tipped swab stick. The *Cocos nucifera* oil extract concentration of 100 mg/ml (undiluted), 50 mg/ml, and 25 mg/ml was filled in three agar wells made on each of the Petri dishes using a 6cm cork borer and incubated at 37 ^oC for 24 hours. After overnight incubation, the clear zones of inhibition were measured and recorded in millimeters (mm) [17].

RESULTS AND DISCUSSION

The data revealed the presence of five *Candida* species namely *C. albicans, C. tropicalis, C. dubliniensis, C. krusei,* and *C. glabrata*. *Candida* species accounted overall prevalence of 98 (32.7%) among pregnant women. The most prevalent *Candida* species was *C. albicans* (41.0%), followed by *C. glabrata* (23.0%), *Candida krusei* (14.0%), *Candida tropicalis* (12.0%), and *C. dubliniensis* (8.0%) as shown in **Table 1**. *Candida* species were more predominant in HVS (43.0%) over ECS (33.0%) and Mid-stream Urine (22.0%) **(Table 1)**. We investigate the antifungal susceptibility profile of *Candida* species **(Table 2)**. All *Candida* species were susceptible to voriconazole within the range of 82.6-100%, *C. dubliniensis* and *C. glabrata* were 75.0% and 100% susceptible to amphotericin B while low susceptibility to amphotericin B 7.1% was recorded against *C. kruesi*. Only *C. krusei* and *C. glabrata* were susceptible to micafungin recording 62.5% and 100%, respectively, while *C. albicans, C. tropicalis,* and *C. dubliniensis* were extremely resistant to micafungin recording 100%. *C. albicans*, *C. kruesi*, and *C. dubliniensis* were susceptible to fluconazole at 70.7%, 64.3%, and 25.0%, respectively, while *C. tropicalis* (100%), *C. glabrata* (100%), and *C. dubliniensis* (75.0%) resistant was displayed against fluconazole. All *Candida* species were extremely resistant to Nystatin recording 100% each. In **Table 3**, the plant-derived antifungal susceptibility patterns of *Cocos nuficera* oil were also studied using the agar-well diffusion technique; all the *Candida* species were 100% susceptible to *Cocos nucifera* oil at 100 mg/ml, 50 mg/ml, and 25 mg/ml (1:0, 1:2, and 1:4 dilutions) concentrations.

Table 1. Percentage occurrence of isolated *Candida* species from clinical specimens

	No. Sampled	*C. albicans* (%)	*C. tropicalis* (%)	*C. dubliniensis* (%)	*C. kruesi* (%)	*C. glabrata* (%)	Occurrence (%)
Mid-stream Urine	100	6 (6.0)	2 (2.0)	3 (3.0)	0 (0.0)	11 (11.0)	**22 (22.0)**
HVS	100	20 (20.0)	7 (7.0)	5 (5.0)	8 (8.0)	3 (3.0)	**43 (43.0)**
ECS	100	15 (15.0)	3 (3.0)	0 (0.0)	6 (6.0)	9 (9.0)	**33 (33.0)**
Total	**300**	**41 (41.0)**	**12 (12.0)**	**8 (8.0)**	**14 (14.0)**	**23 (23.0)**	**98 (32.7)**

Key: HVS-High Vaginal swabs, ECS-Endo Cervical swabs

Table 2. Conventional antifungal agent susceptibility profile of *Candida* species from clinical specimens

	Voriconazole		Amphotericin B		Micafungin		Fluconazole		Nystatin	
	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
C. albicans (n_o = 41)	41 (100)	0 (0.0)	17 (41.5)	24 (58.5)	0 (0.0)	41 (100)	29 (70.7)	12 (29.3)	0 (0.0)	41 (100)
C. tropicalis (n_o = 12)	11 (91.7)	1 (8.3)	0 (0.0)	12 (100)	0 (0.0)	12 (100)	0 (0.0)	12 (100)	0 (0.0)	12 (100)
C. dubliniensis (n_o = 8)	8 (100)	0 (0.0)	6 (75.0)	2 (25.0)	0 (0.0)	8 (100)	2 (25.0)	6 (75.0)	0 (0.0)	8 (100)

C. krusei (n_o = 14)	14 (100)	0 (0.0)	1 (7.1)	13 (92.9)	5 (62.5)	3 (37.5)	9 (64.3)	5 (35.7)	0 (0.0)	14 (100)
C. glabrata (n_o = 23)	19 (82.6)	4 (17.4)	23 (100)	0 (0.0)	23 (100)	0 (0.0)	0 (0.0)	23 (100)	0 (0.0)	23 (100)

Key: R-Susceptible, R-Resistance

Table 3. Plant-derived *Cocos nuficera* oil antifungal activity

Ratio	**1:0**		**1:2**		**1:4**	
Concentration	**100 mg/ml (undiluted)**		**50 mg/ml**		**25 mg/ml**	
Candida Species	**S (%)**	**R (%)**	**S (%)**	**R (%)**	**S (%)**	**R (%)**
C. albicans (n_o = 41)	41 (100)	0 (0.0)	41 (100)	0 (0.0)	41 (100)	0 (0.0)
C. tropicalis (n_o = 12)	12 (100)	0 (0.0)	12 (100)	0 (0.0)	12 (100)	0 (0.0)
C. dubliniensis (n_o = 8)	8 (100)	0 (0.0)	8 (100)	0 (0.0)	8 (100)	0 (0.0)
C. glabrata (n_o = 23)	23 (100)	0 (0.0)	23 (100)	0 (0.0)	23 (100)	0 (0.0)
C. krusei (n_o = 14)	14 (100)	0 (0.0)	14 (100)	0 (0.0)	14 (100)	0 (0.0)

Key: R-Susceptible, R-Resistance

C. albicans (41.0%) was the most prevalent *Candida* species found in the clinical samples of pregnant women in our study. Our observations corroborate with the published report in two earlier studies in Abakaliki South Eastern Nigeria [16, 20], Ethiopia, India, Iran, and Senegal [1, 13, 14, 21]. The high prevalence of *C. albicans* in our study clearly shows their dimorphic, invasiveness, and in-dwelling characteristics in catheter medical devices. Also, hormonal imbalance during pregnancy may increase their successful proliferation and colonization of the host. Earlier published findings show that the growth and adherence of *C. albicans* to the urogenital epithelium is enhanced by an elevated level of estrogen which may increase the risk of vaginal candidiasis. *C. albicans* is the most predominant species that inhabit and causes genital thrush in women [22].

However, the progressive alteration in the prevalence of *C. albicans* over the last decade has enhanced the occurrence of *C. dubliniensis, C. tropicalis C. glabrata,* and *C. kruesi* often described as non-albicans *Candida* species (NAC) [23]. Notably, non-albicans *Candida* species have been implicated to be the common cause of opportunistic fungal infection [1, 2] with increased significant morbidity among immune-compromised patients. Excessive and indiscriminate broad-spectrum antifungal usage, emerging viral diseases, and metabolic disorders are the mainstay for an increase in the occurrence of opportunistic fungal infectious diseases [2, 21].

In our findings, the conventional antifungal agent susceptibility features of *Candida* isolates were studied and *C. krusei* emerged with a 64.3% level of susceptibility to the azole group (fluconazole), which is similar to the result of Elfeky *et al*. [24] who published that 60% of the species were susceptible to the fluconazole but in contrast, *C. krusei* 100% susceptibility to fluconazole has been reported in Ethiopia [1] and elsewhere [25]. Resistant to azole antifungals such as the fluconazole in our study indicate enhanced progression of susceptible to naturally resistant species of *C. tropicalis, C. tropicalis, C. dubliniensis*, and *C. glabrata* due to the excessive use of azole (fluconazole) as a standard agent in the management of yeast infection.

Voriconazole was highly effective against *Candida* species. All *Candida* species were susceptible to the Voriconazole at 100% except two species of *C. glabrata* at 82.6% and *C. tropicalis* at 91.7%. However, the azole antifungal agents are designed for the inhibition of ergosterol synthesis but the variation between the degree of susceptibility to voriconazole and fluconazole against *Candida* species in our study remains unclear. However, our findings opined this discrepancy to excessive and indiscriminate use of fluconazole over time in the treatment of severe invasive and non-life threatening *Candida* infections.

C. glabrata and *C. krusei* were the only *Candida* species susceptible to micafungin at 62.5% and 100%, respectively. Micafungins are designed to inhibit the enzyme that synthesizes β-glucan essential in fungal cell wall synthesis. The resistance demonstrated against this agent by *C. tropicalis, C. dubliniensis*, and *C. albicans* is in contrast with the findings of Seck and colleagues [21] in Dakar Senegal, and also Umamaheshwari and Sumana [14] in southern India where all *Candida* species were 100% susceptible to micafungin. This observation strongly portrays an alarming trend in the epidemiological surveillance of antifungal resistance between different geographical regions. The resistant species may possess virulence determinants such as efflux pumps, that aid in the movement or transportation of antifungal agents out of the fungal cell membrane. This mechanism is part of human cells but is also expressed by yeasts [8] and also sequestration of antifungal agents can be a contributing factor.

C. glabrata (100%), *C. dubliniensis* (75.0%), and *C. albicans* (41.5%) susceptibility to amphotericin B could be linked to the agent's respective size, hydrophobicity, and no interaction or possession of substrate for the efflux pump.

In our result, we observed a reduced susceptibility of *Candida* species to nystatin, micafungin, amphotericin B, and fluconazole. We could correlate this finding with their broad availability for both enteral and parenteral administration and the low cost of the antifungal agent which could serve as the pointer to the looming threat of emerging antifungal resistance that will gradually become a worldwide crisis.

Candida species were 100% susceptible to *Cocos nuficera* oil at 25 mg/ml, 50 mg/ml, and 100 mg/ml (1:4, 1:2, and 1:0 dilutions) concentrations. This plant-derived antifungal agent showed promising *in vitro* antifungal activity against the isolate. Although, the mechanism of action of this plant-derived antifungal agent remains unclear few published studies have attributed its efficacy to the presence of ample quantity of Saturated Aliphatic Monocarboxylic Acid (SAMA) such as palmitic acid, caproic acid, oleic acid, lauric acid, caprylic, etc. [16]. These SAMAs are fatty acids with distinct fungicidal modes of action reported against *C. albicans* [16]. The SAMAs insertion into the fungal bilayer is capable of cell membrane disruption resulting in an increased osmotic gradient causing generalized and indefinite cell disintegration [16]. Our findings have indicated that *Cocos nuficera* oil is a good antifungal agent if properly harnessed for *in vivo* utilization.

CONCLUSION

This study gives an outline of *Candida* species susceptibility to the conventional and plant-derived antifungal agents as voriconazole appeared as a better option for the management of *Candida* infection in this study while *in vitro* susceptibility assay of another conventional antifungal agent such as micafungin, amphotericin B was not distinctive among certain species, their selection for empirical antifungal treatment should rely on the outcomes of this study. Also, *Cocos nuficera* oil plant-derived antifungal agents were effective on *Candida* species. Against this backdrop, further *in vivo* pharmacological studies need proper quantification and application as a veritable alternative to contemporary medicine for *Candida* infection.

There is a paucity of molecular surveillance on antifungal resistance, more studies are needed on molecular and genotyping screening of antifungal resistance pathways that will support the synthesis of novel antifungal agents that will avert antifungal resistance. Guidelines in health care should be geared toward antifungal stewardship programs, prevention, and control of nosocomial and drug resistance spread.

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