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Insights into the Prevalence and Resistance Patterns of *Candida* Species among Diabetic Patients in Cameroon: Potential Paths for Alternative Treatments

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Abstract The study aimed to fill the knowledge gap regarding the prevalence of *Candida* infections in diabetic patients and their sensitivity to antifungal drugs. Clinical samples from diabetic patients were collected at Yaoundé Central Hospital and *Candida* species identified. The ability of these species to form biofilms was assessed, and their susceptibility to usual antifungal drugs, as well as some plant extracts and fractions, was determined. Mycological diagnosis was performed on the collected samples. The *Candida* species isolated and identified were subjected to the crystal violet assay in a microtiter plate to examine their biofilm-forming abilities. The disk diffusion method was used for susceptibility testing for reference antifungals, while the broth dilution method was employed for plant extracts and fractions. These were obtained from the leaves of *Picralima nitida*, *Phragmanthera capitata*, and *Spondia cythereae*, and the roots and fruits of *Garcinia Kola*. The study found that 43.18% of the 396 collected samples were colonized by various *Candida* species, including *Candida albicans, Candida tropicalis, Candida parapsilosis, Candida glabrata, Candida krusei, Candida guillermondii, Candida lusitaniae*, and *Candida ciferrii*. Biofilm production was observed in 46.04% of the isolates, with non-*albicans Candida* species showing a higher rate than *Candida albicans*. The isolates exhibited high resistance to nystatin and ketoconazole, dose-dependent sensitivity to miconazole, and sensitivity to econazole and fluconazole. Multidrug resistance was observed in 24.75% of the isolates. While none of the crude extracts showed activity against the tested *Candida*, six out of 25 fractions (Pnl^B, Pcl^A, Pcl^W, Scl^A, Scl^W, and Gkf^W) displayed fungistatic activities on some strains. These findings offer valuable insights into the prevalence and antifungal resistance patterns of *Candida* species in diabetic patients in Cameroon. They also suggest the potential for alternative treatments using plant fractions.

Keywords: *diabetes, Candida, biofilms, antifungal susceptibility*

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1. Introduction

Diabetes mellitus, a metabolic disorder marked by chronic hyperglycemia due to defects in insulin secretion or action, is a significant public health concern. The World Health Organization and the International Diabetes Federation project that the global adult diabetic population will reach approximately 629 million by 2045 [1]. In Cameroon, as in many other Sub-Saharan African countries, diabetes is a pressing public health issue. Over 567,000 individuals (a prevalence of 6.5%) are living with the disease, experiencing severe complications leading to morbidity, mortality, and high costs [2,3]. Notably, from December 2015 to March 2016, diabetes was linked to an estimated cumulative mortality rate of 8.6% in Cameroon [4].

In diabetic individuals, there's an observed increase in the production of free radicals, associated with hyperglycemia. This increase disrupts the functions of neutrophils, monocytes, and macrophages, leading to impaired adherence, chemotaxis, and phagocytosis, which in turn affects the intracellular killing of microorganisms. It also weakens the immunological response of T-cells and skin reactions to antigens [1,5]. Additionally, certain host

conditions, such as yeast adhesion to epithelial cell surfaces, elevated salivary glucose levels, decreased salivary flow, and microvascular degeneration, have been identified as factors that predispose individuals to fungal colonization and subsequent infection [1]. Consequently, diabetic patients are viewed as immunocompromised and are more prone to opportunistic mycosis, posing a significant public health challenge.

Indeed, *Candida* species have been identified as a risk factor for colonization and/or infection in affected individuals [6]. In China, 55.4% of fungemia cases have been attributed to *Candida* species [7], and a mortality rate of 39% has been detected among diabetic patients [8]. The pathogenicity of *Candida* is facilitated by several virulence factors, one of the most significant being the formation of surface-attached microbial communities known as biofilms [9]. Biofilms can act as reservoirs of agents, allow co-infection with other pathogens, lead to antifungal treatment failure, promote persistent infection, and increase patient mortality [10]. While data on the incidence of candidiasis in diabetics have been published elsewhere [11,12,13], information in Cameroon is scarce due to an underestimation of its significance and a lack of proper diagnostic procedures. A study by Ekpo et al. [14] revealed that diabetes mellitus accounted for 15.1% of candiduria cases among hospitalized patients in the Dschang district hospital in Cameroon. The recovered isolates showed high resistance to polyene antifungals. Azole antifungal compounds have become the primary drugs used for *Candida* infections in the general population. However, their prolonged and empirical use, coupled with the biofilm-forming ability of some *Candida* species, has led to the development of drug resistance [15]. This resistance is associated with issues such as toxicity, cost, and availability, which hinder the efficient use of antifungal drugs.

Given the urgent need to identify whether the recovered isolates form biofilms and to conduct sensitivity tests that will aid in selecting the most effective treatment, it's crucial to explore if the healing properties of traditionally used plants can be harnessed in the quest for new therapeutic strategies against fungal infections. Our focus was on *Picralima nitida*, *Phragmanthera capitata*, *Garcinia Kola*, and *Spondia cythereae*, which are traditionally employed for treating certain infectious and inflammatory conditions [16,17]. We examined and characterized *Candida* species colonizing samples from different diabetic patients at the Yaoundé Central Hospital. We also evaluated their susceptibility to certain antifungal drugs (Ketoconazole, Miconazole, Fluconazole, Econazole, and Nystatin), as well as the aforementioned plant extracts and fractions. This approach could potentially pave the way for more effective treatments for *Candida* infections in diabetic patients.

2. Materials and Methods

2.1. Ethical Considerations and Enrollment of Participants

The study was conducted over a period of 6 months on diabetic patients of both genders attending the Yaoundé Central Hospital for medical follow-up. The patients, whether presenting symptoms of *Candida* infection or not, fulfilled all the inclusion criteria, which included no history of treatment with antibiotics, oral contraceptives, or antifungals during the previous three months. The Centre Regional Ethics Committee for Human Health Research (CRERSH-Ce) granted approval for the study (CEN°0134/CRERSHC/2014). Before sample collection, the purpose and potential benefits of the investigation were explained to the patients who were willing to participate. They then signed an informed consent form. Data related to the patients, such as age, height, weight, and diabetes status (type of diabetes, duration, glycemia), were recorded as part of the study.

2.2. Collection of Specimens

The purpose of this process was to determine the distribution of *Candida* species among different samples and to understand their susceptibility profiles. This information is crucial for the proper management of *Candida* spp. colonization and infection. From the 270 patients involved in the study, a total of 396 specimens were collected. These specimens included urine, stool, oral swabs, vaginal swabs, and skin scrapings, all of which were submitted for mycological diagnosis.

2.3. Mycological Diagnosis and Identification of the Isolated Species

After the macroscopic analysis of the samples, they were divided into small portions. Some of these portions were plated on Sabouraud Dextrose Agar (SDA) medium supplemented with chloramphenicol and incubated for 48 hours at 37ºC. Other portions were used to prepare a smear for Gram staining. In cases where growth was observed, the microorganisms were presumptively identified using a chromogenic culture medium (chrom ID CAN 2 from Biomerieux), followed by phenotypic identification tests such as the germ tube formation test and the chlamydosporulation test. The ID32C biochemical identification kit from Biomerieux was used to confirm the identities of these microorganisms. The identified isolates were then transferred to a cryobank vial containing Sabouraud Dextrose Broth (SDB) supplemented with glycerol to a final concentration of 20%. These samples were homogenized and frozen at -80ºC for future use.

2.4. Biofilm Forming Ability of the *Candida* Species

The in vitro biofilm-forming ability of *Candida* isolates was evaluated using the Crystal Violet (CV) staining assay, which quantifies the total biomass in a microtiter plate. This assay works by allowing the dye to penetrate and bind to negatively charged molecules in the extracellular matrices of mature biofilms, thereby providing information on cell density. In the experiment, a loopful of each isolate grown on Sabouraud Dextrose Agar (SDA) was introduced into Sabouraud Dextrose Broth (SDB) to prepare a suspension equivalent to the 0.5 Mc Farland standard ($1\text{-}5\times10^6$ cells/mL). Two hundred

microliters of this suspension were added to three consecutive wells of a microtiter plate, which was then incubated at 37ºC for 48 hours. To check for sterility and non-specific binding, some wells were filled with broth only. After incubation, the contents of the wells were discarded and the wells were washed three times with 200 µL of phosphate buffer saline (PBS). The wells were then heat-fixed at 60°C for 30 minutes and stained with 200 µL of 0.1% v/v CV for 15 minutes. The plates were rinsed three times with PBS and heat-fixed again. To dissolve the stain, 200 µL of 30% v/v glacial acetic acid was added to the wells for 15 minutes at room temperature. The biomass was quantified by measuring the color intensity, or absorbance, at a wavelength of 590 nm in a microtiter plate reader. The isolates were classified into four groups based on the cutoff value: non-forming biofilm (NBF) (OD ≤ ODcutoff, i.e., OD≤ 0.2), weakly forming biofilm (WBF) (ODcutoff < OD ≤ 2×ODcutoff, i.e., 0.2 < OD ≤ 0.5), moderately forming biofilm (MBF) (2×ODcutoff < OD≤4×ODcutoff, i.e., 0.5 < OD ≤0.9), and strongly forming biofilm (SBF) (OD > 4×ODcutoff, i.e., OD > 0.9). The cutoff value of optical density was calculated using the formula:

Odcutoff = OD average of negative control 3×(standard deviation of ODs of negative control).

2.5. Antifungal Susceptibility Testing

2.5.1. Antifungal Susceptibility Testing of Reference Drugs

The sensitivity of the identified isolates to reference antifungal drugs was assessed using the disc diffusion test [21]. In this process, an inoculum was prepared (10^6 cells/mL), and a cotton swab was dipped into each suspension and streaked across the surface of a 90 mm Mueller-Hinton Agar (MHA) plate supplemented with 2% glucose and 0.5 µg/mL methylene blue. After any excess moisture was absorbed, five types of antifungal discs (Fluconazole (100 µg), Ketoconazole (10 µg), Econazole (10 µg), Miconazole (50 µg), and Nystatin (100 UI)) were placed onto the agar surface using forceps. The plates were then incubated for 48 hours at 37ºC. The diameter of the clear zone (inhibition zone) around each antifungal disc was measured using a caliper. The interpretative breakpoints, indicating whether the isolates were sensitive (S), sensitive dose-dependent (SSD), or resistant (R) to these antifungal agents, were determined according to Zarei et al. [18].

2.5.2. Antifungal Susceptibility Testing of Plant Extracts and Fractions Collection of Plants and Preparation of Crude Extracts and Fractions

In the Yaoundé region, various plant materials were collected, including the leaves of *Picralima nitida*, *Phragmanthera capitata*, and *Spondia cythereae*, as well as the roots and fruits of *Garcinia Kola* (Table 1). These specimens were identified by comparing them to voucher specimens stored at the Cameroon National Herbarium, and they were assigned the following registration numbers: 27839/SRF/CAM for *G. kola*, 1942/SRK for *P. nitida*, 24673/SRF/CAM for *P. capitata*, and 55561/HNC for *S. cythera*. The collected plant materials were dried at room temperature and then ground into a powder using a blender. Crude extracts were prepared by macerating the individual plant materials in 70% ethanol for 72 hours. The organic solvent was then evaporated from the filtrates at 40ºC using a rotary evaporator (IKA, Germany). This process was repeated three times, and the dried extracts were combined, weighed, and the extraction yields were calculated.

The dried ethanol crude extracts, weighing 100 g, were dissolved in distilled water and then partitioned using a separating funnel between five solvents: hexane (1 L), dichloromethane (1 L), ethyl acetate (1 L), butanol (1 L), and water (1 L). Each solvent was then separately evaporated under reduced pressure to yield respective fractions: hexane, dichloromethane, ethyl acetate, butanol, and water. The yield of each fraction was calculated in relation to the weight of the initial crude extract used.

Fungi species: The investigation utilized two clinical strains, *C. parapsilopsis* (320B) and *C. tropicalis* (390S), and three reference strains, *C. albicans* NR29451, *C. albicans* NR2944, and *C. krusei* HM 122, sourced from Bei resources. The selection of the clinical strains was based on their prevalence in the collected samples and their low sensitivities to antifungal drugs.

Inoculum preparation: Three to four colonies of each reference strain and selected clinical isolate, which had been cultured on Sabouraud Dextrose Agar (SDA) for 48 hours, were introduced into a tube containing 5 mL of physiological water. This mixture was homogenized to create a suspension that corresponded to the 0.5 McFarland scale, equivalent to $1x10^6$ cells/mL. This suspension was then diluted to achieve the required working concentration of 10^4 cells/mL.

Fractions, crude extracts, and Fluconazole stock solutions preparation: Each extract or fraction, weighing 100 milligrams, was dissolved in DMSO (20%) to create a stock solution with a concentration of 100 mg/mL. Fluconazole, used as a positive control, was prepared at a concentration of 1 mg/mL by dissolving 2 mg of its powder in 2 mL of sterile distilled water.

Table 1. Local names, collection site, traditional uses and part of plants

Plant species	Local names	Collection site	Traditional uses	Part used
Picralima nitida **1942/SRFK**	Ebam (Ewondo)	Yaoundé	Diarrhoea, gonorrhea, intestinal worms, malaria [16,19]	Leaves
Spondia cytherea **55561/HNC**	Kassimanga (in Bassa)	Yaoundé	Stomachache, diarrhea	Leaves
Phragmenthera capitata **24673/SRF/CAM**	Tsapla (Dschang)	Yaoundé	Diabetes, chlamydia infection, cancer, arthritis, epilepsy, gynecological problems and cardiovascular diseases [17,20]	Leaves
Garcinia kola **27839/SRF-CAM**	Onié (Ewondo)	Melangué 1 (Biwong-Bané)	Liver disorders, hepatitis, diarrhoea, laryngitis, bronchitis, gonorrhea parasitic and microbial infections [16].	Root Fruits

Preliminary screening: The 30 different extracts and fractions were initially screened against the five aforementioned *Candida* strains at a concentration of 5 mg/mL using a 96-well microtiter plate. In brief, 90 µL of Sabouraud Dextrose Broth (SDB) was added to three consecutive wells of the plate, followed by 10 µL of the extract to be tested. This mixture was homogenized, and then 100 µL of the *Candida* suspension was added to each well. After incubating for 48 hours at 37ºC, the effect of each extract and fraction on *Candida* growth was evaluated visually by comparing their growth in the test wells to that in the negative control wells. This experiment was performed twice for accuracy. The extracts or fractions that demonstrated complete inhibition of the *Candida* strains were selected for further testing to determine the Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC).

MICs of plant extracts and fractions: The assay is designed to evaluate the yeast's potential to grow in a medium supplemented with the compounds, fractions, or extracts, following the M27-S4 [22] protocol. In brief, 180 µL of Sabouraud Dextrose Broth was added to the wells in the first row of a 96-well microtiter plate, and 100 µL was added to all remaining wells. Twenty microliters of each extract or fraction (at a concentration of 100 mg/mL) and 100 µL of fluconazole (256 µg/mL) were added to the wells in the first row. From there, a series of seven two-fold dilutions were made by transferring 100 µL of the mixture from the first wells to the second, and so on, until the last, which was discarded. Then, 100 µL of the fungal suspension, calibrated at 10^4 cells/mL, was added to the wells. The plates were then incubated for 48 hours at 37 ºC. The wells in the 12th column, which contained only the medium and inoculum, served as the negative control, while the wells containing fluconazole served as the positive control. The tested concentrations ranged from 64 to 1 µg/mL for the positive control (fluconazole) and from 5000 to 78.125 µg/mL for the extract or fraction. The final inoculum concentration was 5×10^3 cells/mL. The highest dilutions (the lowest concentrations) of the extract or fraction that showed no turbidity (indicating no visible *Candida* growth) compared to the control wells were considered as the Minimal Inhibitory Concentrations (MICs). The tests were run twice and in duplicate. At the end of the incubation period, 50 µL of the broth mixture taken from wells considered as MIC were introduced into wells of another microtiter plate containing 50µL of freshly prepared Sabouraud Dextrose Broth medium. After a further incubation period of 48 hours at 37ºC, the lowest concentration that showed no growth was defined as the Minimal Fungicidal Concentration (MFC). If the fractions were found to have a fungicidal effect on the tested *Candida*, it meant they could kill the fungus. If a resumption of growth was observed, then the fraction was said to have a fungistatic effect, meaning it could inhibit the growth of the fungus.

2.6. Statistical Analysis

Data from the study were recorded in a Microsoft Excel database and statistically analyzed in terms of frequencies and percentages. One-way analysis of variance (ANOVA) was performed using Graphpad software for the statistical analysis. The Chi-square test was utilized to analyze the associations of *Candida* colonization with various factors such as age, sex, duration of diabetes, glycemia, and body mass index. A P value less than 0.05 was considered statistically significant, indicating a strong likelihood that the results were not due to random chance at a 95% confidence interval.

3. Results

3.1. Characteristics of the Studied Population

Our study consisted of 270 diabetic patients, ranging in age from 18 to 90 years (average 54.74±11.86). The gender distribution was uneven, with women making up 55.18% of the population, resulting in a sex ratio of 0.81. A significant portion of this population, 41.85%, were between the ages of 54 and 66. Only one patient had type 1 diabetes, while the rest were diagnosed with type 2 diabetes. We collected 396 samples from these 270 patients, including 94 oral swabs, 28 vaginal swabs, 68 stool samples, 28 skin swabs, and 178 urine samples. *Candida* colonization was found in 146 patients (54.07%) at at least one of the collection sites.

In our series, most patients (64.07%) had been diagnosed with diabetes for more than 5 years, with an average duration of 9.16 years, ranging from 3 months to 49 years. Among the patients colonized by *Candida*, the average Fasting Blood Glucose was 1.81 g/L, ranging from 0.7 to 3.93 g/L, and the average body mass index was 27.14. However, no statistically significant association was found between *Candida* colonization and any of these parameters (sex, age, duration of diabetes, glycemia, BMI) ($P > 0.05$).

3.2. Mycological Diagnosis, *Candida* Identification and Characterization

The collected samples were cultured on both SDA and chromogenic medium, yielding 171 instances of *Candida* growth. This included 51 from urine (44 mono*Candida* and 7 poly*Candida*), 51 from stool (41 mono*Candida* and 10 poly*Candida*), 24 from the oral cavity (22 mono*Candida* and 2 poly*Candida*), 19 from the vagina (17 mono*Candida* and 2 poly*Candida*), and 26 from the skin (23 mono*Candida* and 3 poly*Candida*). Out of these, 147 cases (or 85.96%) had a single *Candida* isolate, while the remaining 24 cases (or 14.04%) exhibited mixed growth of two or more *Candida* isolates at the same or different sites. This resulted in a total of 202 isolates. However, no significant correlation was found between *Candida* colonization and patient parameters such as age, BMI, and fasting blood glucose ($P > 0.05$). The most frequently colonized sites were the skin (92.86%), stool (75%), and vagina (67.86%).

Among the 202 isolates (Table 2), *C. albicans* was the most common, accounting for 108 cases (or 53.47%). This was followed by *C. tropicalis* (29 cases or 14.36%), *C. parapsilosis* (28 cases or 13.86%), *C. glabrata* (16 cases or 7.92%), *C. guillermondii* (11 cases or 5.44%), *C. krusei* (6 cases or 2.97%), *C. lusitaniae* (3 cases or 1.48%), and *C. ciferri* (1 case or 0.5%). The most prevalent species in stool samples was *C. parapsilosis*, while *C. glabrata* was most common in urine samples, and *C. ciferri* was most frequently found on the skin.

Table 2. Distribution of isolates at different collection sites

	Collection site					Total	Percentage (%)
	Urine	Stool	Oral cavity	Skin	Vagina		
Candida albicans	29	29	23	19	8	108	**53.47**
Candida tropicalis	8	10	1	4	6	29	**14.36**
Candida parapsilosis	8	12	0	5	3	28	**13.86**
Candida guillermondii	0	4	5	0	2	11	**5.44**
Candida glabrata	10	3	0	1	2	16	**7.92**
Candida krusei	2	2	0	0	2	6	**2.97**
Candida lusitaniae	2	1	0	0	0	3	**1.48**
Candida ciferrii	0	0	0	1	0	1	**0.50**
Total						**202**	**100**

Table 3. Association of *Candida* species according to collection site

***Candida* species**	**Urine**	**Stool**	**O. cavity**	**Skin**	**Vagina**
albicans+parapsilosis	2	3	0	1	0
albicans +tropicalis	2	5	0	0	1
albicans+glabrata	1	0	0	0	0
albicans +guillermondii	0	0	1	0	1
albicans +tropicalis + glabrata	1	0	0	0	0
tropicalis +glabrata	1	0	0	0	0
tropicalis +parapsilosis	0	1	0	1	0
parapsilosis +lusitaniae	0	1	0	0	0
Total samples with mixed growth	7	10	1	2	2

Table 4. Biofilm formation by various *Candida* species

Candida spp.	**Total number of isolates**	**Nº of biofilm negative**	**Nº of biofilm positive**			**Total Nº of biofilm positive**
			WBF	**MBF**	**SBF**	
Candida albicans	108	64	26	7	11	44 (40.74%)
Candida tropicalis	29	20	4	3	2	9 (31.03%)
Candida parapsilosis	28	14	4	2	8	14 (50%)
Candida guillermondii	11	2	1	3	5	9 (81.82 %)
Candida glabrata	16	8	2	3	3	8 (50 %)
Candida krusei	6	2	1	1	2	4 (66.67%)
Candida lusitaniae	3	2	0	0	1	1 (33.33%)
Candida ciferrii	1	0	0	0	1	1 (100%)

NBF (non-forming biofilm), WBF (weakly forming biofilm), MBF (moderately forming biofilm) and SBF (strongly forming biofilm)

In the analysis of samples with mixed *Candida* growth, *C. albicans* was found in nearly all the samples (Table 3). However, it was absent in three stool samples, one skin sample, and one urine sample

Biofilm forming ability of Candida: Out of the 202 *Candida* examined (Table 4), biofilm positivity was observed in 93 (46.04%). Non-*albicans Candida* showed higher biofilm production frequencies, with 49 (52.69%) compared to *Candida albicans* with 44 (47.31%). Biofilm could not be formed by 109 other *Candida*. The degree of Biofilm Production (BP) was analyzed across different *Candida* species and collection sites. Among the 108 C.*albicans*, 64 (59.26%) were Non Biofilm Formers (NBF), 26 (24.07%) were Weak Biofilm Formers (WBF), 7 (6.48%) were Moderate Biofilm Formers (MBF), and 11 (10.19%) were Strong Biofilm Formers (SBF). Generally, most *C. albicans* were weak biofilm producers, while *C. parapsilosis*, *C. guillermondii*, and *C. glabrata* were primarily strong biofilm producers.

Biofilm production was also evaluated in relation to the collection site (Table 5). The highest biofilm formation was observed in stool samples (53.9%), followed by skin scrapings (46.67%), vaginal swab samples (43.47%), oral swabs (40.74%), and urine samples (40.68%). Additionally, *Candida* species that are strong biofilm formers were commonly found in stool and urine samples.

Table 5. Biofilm formation in various clinical samples

	Number of biofilm producer			
	WBF	**MBF**	**SBF**	**Total (%)**
Urine (n=59)	9	3	12	24 (40.68 %)
Oral swab (n=27)	6	1	4	11(40.74 %)
Skin scraping (n=30)	8	4	2	14 (46.67 %)
Stool (n=63)	13	7	14	34 (53.97 %)
Vaginal swab (n=23)	4	2	4	10 (43.47 %)

NBF (non-forming biofilm), WBF (weakly forming biofilm), MBF (moderately forming biofilm) and SBF (strongly forming biofilm)

3.3. Antifungal Activity

3.3.1. Susceptibility Profiles Against Reference Drugs

Based on the activity interpretative breakpoints criteria of antifungal (Table 6), *Candida* species were more susceptible to econazole and fluconazole (≥50%), while they exhibited a high resistance rate against nystatin and

ketoconazole, regardless of the specimen type. In our study, all *Candida* species showed almost 85% resistance against nystatin and 53% against ketoconazole. However, they were highly dose-dependent on miconazole (73%) but sensitive to econazole (62%) and fluconazole (57.5%). Apart from *C. ciferri*, which showed a dose-dependent behavior against nystatin and good sensitivity to miconazole, other strains were highly resistant to nystatin and ketoconazole. These strains, however, displayed good sensitivity against econazole and fluconazole, with the exception of *C. glabrata*, which had a high resistance to fluconazole. *C. lusitaniae* was found to be resistant to nystatin (100%) and showed a dose-dependent response against miconazole.

Sensitivity testing for antifungal drugs revealed that some *C. albicans*, as well as non-*albicans Candida*, were resistant to three or more antifungals. As a result, these isolates were classified as multidrug-resistant (Table 7).

Out of the 202 *Candida* species obtained, multidrug resistance was observed in 20.37% (22 out of 108) of *C. albicans* and 29.79% (28 out of 94) of non-*Candida albicans*. Among the 50 multidrug-resistant *Candida*, there were 22 (44%) *Candida albicans*, 8 (16%) *Candida glabrata*, 6 (12%) *Candida parapsilosis*, 5 (10%) *Candida tropicalis*, 5 (10%) *Candida guillermondii*, and 4 (8%) *Candida krusei*.

3.3.2. Susceptibility Against Plant Extracts and Fractions

We harvested and treated five plant parts with 70% ethanol, resulting in five crude extracts and 25 fractions. The yields of these extracts and fractions are detailed in the Table 8 below.

Table 6. Global antifungal susceptibility profile of the different *Candida* species

	Nystatine			Miconazole			Ketoconazole			Econazole			Fluconazole		
	S	**DD**	**R**	**S**	**DD**	**R**	**S**	**DD**	**R**	**S**	**DD**	**R**	**S**	**DD**	**R**
Candida albicans (n=104)	0 (0%)	24 (23.1%)	80 (76.9%)	16 (15.4%)	79 (76%)	9 (8.7%)	13 (12.5%)	50 (48.1%)	47 (45.2%)	69 (66.4%)	31 (29.8%)	4(3.9%)	67 (64.4%)	17 (16.4%)	20 (19.2%)
***Candida tropicalis (n*=30)**	0 (0%)	3 (10%)	27 (90%)	12 (40%)	16 (53.3%)	2 (6.7%)	4 (13.3%)	9 (30%)	17 (56.7%)	19 (63.3%)	10 (33.3%)	1(3.3%)	20 (66.7%)	4 (13.3%)	6 (20%)
Candida parapsilosis (n=28)	0 (0%)	1 (3.6%)	27 (96.4%)	5 (17.9%)	21 (75%)	2 (7.1%)	8 (28.6%)	4 (14.3%)	16 (57.4%)	19 (67.9%)	8 (28.6%)	1(3.6%)	15 (53.6%)	6 (21.4%)	7 (25%)
Candida guillermondii (n=11)	0 (0%)	1 (9.1%)	10 (90.9%)	1 (9.1%)	8 (72.7%)	2 (18.2%)	1 (9.1%)	3 (27.3%)	7 (63.6%)	5 (45.5%)	4 (36.4%)	2(18.2%)	4 (36.4%)	2 (18.2%)	5 (45.5%)
Candida glabrata (n=17)	0 (0%)	0 (0%)	17 (100%)	1 (5.9%)	15 (88.2%)	1 (5.9%)	0 (0%)	1 (5.9%)	16 (94.1%)	9 (52.9%)	4 (23.5%)	4(23.5%)	4 (23.5%)	5 (29.4%)	8 (47.1%)
Candidakrusei (n=6)	0 (0%)	0 (0%)	6 (100%)	0 (0%)	4 (66.7%)	2 (33.3%)	1 (16.7%)	1 (16.7%)	4 (66.7%)	1 (16.7%)	5 (83.3%)	0(0%)	2 (33.3%)	0 (0%)	4 (66.67%)
Candidalusitaniae (n=3)	0 (0%)	0 (0%)	3 (100%)	0 (0%)	3 (100%)	0 (0%)	0 (0%)	2 (66.7%)	1 (33.3%)	1 (33.3%)	2 (66.7%)	0(0%)	2 (66.7%)	1 (33.3%)	0 (0%)
Candida ciferrii (n=1)	0 (0%)	1 (100%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	1 (100%)	0 (0%)	0(0%)	1 (100%)	0 (0%)	0 (0%)
Total (n=200)	**0 (0%)**	**30 (15%)**	**170 (85%)**	**36 (18%)**	**146 (73%)**	**18 (9%)**	**32 (16%)**	**62 (31%)**	**106 (53%)**	**124 (62%)**	**64 (32%)**	**12 (6%)**	**115 (57.5%)**	**35 (17.5%)**	**50 (25%)**

S: Sensitive; **DD**: Dose Dependent; **R**: Resistant

Table 7. Multiple resistance patterns of *Candida* isolates amongst diabetic patients

	Antibiogram patterns					
	R0	**R1**	**R2**	**R3**	**R4**	**R5**
Candida albicans (n=108)	22(20.4%)	40(37.0%)	24(22.2%)	17(15.7%)	4(3.7%)	1(0.9%)
Candida parapsilosis (n=28)	0(0%)	13(42.9%)	9(32.1%)	4(14.3 %)	1(3.6%)	1(3.6%)
Candida tropicalis (n=29)	3(10.3%)	7 (24.1%)	15(51.7%)	3(10.3%)	1(3.5%)	1(3.5%)
Candid aguillermondii (n=11)	0(0%)	5(45.5%)	1(9.1%)	3(27.3 %)	0(0%)	2(18.2%)
Candida glabrata (n=16)	0(0%)	1(6.3%)	7(43.8%)	8(50%)	0(0%)	0(0%)
Candida krusei (n=6)	0(0%)	2(33.3%)	0(0%)	2(33.3%)	2(33.3 %)	0(0 %)
Candida lusitaniae (n=3)	0(0 %)	1(33.3%)	2(66.7%)	0(0%)	0(0%)	0(0%)
Candida ciferrii (n=1)	0(0%)	1(100%)	0(0%)	0(0%)	0(0%)	(0%)
Total (n=202)	**24(11.9%)**	**70(34.7%)**	**58(28.7%)**	**37(18.3%)**	**8(3.96%)**	**5(2.48%)**

R0 = No antifungal resistance R1 = Resistant to one antifungal agent R2 = resistant to two antifungal agent R3 = Resistant to three antifungal agent R4 = Resistant to four antifungal agent R5=Resistant to five antifungals

Table 8. Yields of crude extracts and fractions

	Yield (%)					
	Crude extract	**Hexane**	**Dichloromethane**	**Ethylacetate**	**Butanol**	**Water**
Picralima nitida	13.78	37.94	19.38	10.95	8.77	26.07
Spondia cythereae	10.78	26.62	4.94	14.66	32.48	20.01
Phragmanthera capitata	8.41	28.89	10.93	7.47	16.32	36.39
***Garcinia kola* root**	22.45	15.07	7.45	26.27	30.89	20.89
***Garcinia kola* fruit**	12.82	23.40	18.72	14.04	15.60	28.08

A preliminary screening was conducted with 30 extracts at a concentration of 5 mg/mL against 2 *Candida* isolates and 3 reference strains. The results showed that among the extracts and fractions of *Picralima nitida*, only Pnl^{B} exhibited activity against 2 strains at 5 mg/mL. The crude extract and fractions of *Garcinia kola* root did not show any effect on the tested *Candida*. However, while the crude extract of *Phragmanthera capitata* did not show any activity against *Candida*, two of its fractions (Pcl^{A}, Pcl^{W}) did. PclA was active against 4 *Candida* species, while Pcl^{W} was active against only 1 species (Table 9). A similar observation was made for *Spondia cythereae*, specifically for its ethylacetate and aqueous fractions.

Table 9. MIC (µg/mL) of the selected fractions

	Candida parapsilosis 320B	*Candida tropicalis* 390S	*Candida albicans* NR29451	*Candida albicans* NR29444	*Candida krusei* HM 122
***Pcl*^{A}**	ND	5000 ± 0.00	5000 ± 0.00	5000 ± 0.00	5000 ± 0.00
***Pnl*^{B}**	ND	ND	5000 ± 0.00	ND	5000 ± 0.00
***Pcl*^{W}**	5000 ± 0.00	ND	ND	ND	ND
***Scl*^{W}**	5000 ± 0.00	ND	ND	ND	ND
***Scl*^{A}**	5000 ± 0.00	ND	ND	ND	ND
***Gkf*^{W}**	5000 ± 0.00	ND	ND	ND	ND
Fluconazole	8.0 ± 0.00	2.0 ± 0.00	8.0 ± 0.00	8.0 ±0.00	6.5 ± 1.50

*Pn*l: ethanol extract of leaves of *P. nitida*; *Sc*l: ethanol extract of leaves of *S. cytherea*; *Pc*l: ethanol extract of leaves of *P. capitata*; *Gk*r: ethanol extract of root of *G. kola*; *Gk*f: ethanol extract of fruit of *G. kola*; H: hexane; D: dichloromethane; A: ethyl acetate; B: butanol; W: water; ND: Not Determined

In the process of determining the antifungal parameters (MIC and MFC) of the selected fractions, we found that none of the fractions were active at concentrations below 5 mg/mL. The fraction Pcl^{A} showed a MIC of 5 mg/mL against *C. tropicalis* 390S, *C. albicans* NR29451, *C. albicans* NR29444, and *C. krusei* HM 122. Against C. parapsilosis 320B, four fractions (Pcl^{W}, Scl^{W}, Scl^{A}, Gkf^{W}) exhibited a MIC of 5 mg/mL. However, against *C. albicans* NR29451 and *C. krusei* HM 122, only Pnl^{B} demonstrated a MIC of 5 mg/mL. During the tests evaluating the effect of fractions on *Candida*, we noticed the growth of yeast cells when they were transferred into freshly prepared Sabouraud Dextrose Broth. This observation indicates that the active fractions have a fungistatic effect.

4. Discussion

In this study, we found that the overall prevalence of *Candida* colonization among diabetic patients was 54.07%. This high rate can be attributed to factors such as high sugar concentrations in tissues and low salivary secretions. While *Candida* can colonize various body sites, most studies have focused on one site at a time. For instance, Manfredi [23] reported a 62.8% colonization rate in the oral cavity of diabetic patients, while Goswami et al. [24] reported a 46% incidence of vaginal *Candida* infection in adult diabetic patients. In our study, the overall prevalence of candiduria in diabetic patients was 28.65%. Similar findings have been reported with rates of 30% in Brazil from diabetic patients [25]. However, lower rates of isolation were reported in Pakistan (10.2%) [26] and in Ethiopia, where Yismaw et al. [13] in their cross-sectional study, detected candiduria in 17.1% of asymptomatic type 2 diabetic patients. Few researchers have focused on the presence of *Candida* in the stool of diabetics. Soyucen et al. [27] demonstrated their occurrence in the feces of children with type 1 diabetes using microbiological culture methods, while Gosiewski et al. [28] did the same work but in adult type 2 diabetics' patients using qPCR. Among the 202 *Candida* species identified, *Candida albicans* was the most commonly detected (53.47%), while non-*Candida albicans* represented 46.53% of the species obtained from the 396 analyzed samples. The increased number of non-*Candida albicans* species identified can reflect both the improvement of diagnostic methodologies and their major capability to persist in the host with respect to C. *albicans*. In samples with mixed *Candida* growth, *Candida albicans* was present in all samples, with the exception of 3 samples from stool, one from urine, and one from the skin. Many reasons have been put forward to explain why *Candida albicans* is found in the majority of samples with mixed growth. Among them is the reverse conversion of C. *albicans* from unicellular yeast cells to either pseudohyphal or hyphal growth, important for tissue invasion and resistance to phagocytosis [29], and finally the possession of virulence factors (hydrolytic enzymes, biofilm formation, adhesins for adherence to host tissues). Though these virulence factors are clinically significant, it is important to note that their expressions differ from one species to another and even within the same species, as is the case in this study, where not all *Candida* species isolated could form biofilm.

In our examination of 202 clinical *Candida* samples, we found that 93 (46.04%) were positive for biofilm formation. Non-*albicans Candida* species accounted for 49 (52.13%) of these, while *Candida albicans* accounted for 44 (40.74%). The remaining 109 isolates did not form biofilm. This suggests an increasing trend in biofilm production by non-*albicans Candida* species. Our findings align with those of Marak and Dhanashree [30], who reported biofilm production rates of 57.14% among non-*Candida albicans* species and 39.02% among *C. albicans*. We also found that biofilm production varied not only by *Candida* species but also by infection site, corroborating the observations of Mohandas and Ballal [9]. Indeed, managing the damage caused by these virulent species requires the careful selection of appropriate antifungals. While azoles are commonly used to treat fungal infections, other groups of antifungals may also be considered, depending on the type of infection, the affected anatomical site, and the sensitivity profile of the involved species. Our investigation revealed that the *Candida* species under study were more susceptible to econazole and fluconazole (with susceptibility rates of 50% or higher) than to nystatin and ketoconazole, to which they showed a high resistance rate, regardless of the specimen type. An increasing resistance to nystatin among *Candida* isolates has been observed in various locations: Gauteng, South Africa (67%), Buea, Cameroon (68% and 80%), and Kenya (35.6%) [31,32,33,34]. *C. krusei*, *C. guillermondii* and *C. glabrata* have shown high resistance rates to fluconazole and ketoconazole. A similar resistance rate

(42.9%) was found for *C. glabrata* against fluconazole in a study by Khadka et al. [35]. This aligns with the view that *C. glabrata* and *C. krusei* are the species most likely to develop resistance to fluconazole and other azoles [36,27]. Despite *C. krusei* being known for its intrinsic resistance to fluconazole, several theories have been proposed to explain the rising resistance of *Candida* to antifungal agents. Several factors contribute to the growing resistance of *Candida* to antifungal agents. First, the affordability and accessibility of nystatin have resulted in its overuse as a topical ointment or suppository. Second, the lack of regulation or poor regulation of antimicrobial medication sales in Africa, compounded by the influx of counterfeit drugs with minimal or no active ingredients, often found in both pharmacies and street markets. Lastly, the practice of prescribing antimicrobial medications based solely on clinical symptoms without a microbiological diagnosis [34,38]. Given the rise of antifungal drug resistance among both immunocompetent and immunocompromised individuals, it's crucial to explore and develop innovative therapies to alleviate the burden associated with these infections.

Exploring medicinal plants is a promising approach to discovering new antifungal drugs. However, it was found that all crude extracts lacked activity against the tested yeasts at a concentration of 5 mg/ml. This aligns with the findings of Ohikhena et al. [39], who reported that *Candida albicans* was not sensitive to organic solvent extracts (acetone, ethanol, and methanol) of *Phragmanthera capitata* at concentrations from 0.078 to 10 mg/mL. Interestingly, the aqueous and ethyl acetate fractions of *P. capitata* showed good activity at 5 mg/mL, inhibiting 1 and 4 out of the five tested fungi, respectively. Similarly, the ethylacetate and aqueous fractions of *Spondia cytherea* (Scl^{A}, SclW), the aqueous fraction of *Garcinia kola* fruit pulp (Gkf^{w}), and the butanol fraction of *Picralima nitida* (Pnl^{B}) showed activity on 1 and 2 species, respectively. These results suggest that the crude extracts contain small quantities of antifungal compounds, which have been concentrated in fractions during the partitioning process. The lack of activity of *P. nitida* crude leaf extract on *Candida* species at 5 mg/mL aligns with the findings of Ubulom et al. [40], who reported no activity on C. *albicans* at 25 mg/mL, but a very low activity of the aqueous and ethanolic leaf extracts of *P. nitida* at concentrations equal to or above 50 mg/mL. The observed differences in susceptibility could be due to variations in the preparation of the plant extract, the antifungal susceptibility method used, and the concentration of the crude extract. The crude extracts of *Garcinia kola*'s stem roots and fruit pulp showed no antifungal activity against all tested *Candida* species. Most existing studies on *G. kola*'s biological activities have focused on its seeds. While there are a few studies on *G. kola*'s antifungal activity, reports on the activity of its roots, stem barks, and leaves are scarce. However, it was observed that different fractions of *Garcinia kola* fruits inhibited the growth of a *C. albicans* isolate (CF1) at concentrations between 20 to 25 mg/mL [40].These findings support the ethno-pharmacological use of these active plants in treating gastrointestinal infections and other opportunistic diseases in humans and animals. As such, they could potentially serve as alternative therapies to address the resistance issues often encountered with conventional antifungals.

5. Conclusion

Candida colonization was found to be prevalent among diabetic patients, with C. *albicans* being the most commonly detected species in all samples. The production of biofilm varied based on the species and the site of collection, with non-C. *albicans* species producing stronger biofilms than C. *albicans*. Resistance to nystatin and ketoconazole was seen in approximately 85% and 53% of all *Candida* species, respectively. However, these species showed a high dose-dependency on miconazole, while being sensitive to econazole and fluconazole. Both C. *albicans* and non-C.*albicans* species exhibited multidrug resistance. Therefore, characterizing and determining the susceptibility profile of *Candida* species is crucial for effective candidiasis management. Moreover, some fractions demonstrated fungistatic activities against certain *Candida* strains, suggesting potential alternative antifungal treatments.

Conflict of Interest

The authors declared the non-existence of conflict of interest

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