



Efficacy of Extracts of *Cyanthillium Cinereum*, *Khaya senegalensis* and *Lippia multiflora* on *Candida* Strains Isolated From Urine Samples in Benin (West Africa)

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The search for new bioactive molecules with antifungal properties to combat resistance to classical antifungals represents a great challenge. This study aimed to explore the virulence factors and resistance profile of *Candida* species isolated from urine samples in Benin and the *in vitro* efficacy of organic extracts of *Cyanthillium cinereum* (L.) H. Rob., *Lippia multiflora* Moldenke and *Khaya senegalensis* (Desv.) A. Juss. on the growth of these *Candida* spp. The study focused on *Candida* strains isolated from urine samples collected from patients admitted to the bacteriological analysis laboratories of hospitals in Southern Benin. The sensitivity of these strains to classical antifungal agents was determined by the simple diffusion method. Their pathogenicity was investigated *via* several virulence factors (gelatinase, hemolysin, hydrophobicity, adhesin, biofilm and lecithinase). The *in vitro* efficacy of the aqueous, ethanolic and hydro-ethanolic extracts of the plants on *Candida albicans* ATCC 90028 and on six clinical strains was evaluated by the method of determination of the inhibition diameters. The results obtained showed that 51 different *Candida* strains were isolated from the collected urine samples with a respective predominance of *Candida albicans* (52.94%) and *Candida glabrata* (17.64%) species. All identified species were sensitive to amphotericin B and nystatin but 20% are resistant to fluconazole and present 15 different resistance profiles. Six different virulence factors were identified with a high frequency of hydrophobicity (96.08%) and adhesin (94.12%). Antifungal tests revealed that at 100 mg/mL the plant extracts were active on the tested strains with better activity for *Cyanthillium cinereum* and *Khaya senegalensis*. *Cyanthillium cinereum*, *Khaya senegalensis* and *Lippia multiflora* showed antifungal activity on virulent *Candida* strains suggesting the possibility to explore them further for the discovery of new antifungal molecules.

Keywords: *Cyanthillium cinereum*, *Khaya senegalensis*, *Lippia multiflora*, antifungal susceptibility, virulence factors, *Candida* spp

1 INTRODUCTION

Candidiasis is the most frequent fungal infection in human pathology (1). Affecting millions of women each year, candidiasis is considered as a major public health problem (2). This benign affection has a very negative impact on life quality and high health costs (3). Fungal pathologies have various degrees of severity, ranging from superficial and benign infections to invasive and fatal infections (4). According to some epidemiological data, their incidence has increased in the last two decades due to the pathological state of immunocompromised patients, invasive medical procedures and therapy with broad spectrum antibiotics (5).

Candidiasis is caused by different species of *Candida* with *Candida albicans* as the main species isolated from infected patients. Its contamination is almost exclusively endogenous and its importance depends on the location of the responsible yeast species (5).

Urinary tract candidiasis is a most common nosocomial fungal infection worldwide (6). Usually asymptomatic, it is frequently seen in hospitalized patients and can be due to cystitis, pyelonephritis, prostatitis, epididymo-orchitis or disseminated candidiasis (7). Major risk factors include diabetes mellitus, use of broad-spectrum antibiotics, urinary tract obstruction, viral infections, admission to an intensive care unit, among others (7, 8). *Candida albicans* is the main cause of this nosocomial infection. *Candida* UTIs can be caused by hematogenous spread following candidemia, or by retrograde route *via* the urethra (9). The presence of *Candida* species in the urine of asymptomatic patients does not warrant antifungal therapy except for neutropenic patients, very low birth weight infants, and patients undergoing urologic procedures.

The management of urinary candidiasis is effectively done with fluconazole, which is the antifungal agent of choice for this type of nosocomial infection. However, amphotericin B or flucytosine can also be used for this purpose in rare cases (10, 11). In recent years, the increase in urinary tract candidiasis has led to the emergence of *Candida* species that are resistant to conventional antifungals (6). Thus, *Candida glabrata*, *Candida krusei* strains are increasingly associated with urinary candidiasis (12). Some authors had even reported cross-resistance of *Candida albicans* to itraconazole and fluconazole (13, 14). These *Candida* species have the potential to develop antifungal resistance either intrinsically or during treatment (15). The emergence of fungal infections is favored by several virulence factors responsible for the degree of pathogenicity of *Candida* species. Nowadays, the emergence of new resistant and virulent *Candida* species that are virtually untreatable has further highlighted this public health problem (16, 17).

Faced with this resistance observed nowadays to classical antifungal drugs, even those of last resort (fluconazole for example) used for the treatment of candidiasis, it is necessary to resort to safe and effective alternative solutions. The search for new bioactive molecules with antifungal properties to combat resistance to classical antifungals represents a great challenge for researchers in the field of biomedical research. The use of medicinal plants is the first reflex of most populations, especially those in developing countries, for socio-cultural and

economic reasons, and is one of the most promising alternative solutions. Indeed, in recent years, scientific studies conducted on a large number of medicinal plants have identified about 7,000 natural bioactive compounds currently used in modern medicine, which increases the global market value of medicinal plant products (18). Over 90% of today's therapeutic classes are derived from a prototype natural product, the discovery of which has led to significant changes in the practice of modern medicine (19, 20).

The flora of Benin (a West African country), rich in 2807 plant species, offers the possibility of using many medicinal plants in the treatment of various pathologies (21). Several plant species including *Cyanthillium cinereum* (L.) H. Rob., *Khaya senegalensis* (Desv.) A. Juss. et *Lippia multiflora* Moldenke are used in the management of candidiasis in Benin (22). However, very little scientific evidence exists on the efficacy of plants against *Candida* strains as well as on the resistance and virulence profiles of *Candida* strains.

This study aimed to explore the virulence factors and resistance profile of *Candida* species isolated from urine samples in Benin and the *in vitro* efficacy of organic extracts of *Cyanthillium cinereum*, *Lippia multiflora* and *Khaya senegalensis* on the growth of these *Candida* spp.

2 MATERIAL AND METHODS

2.1 Isolation and Identification

Candida strains were isolated from urine samples collected from patients sent to the laboratory for urine cytobacteriological examinations + antibiotic susceptibility testing in various bacteriological analysis laboratories in hospitals in southern Benin. Each strain was identified according to the methodology described by Khan et al. (23) and Pandey et al. (24). For this purpose, macroscopic examination was performed on the strains obtained on Sabouraud agar supplemented with 0.5% chloramphenicol (SDA+C). The phenotypic identification of the strains was done based simultaneously on the aspects of the colonies obtained on the differential media ChromAgar and Tetrazolium Reduction Medium. The ability of yeast to produce germ tubes was studied by the blast test.

2.2 Identification by PCR

Candida spp. strains were also identified by restriction fragment length polymorphism (RFLP) PCR using the *MspI* restriction enzyme according to the methods of Dehghan et al. (25) and Hamzehee et al. (26).

2.2.1 DNA Extraction and PCR Reaction

DNA extraction was performed using 24 hours young colonies isolated on YEPD medium. The chloroform-phenol technique described by Silva et al. (27) has been used. The PCR was done in two steps: the first step consists in amplifying the ITS1 and ITS4 region named ITS1-ITS4 and the second was digesting the PCR product of the ITS region with restriction enzyme. For the first step, total DNA amplification was performed using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-

TCCTCCGCTTATTGATATGC-3'). The reaction mixture, with a total volume of 25µL, consisted of 10µL of master mix (Invitrogen), 12µL of water without DNA, 1µL of each primer and 1µL of the DNA of the yeast to be identified. The PCR was performed in a thermal cycler (PeQlab, Germany) with the following amplification conditions: 1 cycle of DNA denaturation of 5 min at 95°C, followed by 35 hybridization cycles of 30s at 94°C, 1 min at 56°C and 1 min at 72°C and a final extension step at 72°C for 10 minutes. A positive and negative control consisting of respectively a reference strain of *Candida albicans* ATCC 90028 and DNase, RNase free water were used.

2.2.2 Digestion With Msp1 Enzyme and Electrophoresis

Each PCR product was then individually digested in an aliquot with the MspI enzyme (R0106S, England Biolabs). Indeed, 10U of Msp1 enzyme corresponding to approximately 1µL of enzyme was added to 20 µl of the PCR product contained in an aliquot with 9µL of a buffer solution making a final reaction volume of 30 µl. The mixture was incubated at 37°C for 30mn and then the PCR products (ITS) and digestion products were then migrated onto agarose gels prepared at 1.5% in 0.5x TBE buffer stained with ethidium bromide, through an electrophoresis chain for 30 minutes at 100V. The observed band patterns were then observed and the observed amplicon sizes for each strain were noted and used to differentiate *Candida* species (28). The ITS1-ITS4 region size were presented in **Table 1**.

2.3 Antifungal Susceptibility of *Candida* Strains

The susceptibility of isolates to antifungal agents was determined by the agar diffusion method (23, 29). The inhibition diameters were measured and interpreted to establish the resistance profile of each strain. A reference strain of *C. albicans* ATCC 90028 was used for quality assurance (Ref. 0264P Microbiologics, Paris, France). The antifungals used were Amphotericin B, Nystatin, Fluconazole, Kétoconazole, Clotrimazole and Itraconazole.

2.4 Research for Virulence Factors

Several virulence factors were investigated from the fungal suspensions prepared at approximately 10⁷UFC/ml (according the method of Noumi et al. (30)). Briefly, 2mL of sterile YEPD broth was inoculated with a young colony obtained on SDA + chloramphenicol agar and incubated at 30°C. After 18h of incubation, the culture was then centrifuged and washed twice with sterile PBS at 3000g for 5 minutes. After removing the supernatant from the last wash, 1ml of PBS was added to the pellet and resuspended by vortexing. The resulting fungal

suspension was diluted to obtain a solution with an optical density between 0.4 and 0.5 corresponding to a cell concentration of about 10⁷ CFU/ml. Hydrophobicity, stickiness, biofilm, lecithinase, gelatinase and hemolysin were the virulence factors investigated.

2.4.1 Hydrophobicity Activity

Method described by Zanni et al. (31) which consists of measuring the adhesion of yeast to hydrocarbons such as cyclohexane, was used to evaluate the hydrophobicity of the yeast cell surface (CSH). The Optical Densities of the fungal suspensions were read and adjusted between 0.4 and 0.5 (A0). Then, 200µl of each fungal suspension to be tested was transferred into two wells of a sterile microplate to which 60µl of cyclohexane (BDH Laboratories LTD, England) was added. In the two negative control wells, the *Candida* suspension was replaced by distilled water. The plate was shaken vigorously for 3 min and then allowed to stand for 20 min at room temperature in a hood. Finally, 100µL of the aqueous phases from the reaction between the fungal suspensions and cyclohexane were transferred to a new microplate and the optical densities (A1) read at 620nm with the plate reader. The percentage of cells adhering to the cyclohexane layer (%CSH) was determined by the following formula:

$$\% \text{ CSH} = (1 - A1/A0) \times 100$$

The percentage of CSH was used to assess the importance of cell surface hydrophobicity following the criteria specified by El-Houssaini et al. (32)

2.4.2 Adhesin and Biofilm Activities

The power of adhesion to polystyrene and the ability of isolates to form a biofilm on it were evaluated in this study according to the method of Zanni et al. (31) modified. Thus, we had two sterile microplates (96 wells) (one for adhesion and the other for biofilm). One hundred microliters of RPMI 1640 medium were transferred to the test and negative control wells of a sterile microplate. 100µL of fungal suspension and 100µL of PBS solution, were added to the test and two negative control wells respectively. The treated plates were incubated at 37°C for 2 hours.

The adhesion plate was removed and the wells were washed twice with PBS solution and then stained for 1 min with 100µL of 1% diluted crystal violet. The stained wells were then washed twice with PBS solution. The treated microplate was left at room temperature for 20 min and the wells were destined for 30 min with 100µL of alcohol-acetone mixture. The supernatants were transferred to a new microplate and their optical densities were read at 560nm with a microplate reader (Infinite F200 Pro, Tecan 504).

For the biofilm plate (the second microplate), the wells were washed every 24h of incubation with sterile PBS solution and RPMI medium renewed at each occasion. The incubation time was 66h. The last wash before staining was performed after the incubation time. The adhesion and biofilm formation powers of

TABLE 1 | Size of ITS1-ITS4 region of *Candida* species before and after digestion with MspI.

<i>Candida</i> species	ITS1-ITS4 region	Base pair sizes after MSP1
<i>Candida albicans</i>	540	300-240
<i>Candida glabrata</i>	900	570-330
<i>Candida krusei</i>	510	260-250

the strains were determined from the optical densities read and then interpreted according to the criteria specified by Noumi et al. (30).

2.4.3 Lecithinase Activity

The production of phospholipase or lecithinase and gelatinase was investigated according to the methodology of El-Houssaini et al. (32) and that of Elavarashi et al. (33). 10 μ L of the prepared fungal suspension was seeded onto the agar and incubated at 37°C in the oven for 5-7 days. The presence of an opaque zone around the fungal colony indicates the production of lecithinase. The altering power of this enzyme (Pz) was evaluated according to the formula: $Pz = D0/Dt$ Where, D0 = colony diameter and Dt = colony diameter + halo (large diameter). The different interpretations were made.

2.4.4 Gelatinase

Gelatinase production was demonstrated according to the method described by Elavarashi et al. (33). 4000 μ L of gelatin-enriched nutrient broth in a hemolysis tube, were seeded with a young colony of the strain to be studied previously isolated on YEPD medium. The inoculated tubes were incubated at room temperature (25°C) and the reactions were read every 24 hours for two weeks after 30 minutes of refrigeration at 4°C. A strain of *Staphylococcus aureus* was used as a positive control. A tube containing only the unseeded nutrient broth was used as a negative control.

- A positive result means that the medium liquefies after cooling in the refrigerator.
- A negative result means that the medium solidifies after cooling

2.4.5 Hemolysin

The investigation of hemolysin production by the strains was done by culturing them on 7% fresh blood (GSL) enriched agar medium (24, 32). Indeed, 10 μ L of the prepared fungal suspension was seeded on GSL medium and then incubated at 37°C for 48h in CO₂ enriched atmosphere (5%). The presence of a greenish halo or white halo around the fungal colony shows the presence of hemolysin (alpha or beta respectively).

2.5 Antifungal Activity of Plant Extracts

2.5.1 Medicinal Plants

The medicinal plants used in this study were selected from an ethno pharmacological survey conducted in Benin on medicinal plants used in the traditional treatment of mycoses (22). These plants have been identified in the national herbarium of Benin at the University of Abomey-Calavi. These plants were collected in Houèto in the commune of Abomey-Calavi. **Table 2** presents the plant material and the parts used. Three types of extracts

(aqueous, hydroethanolic and ethanolic) were obtained following the protocol described by Klotoé et al. (34).

2.5.2 Phytochemical Screening of the Studied Plants

A qualitative screening of the three studied plants was carried out in order to assess the main chemical groups present according to the precipitation and colouring reactions described by Houghton and Raman (35). The main groups investigated were polyphenolic compounds (flavonoids, total phenols, tannins), anthocyanins, saponosides, reducing compounds, mucilages, and alkaloids. In the extracts produced, total polyphenols were quantified using the commercial Folin Ciocalteu reagent. Total polyphenol levels were determined using the equation from the calibration curve of gallic acid (0-200 μ g/mL) taken as reference standard. Samples were prepared in triplicate for each analysis. Total polyphenol content was determined in mg gallic acid equivalent/g extract (mg GAE/g) by the formula used by Ahmed et al. (36):

$$TPT = (X \times V) / m$$

With TPT, the Total Polyphenol Content, X the Gallic Acid concentration in mg/mL; V the volume of extract used in mL and m the mass of the extract in grams.

2.5.3 Antioxidant Activity of the Extracts of the Studied Plants

The antioxidant activity of the extracts was evaluated by the DPPH free radical scavenging assay which is one of the most widely used methods for the evaluation of antioxidant activities of herbal extracts. DPPH (2,2- Diphenyl-1-picrylhydrazyl) is a stable purplish colored free radical that absorbs at 517 nm. In the presence of anti-free radical compounds, the DPPH radical is reduced and changes its color to yellow. The method adopted in this study is that of Klotoé et al. (34). Thus 100 μ L of different concentrations of each extract is added to 1900 μ L of the ethanolic solution of DPPH (0.4 mg/mL). The blank is prepared by mixing 100 μ L of the extraction solvent with 1900 μ L of the DPPH solution. After incubation in the dark for 1 hour at room temperature, absorbance readings were taken at 517 nm using a MINDRAY spectrophotometer (BA-88-A). The recorded optical densities were used to calculate the percentage of DPPH radical scavenging which is proportional to the antioxidant power of the sample. Vitamin C and BHT were used as reference standards. Samples were prepared in triplicate for each analysis. The percentage of DPPH radical scavenging was determined by the formula:

$$P = \frac{Ab - Ae}{Ab} \times 100$$

P: Percentage of trapping; Ab: Absorbance of blank; Ae: Absorbance of sample

TABLE 2 | Different parts of the plants used and the identification numbers in the national herbarium.

Medicinal Plants	Botanical Family	Parts Used	Identification number
<i>Cyanthillium cinereum</i> (L.) H. Rob.	Compositae	Whole Plant	YH 361/HNB
<i>Khaya senegalensis</i> (Desv.) A. Juss.	Meliaceae	Bark	YH 382/HNB
<i>Lippia multiflora</i> Moldenke	Verbenaceae	Leafy stem	AA 6750/HNB

2.5.4 Sensitivity of *Candida* Strains to Plant Extracts

For the sensitivity test, seven *Candida* strains isolate for urines were used. These were six clinical strains of *Candida* and a reference strain of *Candida albicans* ATCC 90028. A stock concentration of 100 mg/ml of each of the nine extracts were prepared and tested separately on the *in vitro* growth of a reference strain of *Candida albicans* ATCC 90028 by the diffusion method on Muëller-Hinton agar supplemented with 2% glucose and 0.5% Methylene Blue (MHGB) (37). The fungal suspensions were prepared with Yeast Extract Peptone Dextrose (YPD or YEPD) broth. After inoculating the agar with the fungal suspension, 100 µl of each type of extract from the three plants was sterilely transferred onto Whatman paper cut into small pieces and previously deposited on the agar surface. The same procedure was followed for distilled water and fluconazole (100µg/mL) which served as negative and positive controls respectively. After 24 hours of incubation, the diameters of inhibition observed around Whatman paper were measured and interpreted. The most active plant is the one whose extracts were active on the strain with the largest inhibition diameters. The most active plant extracts were thus identified and tested following the same process on previously characterized clinical *Candida* strains. The anti-*candida* activity of the extracts was tested on the majority resistance profiles of the three most isolated *Candida* species (Table 4).

2.5.5 Determination of the Minimum Inhibitory Concentration

The Minimum Inhibitory Concentrations (MIC₅₀) of the effective extracts were determined following the methodology of Okou et al. (37) in 96-wells microplates. A stock solution of these extracts was prepared at a concentration of 100 mg/ml in distilled water. Fluconazole at 100mg/ml was used as the reference antifungal agent. 100µl of a fungal suspension made with Yeast Extract Peptone Dextrose (YEPD) (38) broth was placed in the first eight wells. The first two wells of the microplate served as positive control (YEPD broth with yeast) and reference (YEPD broth with yeast mixed with 100µl of fluconazole

solution), respectively. The ninth well is the negative control containing only YEPD broth without yeast. Then, 100 µl of the stock extract solution was added to the third well. Five serial two-for-two dilutions were then performed starting from the third well to well 8 where the additional 100 µl was discarded. For each extract the same procedure was followed for six different *Candida* strains. The microplates were covered with aluminium foil and placed for 24 hours in an oven at 37°C. The MIC₅₀ corresponds to the minimum concentration where 50% of the yeasts are inhibited (39). They were estimated by counting the number of yeast using the Malassez cell under a microscope with an with the X40 lens.

2.6 Data Processing and Statistical Analysis

Data were entered using Microsoft Excel 2010. Data analysis was done using Graph Pad Prism version 8. Quantitative variables are presented as mean and standard deviation. Qualitative variables are presented as percentages. Probit analysis was used for the determination of the IC₅₀ and MIC of the most active extract. Student's t test was used to compare the means. The significance level was set at 5%.

3 RESULTS

3.1 Isolation, Identification and Antibiogram of *Candida* Strains

A total of 51 *Candida* strains were isolated and identified from urine samples collected from patients sent to the laboratory for urine cytobacteriological examination. **Figure 1** shows that *Candida albicans* (52.94%) was the most identified species, followed by *Candida glabrata* (17.64%). **Figures 2, 3** show electrophoresis images of the PCR product of the ITS1-ITS4 region and the PCR product of the digestion of the DNA of *Candida* isolates, respectively.

The study on sensitivity of the isolated *Candida* to antifungal drugs showed that all the isolated species are sensitive to

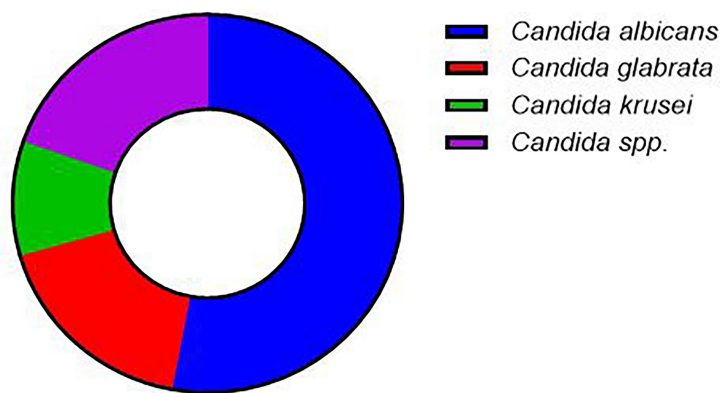


FIGURE 1 | Frequency of identification of different *Candida* species from urine samples.

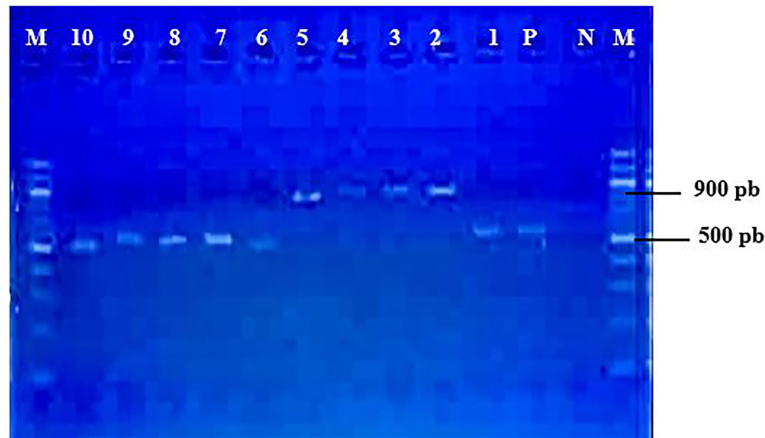


FIGURE 2 | Gel images showing migration band profiles of the ITS1-ITS4 region of *Candida* strains. (M): Gene ruler 100pb; (N): Negative control; (P): Positive control (*Candida albicans* ATCC 90028); (1, 6, 7, 8, 9, 10): *Candida albicans*/*Candida krusei*; (2, 3, 4, 5): *Candida glabrata*.

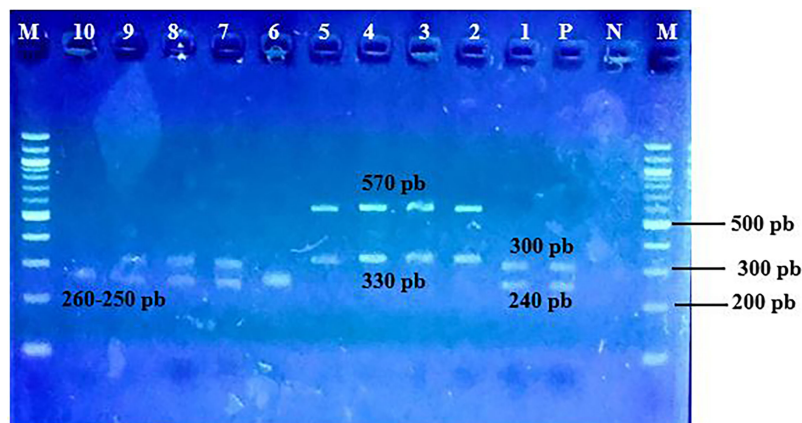


FIGURE 3 | Gel images showing *MspI* restriction fragment profiles of the ITS1-ITS4 region of *Candida* strains. (M): Gene ruler 100pb; (N): Negative control; (P): Positive control (*Candida albicans* ATCC 90028); (1, 7, 8, 9): *Candida albicans*; (2, 3, 4, 5): *Candida glabrata*; (6, 10): *Candida krusei*.

amphotericin B and nystatin, two antifungal drugs of the polyene family and the most used against candidiasis. For the other antibiotics, namely, fluconazole, ketoconazole, clotrimazole and itraconazole, the *Candida* strains showed low levels of intermediate resistance. Only 3.70% of *Candida albicans* strains were resistant to clotrimazole. No *Candida glabrata* strains were resistant to any of the antibiotics tested but showed intermediate resistance levels of 22.22% to 55.55% to fluconazole, ketoconazole, clotrimazole and itraconazole. Eighty percent of *Candida krusei* strains were resistant to fluconazole (**Table 3**).

Table 4 show the antibiotic resistance profiles of *Candida albicans*, *Candida glabrata*, *Candida krusei* and *Candida* spp. strains. *Candida albicans* strains showed 8 different antibiotic resistance profiles, with the most dominant being AmB^S Nys^S Flu^S Ket^S Ctr^S Itr^I. The other strains, notably *Candida glabrata*

and *Candida krusei*, each showed 5 different antibiotic resistance patterns.

3.2 Research of Virulence Factors

The study of virulence factors showed that the majority of the isolated strains showed hydrophobicity (96.08%), hemolysin (90.20%) and adhesin (94.12%) activity. Of all the virulence factors investigated, only gelatinase was absent in *Candida krusei* strains. Regarding the hemolytic power of *Candida* isolated from urine, most strains can degrade haemoglobin. Both types of hemolysis (alpha hemolysis and beta hemolysis) were observed with a predominance of beta hemolysis in all strains (**Table 5a**). In addition, all *Candida* species showed lecithinase activity. Regarding the powers of adhesion, hydrophobicity and biofilm activity, *Candida* isolates were found to be highly adherent

TABLE 3 | Resistance profile of the isolated *Candida* strains to the tested antifungal agents.

<i>Candida</i> species	Numbers	Interpretation of inhibition diameters	Antifungals					
			AMB	NYS	FLU	KET	CLT	ITR
<i>Candida albicans</i>	27	Sensitive (%)	100	100	85.18	92.59	77.78	25.92
		Intermediary (%)	0.00	0.00	14.81	7.41	18.52	74.07
		Resistant (%)	0.00	0.00	0.00	0.00	3.70	0.00
<i>Candida glabrata</i>	09	Sensitive (%)	100	100	77.78	77.78	77.78	44.44
		Intermediary (%)	0.00	0.00	22.22	22.22	22.22	55.55
		Resistant (%)	0.00	0.00	0.00	0.00	0.00	0.00
<i>Candida krusei</i>	05	Sensitive (%)	100	100	0.00	40.00	80.00	20.00
		Intermediary (%)	0.00	0.00	20.00	60.00	20.00	80.00
		Resistant (%)	0.00	0.00	80.00	0.00	0.00	0.00
<i>Candida</i> spp.	10	Sensitive (%)	100	100	70.00	100	70.00	50.00
		Intermediary (%)	0.00	0.00	30.00	0.00	30.00	50.00
		Resistant (%)	0.00	0.00	0.00	0.00	0.00	0.00

AMB, Amphotericin B; NYS, Nystatin; FLU, Fluconazole; KET, Ketoconazole; CLT, Clotrimazole; ITR, Itraconazole.

TABLE 4 | Resistance profile of *Candida* strains to antifungal agents.

Profils	Percentage (%)			
	<i>Candida albicans</i>	<i>Candida glabrata</i>	<i>Candida krusei</i>	<i>Candida</i> spp.
Profil 1 : Am ^B S Nys ^S Flu ^S Ket ^S Ctr ^S Itr ^S	22.22	44.44	-	50.00
Profil 2 : Am ^B S Nys ^S Flu ^S Ket ^S Ctr ^S Itr ^I	40.74	22.22	-	10.00
Profil 3 : Am ^B S Nys ^S Flu ^S Ket ^S Ctr ^I Itr ^I	18.52	-	-	10.00
Profil 4 : Am ^B S Nys ^S Flu ^S Ket ^I Ctr ^S Itr ^I	3.70	-	-	-
Profil 5 : Am ^B S Nys ^S Flu ^I Ket ^S Ctr ^S Itr ^I	3.70	-	-	10.00
Profil 6 : Am ^B S Nys ^S Flu ^I Ket ^I Ctr ^S Itr ^I	3.70	11.11	20.00	-
Profil 7 : Am ^B S Nys ^S Flu ^I Ket ^S Ctr ^S Itr ^S	3.70	-	-	-
Profil 8 : Am ^B S Nys ^S Flu ^I Ket ^S Ctr ^R Itr ^I	3.70	-	-	-
Profil 9 : Am ^B S Nys ^S Flu ^S Ket ^S Ctr ^I Itr ^I	-	11.11	-	-
Profil 10 : Am ^B S Nys ^S Flu ^I Ket ^I Ctr ^I Itr ^I	-	11.11	-	-
Profil 11 : Am ^B S Nys ^S Flu ^R Ket ^S Ctr ^S Itr ^S	-	-	20.00	-
Profil 12 : Am ^B S Nys ^S Flu ^R Ket ^S Ctr ^S Itr ^I	-	-	20.00	-
Profil 13 : Am ^B S Nys ^S Flu ^R Ket ^I Ctr ^S Itr ^I	-	-	20.00	-
Profil 14 : Am ^B S Nys ^S Flu ^R Ket ^I Ctr ^I Itr ^I	-	-	20.00	-
Profil 15 : Am ^B S Nys ^S Flu ^I Ket ^S Ctr ^I Itr ^I	-	-	-	20.00

(AMB, Amphotericin B; NYS, Nystatin; FLU, Fluconazole; KET, Ketoconazole; CLT, Clotrimazole; ITR, Itraconazole; S, Sensitive ; R, Resistant ; I, Intermediate)

TABLE 5A | Frequency of *Candida* strains producing the virulence factors.

<i>Candida</i> species	Numbers	Gelatinase	Hemolysin activity			Lecithinase activity			
			% α-Hemolysis	% β-Hemolysis	Total	% Low	% Moderate	% High	Total
<i>Candida albicans</i>	27	40.74	29.62	37.03	66.67	48.14	3.70	37.03	88.88
<i>Candida glabrata</i>	9	77.77	22.22	55.55	77.78	33.33	0.00	55.55	88.88
<i>Candida krusei</i>	5	0.00	20	80	100	40	0.00	60	100
<i>Candida</i> spp.	10	40.00	20	20	40	50	0.00	20	70
Total	51	43.13%	25.49	41.17	66.67	24	1.96	39.21	86.27

Bold values indicates, Row (Frequency of *Candida* strains for each virulence factors), Column (Total of the frequency for each species).

(94.12%), hydrophobic (96.08%) and biofilm activity (90.20%). All *Candida glabrata* and *Candida krusei* strains were 100% hydrophobic and biofilm producer (Table 5b).

3.3 Phytochemical Screening and Total Polyphenol Content of the Plant Extracts Studied

The phytochemical analysis of the compounds of the three plants studied reveal the presence of several chemical compounds. These

are tannins, flavonoids, anthocyanins, leuco anthocyanins, saponosides, reducing compounds, alkaloids, mucilages and sterols. These different data are presented in the table below. The sign (+) indicates a positive reaction and the sign (-) indicates a negative reaction (Table 6).

The results of the quantification of total polyphenols in the extracts produced are presented in Table 7. From this table it is to be noticed that the aqueous extracts of the studied plants presented a not significantly high content of total polyphenols

TABLE 5B | Frequency of *Candida* strains producing the virulence factors (Second part).

<i>Candida species</i>	Numbers	Adhesin activity				Hydrophobicity activity				Biofilm activity			
		% Low	% Moderate	% High	Total	% Low	% Moderate	% High	Total	%Low	% Moderate	% High	Total
<i>Candida albicans</i>	27	51.85	37.04	11.11	100	11.11	15.52	66.67	96.30	40.74	40.74	3.70	85.18
<i>Candida glabrata</i>	9	88.89	11.11	0.00	100	11.11	0.00	88.89	100	77.78	22.22	0.00	100
<i>Candida krusei</i>	5	80	20.00	0.00	100	0.00	20.00	80.00	100	100	0.00	0.00	100
<i>Candida spp.</i>	10	50.00	20.00	0.00	70.00	0.00	20.00	70.00	90.00	70.00	20.00	0.00	90
Total	51	60.78	27.45	5.88	94.12	7.84	15.67	72.55	96.08	58.82	29.41	1.96	90.20

Bold values indicates, second column (total numbers of candida strains); 6, 10 and 14 column (Total of the frequency for each species) respectively.

TABLE 6 | Phytochemical composition of the three plants used.

Chemical groups	<i>Khaya senegalensis</i>	<i>Lippia multiflora</i>	<i>Cyanthillium cinereum</i>
Tanins	+	+	+
Catechic Tannins	-	-	+
Gallic tannins	+	+	-
Flavonoids	+	+	+
Anthocyanins	+	+	+
Leuco	+	+	+
Anthocyanins	+	+	+
Saponosids	+	-	-
Reducing compound	-	+	+
Sterols/terpenes	+	+	+
Mucilages	+	-	-
Alkaloids	+	+	+

TABLE 7 | Polyphenol content of the different plant extracts.

Plants	Extracts	Polyphenol content (mgEAG/gMS)	Ecart type
<i>Khaya Senegalensis</i>	Aqueous	2692.23301	12.3572059
	Hydro-ethanolic	2259.2233	38.4446405
<i>Lippia multiflora</i>	Ethanolic	2170.87379	94.7385785
	Aqueous	2556.79612	128.377639
<i>Cyanthillium cinereum</i>	Hydro-ethanolic	2250	19.9088317
	Ethanolic	2550.48544	118.079967
	Aqueous	2289.80583	32.2660376
	Hydro-ethanolic	2405.33981	73.4567239
	Ethanolic	2550.48544	118.079967

compared to the hydro-ethanolic and ethanolic extracts (P<0,05) except in the case of *Cyanthillium cinereum* for which the ethanolic extract is higher.

3.4 Antioxidant Activity of the Studied Plant Extracts

All the tested extracts reduced the DPPH radical in variable proportions. This inhibition of the DPPH radical translating the antioxidant activity of the extracts is proportional to the increase of the concentration of the extracts. The DPPH radical inhibitory power of the different extracts and reference standards is expressed in IC₅₀ (Table 8). Since the IC₅₀ is inversely proportional to the antioxidant potential of the extract, the lower the IC₅₀ value, the better the antioxidant power of the extract.

TABLE 8 | Antioxidant activity of plant extracts expressed in IC₅₀.

Plants	Extracts	IC ₅₀ (mg/mL)
<i>Khaya Senegalensis</i>	Aqueous	0.079
	Hydro-ethanolic	0.036
<i>Lippia multiflora</i>	Ethanolic	0.017
	Aqueous	0.0056
	Hydro-ethanolic	0.0049
<i>Cyanthillium cinereum</i>	Ethanolic	0.0042
	Aqueous	0.053
	Hydro-ethanolic	0.016
	Ethanolic	0.053

3.5 Antifungal Activity of Plant Extracts

The study of the sensitivity of the aqueous, hydro-ethanolic and ethanolic extracts of *Cyanthillium cinereum*, *Khaya senegalensis* and *Lippia multiflora* on the reference strain of *Candida albicans* ATCC 90028 showed a good sensitivity of the extracts of *Cyanthillium cinereum* and *Kaya senegalensis*. The largest inhibition diameters were obtained with the extracts of *Cyanthillium cinereum* particularly the hydro-ethanolic (Figure 4).

Figure 5 shows the effect of aqueous, hydroethanolic and ethanolic extracts of *Cyanthillium cinereum* on the *in vitro* growth of clinical strains of *Candida* isolated from urinary tract infections. From this figure, it appears that all the extracts were active on all the strains studied with variable inhibition diameters depending on the extract and the strain. The greatest diameters of inhibition were obtained with the aqueous extract of this plant on most of the strains except for the strains of *Candida krusei* k1 and *Candida glabrata* g1. Regarding *Khaya senegalensis*, these extracts were active on four different clinical strains (*Candida krusei* k1 and k2, *Candida glabrata* g1 and *Candida albicans* a1). The aqueous extract was more active. The hydro-ethanolic extract was not active on any *Candida albicans* strain and on the *Candida glabrata* g2 strain (Figure 6). The aqueous extracts of the two plants (*Cyanthillium cinereum* and *Khaya senegalensis*) were used to determine the Minimum Inhibitory Concentrations (MIC50) on each clinical strain. The MIC50 obtained varied from one strain to another and according to plant. In general, *Cyanthillium cinereum* presented the best anti-*Candida* activity (MIC50 low) compared to *Khaya senegalensis* (MIC50 high) (Table 9). The best anti-*Candida* activity of *Cyanthillium cinereum* was obtained on *Candida albicans* a1 and *Candida glabrata* g1.

4 DISCUSSION

The present study is part of the search for new sources of bioactive molecules useful to fight resistance to classical

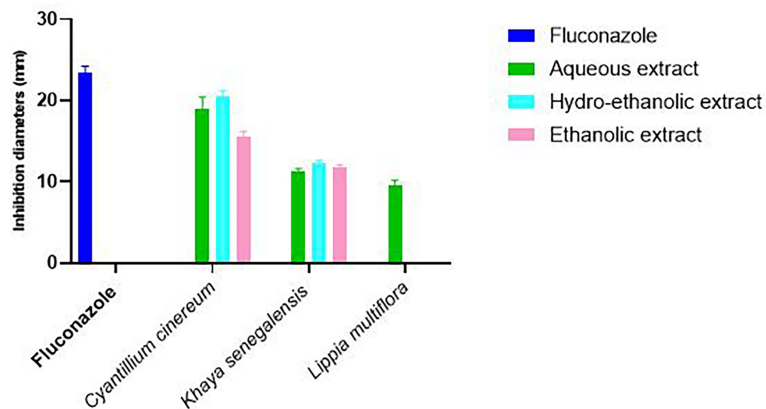


FIGURE 4 | Effect of plant extracts on *Candida albicans* reference strain ATCC 90028.

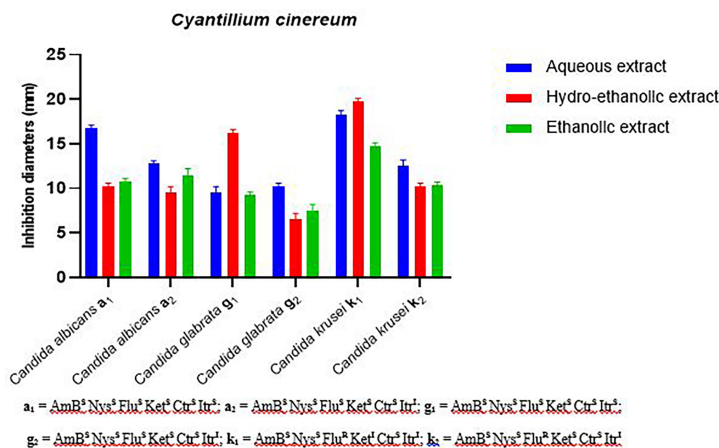


FIGURE 5 | Effect of *Cyantillium cinereum* extracts on the characterized clinical strains.

antifungal agents. It aimed at exploring the virulence factors and the resistance profile of *Candida* species isolated from urine samples in Benin and the *in vitro* efficacy of organic extracts of *Cyantillium cinereum*, *Lippia multiflora* and *Khaya senegalensis* on the growth of *Candida* spp. strains.

The search for *Candida* strains in urine samples from patients in South Benin resulted in the isolation of 51 different *Candida* species. *Candida albicans* (52.94%) was the most identified species followed by *Candida glabrata* (17.65%). The high proportion of *Candida albicans* species found in this study was also mentioned by several reports in the scientific literature (1, 40; 41) for candidiasis in general and urinary candidiasis (6). Indeed, urinary tract candidiasis is known as the most frequent nosocomial fungal infection worldwide (42).

The study of the susceptibility of isolates to antifungal agents showed that all species were susceptible to amphotericin B and nystatin. This sensitivity of the strains to nystatin and amphotericin B obtained in the present study contrasts with

the data reported by Ekpo et al. (43) related of *Candida* strains isolated from urine samples in Cameroon. These differences could be justified by the origin of the strains, the study area, self-medication and the misuse of chemicals against vaginal infections. However, variable levels of resistance have been obtained to other classical antifungal agents (fluconazole, ketoconazole, itraconazole, clotrimazole). Furthermore, the *Candida* species isolated presented different patterns of resistance to antifungal drugs. Similar data reported a variety of resistance of *Candida* strains to antifungal drugs (23, 44). Jensen et al. (45) explained that this observed resistance would be due to the induction of drug efflux pumps and sequestration of antifungal agents. The great variability of resistance to antifungal drugs could be linked to the inactivation of proteins essential to ergosterol biosynthesis, leading to ergosterol depletion (target of amphotericin B) and the formation of other sterols (46)

The search for some virulence factors showed that the *Candida* strains isolated from urine in this study possess

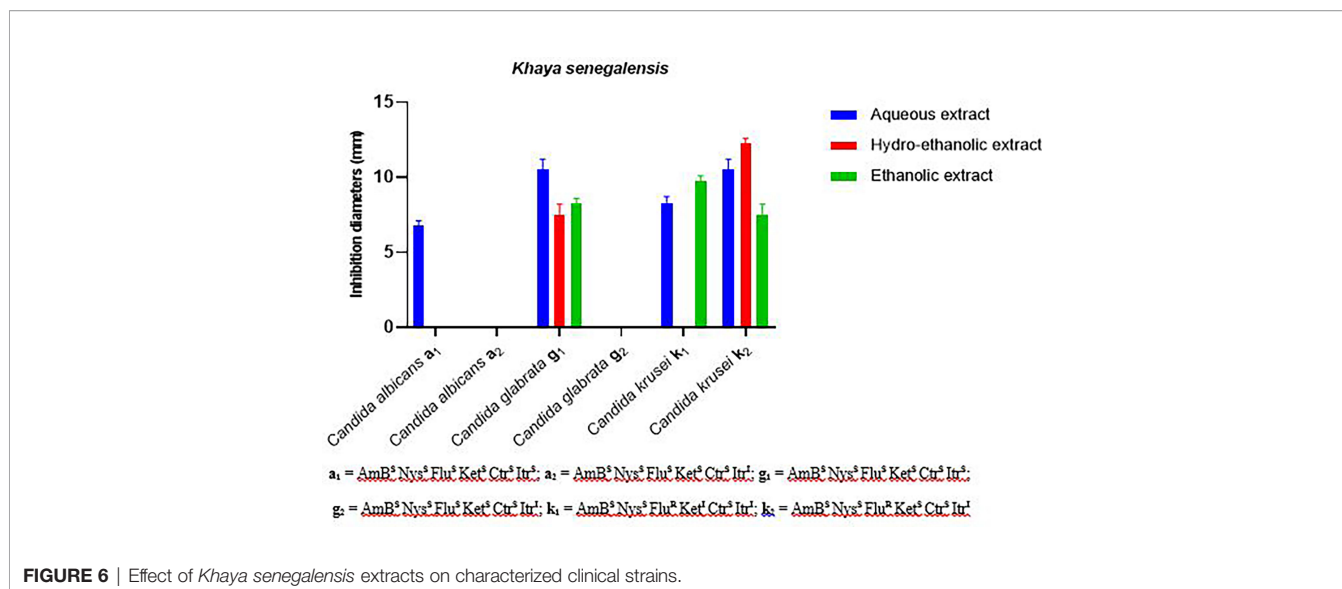


TABLE 9 | Minimum inhibitory concentration (MIC 50) of the aqueous extract *Cyanthilium cinereum* and *Khaya senegalensis* on the characterized clinical strains.

<i>Candida</i> strains	MIC 50 (mg/ml) <i>C. cinereum</i>	MIC 50 (mg/ml) <i>K. senegalensis</i>
<i>Candida albicans</i> a ₁	2.08	77.30
<i>Candida albicans</i> a ₂	62.42	94.11
<i>Candida glabrata</i> g ₁	3.71	76.79
<i>Candida glabrata</i> g ₂	85.36	81.01
<i>Candida krusei</i> k ₁	48.48	135
<i>Candida krusei</i> k ₂	29.08	67.91

several virulence factors such as hemolysin production, biofilm, adhesin, lecithinase, gelatinase and hydrophobicity activity. *Candida albicans* biofilm formation reflects a powerful resistance structure that is installed by many pathogens such as *Candida albicans* to resist antimicrobials (47, 48). According to Pandey et al. (24), the production of hydrolytic enzymes by *Candida albicans* would increase yeast adhesion. Lecithinase being a phospholipase, its activity by *Candida albicans* could destroy cell lipids, one of the main components of the membrane (49) and promote oxidative stress (50–52).

The antifungal tests carried out on the plants studied show a variable activity depending on the plant, the type of extract and the strain tested. Indeed, on *Candida albicans* ATCC 90028, *Cyanthilium cinereum* and *Khaya senegalensis* were more active in inhibiting the growth of this strain. On clinical strains selected, the extracts of these plants showed anti-*Candida* activity depending of the plant. *Khaya senegalensis*, presented a low activity anti-*Candida* (MIC 50 high) compared to *Cyanthilium cinereum* (MIC50 low). The best activity of *Cyanthilium cinereum* was obtained on *Candida albicans* a₁ and *Candida glabrata* g₂. This antifungal activity would be related to the bioactive secondary metabolites such as flavonoids, tannins, coumarins, anthocyanins, leuco anthocyanins identified in the plant (53–56). Belila and Ounis (57) report that alkaloids possess a broad spectrum of biological

activities including antibiotic properties. However, the lowest anti-*Candida* activity of the *Khaya senegalensis* will be related of the resistance profile and pathogenicity of the tested strains.

The scientific data generated by the present study highlight the possibility of using the *Cyanthilium cinereum* as probable sources of new bioactive molecules with anti-*candida* properties for an efficient fight against antifungal drugs resistance.

In conclusion, this study revealed that several *Candida* species are involved in urinary tract infections. The *Candida* species isolated from urine are all-sensitive to amphotericin B, nystatin, and show 15 different resistance profiles. These bacteria also possess several virulence factors, the most represented of which are adhesin, hydrophobicity and Biofilm. *In vitro* antifungal tests of aqueous, ethanolic and hydro-ethanolic extracts of *Khaya senegalensis*, *Cyanthilium cinereum* and *Lippia multiflora* revealed a variation in antifungal power depending on the plant and the type of extract. *Cyanthilium cinereum* have a good *in vitro* activity on the growth of clinical and virulent of *Candida* strains. These results permit new research perspectives on the mechanisms of action of the antifungal effect of the studied plants.

AUTHORS CONTRIBUTIONS

All authors have actively contributed to the manipulation and writing of this article

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Ethics Committee of Research Unit in

Applied Microbiology and Pharmacology of Natural Substances of University of Abomey-Calavi N° 002_CE_URMAPha_UAC. The patients/participants provided their written informed consent to participate in this study

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