Biological properties and antifungal activity of Acalypha wilkesiana, Mitracarpus hirtus, Senna alata on Candida strains isolated from hospital stool samples in Southern Benin

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Abstract

Candidiasis is one of the opportunistic diseases that occur following HIV/AIDS infection. Currently, the management of candidiasis is problematic due to the emergence of resistance to classical antifungal drugs. The search for alternative solutions for the discovery of new bioactive molecules is necessary. This study aims to explore the antifungal properties of Mitracarptus hirtus, Acalypha wikesian and Senna alata on Candida strains isolated from stools of people living with the acquired immunodeficiency virus and collected in some hospitals in Benin and then confirmed by molecular biology techniques at the Research Unit in Microbiology and Pharmacology of natural substances. The strains were tested for different resistance factors. The main phytochemical groups of the plants were searched by staining methods from the dry matter of the studied plants. The determination of total polyphenols and the evaluation of the antioxidant activity of the extracts by the DPPH radical scavenging test were carried out by spectrophotometric methods. The in vitro antifungal activity of the aqueous and hydroethanol extracts of these plants was then evaluated on the strains. The antifungal test data revealed that of the three plants tested, only Mitracarptus hirtus showed inhibitory activity on the growth of the reference strain of Candida albicans ATCC 90028. The hydroethanol extract of this plant was active on all clinical strains with a better effect at the concentration of 100 mg/ml. The data obtained also highlighted the richness in total polyphenols and the anti-free radical activity of the plant extracts studied. This study proved particularly the anti-candidus properties of the hydro-ethanolic extract of the leafy stem of Mitracarptus hirtus. Further studies will generate data on the in vivo efficacy and mechanism of action of the anti-candidosis effect of Mitracarptus hirtus.

Background

Immunosuppression such as that caused by the human immunodeficiency virus (HIV) reminds and highlights the important function of the immune system. Its collapse in people living with HIV (PLWHIV) is usually the cause of the emergence of opportunistic infections [1]. Widespread use of cotrimoxazole for prophylaxis in people living with HIV (PLWHIV) is sometimes the basis for the elimination of the normal bacterial flora and promotes the proliferation of gastrointestinal opportunistic fungi [2]. It is now scientifically recognized that *Candida* is an opportunistic fungus of the gastrointestinal tract of people living with HIV (PLWHIV) [2]. In addition to the phenomenon of gastrointestinal colonization, the numerous cases of treatment failure related to the emergence and increase of *Candida* strains resistant to existing synthetic antifungals is a real problem in the monitoring of PLWHIV [3]. The species spectrum and resistance of *Candida* isolates to currently available antifungal drugs is therefore a very relevant factor as it has important implications for morbidity and mortality [4]. To date, there is no cure for HIV/AIDS. As a result, the control of opportunistic pathogens associated with this pandemic remains the safest way to manage the infected individuals who have the disease [4]. This poses a crucial public health problem and alternative solutions are needed. Favourably, today, many plant species with medicinal properties used in the treatment of various ailments have been identified [5]. The ability of several medicinal plant extracts to reduce the growth of fungal species has also been highlighted in many studies [6]. Thus, the use of plants with antimicrobial activity could be an excellent control alternative to known synthetic drugs. *Acalypha wilkesiana, Mitracarptus hirtus* and *Senna alata* are three plant species, indicated in traditional medicine recipes for the treatment of candidiasis in southern Benin [5]. Unfortunately, very little scientific data exists at this stage on the antifungal properties of the three selected plants. This study aims to explore the antifungal properties of these plants on Candida strains isolated from stools collected from PLWHIV.

Materials And Methods

Plant material

The plant material used in this study consisted of three plants including *Acalypha wilkesiana* (AA6752HNB), *Senna alata* (YH410HNB) and *Mitracarpus hirtus* (YH388HNB) collected in the commune of Abomey-Calavi (Benin) in their natural habitat and authenticated at the National Herbarium of Benin.

Biological material

The biological material consisted of eight *Candida* isolates collected from stool of people living with HIV (PLWHIV) and a reference strain of *Candida albicans* ATCC 90028 (Ref. 0264P Microbiologics, Paris, France).

Phenotipic identification of *Candida* strains

Phenotypic identification of the strains was performed using the technique of Ghaddar et al. [7].

Identification of *Candida* strains on chromogenic media ChromAgar and Tetrazolium Reduction Medium

The method of Giri and Kindo. [8] was used. Indeed, the coupled aspects of two chromogenic media were concomitantly noted. These chromogenic media allow the identification of species of the genus *Candida* from the different colors, morphologies, textures presented by the colonies. Thus, a fraction of isolated colonies of each strain was plated on Chromagar Candida (Conda, Spain) and Tetrazolium Reduction

Medium (TRM), then the cultures were incubated at 37°C for 48h. Aspects of the derived colonies were recorded, and the strains identified. The following correspondence table (Table I) was used for strain classification.

Table I: Identification aspects of Candida strains on ChromAgar (Conda, Spain) and on TRM medium

Candida strains	Colony appearance	
	ChromAgar	**TRM**
Candida albicans	Petites colonies vert clair	Small to medium pinkish colonies
Candida glabrata	Small pinkish-gray colonies rosâtre	Small shiny pink colonies
Candida Parparapsilosis	Pink-orange colonies	Pink-orange colonies
Candida krusei	Large pale pink colonies	whitish outline Small to medium pinkish and dry colonies
Candida tropicalis	Blue or metallic blue	violet colonies Dry brown to violet colonies
Candida spp.	small to medium pink colonies	purple colonies

Blast test (filamentation test)

Each isolate was cultured in blood serum (filamentation test) and incubated at 37°C. This simple test is used to study the capacity of yeasts to produce germ tubes, which is considered a virulence trait. Indeed, it is performed by emulsifying a portion of the yeast colony to be studied in 0.5ml of human blood serum contained in a test tube and incubated for 3h [9]. Then, a drop of the suspension is observed between slide and coverslip under a light microscope at X40 objective after the incubation time. A positive result is the appearance of filament starting from a cell without constriction. It allows to differentiate *Candida albicans* from *non-Candida albicans.*

Research of virulence factors

Several types of virulence factors were investigated including phospholipase production, gelatinase production, hemolysin production, biofilm production or RCA test, adhesion power and hydrophobicity power. For these tests, a suspension was prepared by taking an isolated fresh colony obtained on SDA + chloramphenicol agar in 2ml of sterile YEPD broth and incubated at 30°C. After 18h incubation, the culture was centrifuged and washed twice with sterile PBS at 3000g for 5 minutes. After removing the supernatant in the last wash, 1ml of PBS was added to the pellet and resuspended by vortex. 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.

Production of phospholipases or lecithinase

The fungal suspension made at 107 CFU is inoculated on a petri dish containing SDA, yeast extract, NaCl, $CaCl_2$ added with 10% egg yolk. The dish is placed at 37°C in the oven for 120h to 7 days. An opaque zone around the grown colony leads to a positive result. The ratio of the colony diameter to the opaque zone is compared with the reading grid described by Sriphannam et al. [10].

- Pz < 0.40 corresponds to high production,

- Pz between 0.41-0.60 corresponds to moderate production,

- Pz between 0.61-0.80 corresponds to low production,

- Pz between 0.81-0.99 corresponds to a very low production,

- Pz =1 corresponds to no production,

Gelatinase production

The gelatinase production assay was put according to Elavarashi et al. [11]. An 18h young colony isolated on YEPD (Yeast Extract Peptone Dextrose) agar was taken, seeded into 4 ml of sterile nutrient gelatin agar (5 ml special peptone 3g/l beef extract and 120g/l gelatin) in tube

and incubated at room temperature (25 ^{o}C) for 2 weeks and examined at the regular time interval to check the liquefaction of gelatin. A strain of Staphylococcus aureus was used as a positive control, the negative control is made up of tubes containing only the agar medium. Gelatin liquefies at over 28 ^{o} C generally, to confirm gelatin liquefaction due to gelatin production, test and control tubes were placed at 4 ^{o}C in the refrigerator for 30 minutes. The tubes were tilted to observe the liquefaction of the gelatin. The hydrolyzed gelatin remains liquid even after gelatin production is demonstrated by cold exposure while the uninoculated tubes remained in the solid state. Thus we have gelatinase positive : liquefied medium and gelatinase negative : solid medium.

Hemolysin research on blood agar

This research of hemolysin production by the strains is done on fresh blood agar and incubated at 37o C for 48h. The test is positive if there is a clear zone around the colony.

Assessment of adhesion strength and biofilm formation

At this level, a cell suspension is prepared according to the modified method of Noumi et al. [12]. Indeed, it was performed a dilution of the fungal cell concentrate in PBS (centrifugation pellet + 500µL of PBS) and in RPMI 1640 medium to have the density of the suspension obtained adjusted to 0.38 at 520 nm corresponding to 10^{7}UFC/ml (50µL of the PBS suspension). This cell concentration is the one required for the evaluation of adhesion and biofilm formation power according to Nakamura-Vasconcelos et al. [13]. Four plates (96 wells) with flat bottom were used two by two for adhesion power and biofilm formation respectively with RPMI 1640 medium and YEPD medium. For each type of medium, the attached plating plan was followed. Introduction into wells A1 and A2 (negative control). Introduction of 200µL of sterile RPMI in wells A3 and A4 (or sterile YEPD) as a second negative control. Three consecutive wells are used for one strain. Thus, the first sample occupied wells A5, A6 and A7. In the first well A5, it was transferred 200µL of the suspension made with PBS. As for the next two wells A6 and A7, 200µL of the suspension made with RPMI 1640 medium (or YEPD) was transferred. The treated plates were incubated for 2 hours at 37°C on a rotary shaker at a speed of 75 rpm. After the incubation time, the RPMI 1640 medium was aspirated, and the non-adherent cells were washed twice with PBS.

Adhesion power

It was transferred in all the wells of one of the plates initially prepare 100µL of a 1% Crystal violet solution to stain them. After the minute of action of the dye, the wells were immediately washed with PBS and dried in the oven for 1h. The evaluation of the adhesion is done either directly by comparing the stains of the wells or indirectly by reading the OD (570 nm) of the supernatant of the well wash solution to which 200µL of glacial acetic acid or alcohol-acetone has been added. Thus, the following classifications were considered by comparing the optical densities read according to Noumi et al. [12]. The power of adhesion was determined according to the following criteria. Non-adherent (0) = OD $\leq$ Doc ; Low adhesion (+) = ODc < OD $\leq$ 2 x ODc ; Medium adhesion (++) = 2 x ODc < OD $\leq$ 4 x ODc, High adherence (+++) = 4 x DOc < DO.

Biofilm formation

Quantitative assessment of biofilm formation was done according to the modified protocol of Noumi et al. [12]. Considering here the second plate, a total of 100µL of YNB medium was transferred to each washed well using a pipette. The plate was incubated at 37°C on a shaker at 75rpm. During the 66 hours that biofilms were growing, the used medium was renewed daily by suctioning it with a new medium. The control wells contained only YNB medium. The wells were washed twice with PBS. Biofilms formed were quantified by colorimetry. Indeed, 100µL of a 1% Crystal violet solution was added to all wells to stain any biofilms formed. After one minute the dye was completely removed and 200µL of alcohol-acetone mixture was added to decolorize. The biofilms formed were quantified directly by comparing the shades of the wells and indirectly by reading the OD of the supernatant of the well wash solution at 570 nm with a spectrophotometer. All tests were repeated three different times. As in the case of adhesion power, the yeasts tested were classified on the basis of the same classification scale.

Assessment of cell surface hydrophobicity (CSH)

The hydrophobicity of *Candida* strains was measured using the protocol described by several authors ([14] ; [12]). It consists in measuring the adhesion of yeast to hydrocarbons, such as cyclohexane. Thus, as in the protocol for biofilm formation, suspensions of the different strains to be tested were prepared with phosphate buffered saline (PBS) and concentrated to obtain a solution with a density corresponding to OD620 varies between 0.4 and 0.5 (A0). For host cell surface adhesion assays, 100µL of each fungal suspension (obtained by diluting in 500µL the centrifugation pellet) was transferred to the wells of a sterile microplate (96 wells). Then, 300µL of cyclohexane was added to the wells except for the first three wells which served as negative control wells. After this treatment, the microplate was shaken for 3 minutes and left to rest for 15 to 20 minutes at room temperature. The aqueous phase is finally transferred to a new microplate and the ODs read at 620nm (A1) using a plate reader. The absorbances (A1) correspond to the measured OD of the test wells and are compared to those obtained for the control wells (negative control) OD(A0). The percentage of cells in the cyclohexane layer (stuck cells) denoted by %CSH was used to estimate the amount of

absorption of the cyclohexane layer at 620 nm (A1). It was compared to that obtained before the mixing procedure (A0) of hydrophobicity using the serial dilutions of yeast mixtures will be set up on CHROMagar *Candida* to differentiate and recognize them. The presence of budding yeast cells with pseudohyphae under direct microscopy and yeast growth were considered as *Candida* strains.

Susceptibility of strains to modern antifungal agents

The sensitivity of isolates to antifungal drugs was determined by the agar diffusion method [15] (. A suspension with a density of about 0.5 Mc Farland was prepared from young colonies of the isolates to be tested. The suspension thus prepared was plated on Mueller Hinton khanagar supplemented with 2% glucose and 0.5% methylene blue (MHGB) by flooding. The excess suspension was carefully removed and the surface of the agar plates was dried before the antifungal impregnated discs were deposited. The treated MHGB agar plates were then incubated at 37°C for 24 h and the inhibition diameters were measured with a flat ruler.

Interpretations of the inhibition diameter measurements were made according to the grid in the table II [15].

Table II: Interpretation of inhibition diameter measurements

Antifungals	Disc concentration (mg)	Activity zone (mm)		
		Sensitive	Intermediate/ SDS	Resistant
Nystatine	100	≥15	10-14	≤10
Fluconazole	100	≥ 20	10-19	≤ 10
Amphotéricine B	50	>10	=10	< 10
Clotrimazole	10	≥ 20	10-19	≤ 10
Ketoconazole	10	≥ 20	10-19	≤ 10
Itraconazole	10	≥ 20	10-19	≤ 10

Sensitivity of *Candida* strains to plant extracts

Mueller Hinton agar supplemented with 2% glucose and 0.5% Methylene Blue (MHGB) and Yeast Extract Peptone Dextrose (YPD or YEPD) broth were prepared and sterilized. Three young 24-hour colonies of the test strain, grown on SDA + Chloramphenicol agar was picked and emulsified in 5mL of 9‰ saline. The resulting Candida suspensions were adjusted to density 0.5 on the Mc Farland scale corresponding to 1.5 x 105 CFU/mL. 200 mg of each extract was weighed and dissolved completely in 1mL of sterile distilled water. The resulting suspension was then thoroughly vortexed, sterilized by filtration using a syringe to which a millipore filter gauge (0.20µm and 0.22µm) is fitted, and stored in a sterile 50mL dark bottle in the refrigerator before each use. The most active extracts on inhibition of fungal strains were determined in agar medium. Three Mueller-Hinton agar plates supplemented with 2% (2 g per 100 ml) Glucose and 0.5% Methylene Blue (MHGB) were plated with the *Candida* suspension for each type of extract. Five 7 mm diameter wells were then sterilely made in the agar media using Pasteur pipettes. The three peripheral wells (two for the extracts and one for the positive control) were used as test wells and the central well used for the negative control. Thus, 100µL of each extract dilution (aqueous and hydroethanol extracts) of *Acalypha wilkesiana* Müll. & Arg., *Senna alata* L. and *Mitracarpus hirtus* (L.) DC prepared at 200 mg/mL were respectively transferred sterilely into two consecutive wells. 100 µL of Fluconazole (100µg/mL) was transferred to the last peripheral well and 100 µL of distilled water to the center well (negative control well). The treated plates were covered, left for two hours on the bench (to facilitate the diffusion of the different extracts) and then incubated at 37°C for 24 hours. The

inhibition diameters observed around the wells after incubation were then measured for each type of extract studied. The most active extract corresponds to the one with the largest inhibition diameter.

Determination of the Minimum Inhibitory Concentration (MIC)

The antifungal activity of the effective extracts was determined following the methodology of Wayne, [17]. A stock solution of these extracts was prepared at the concentration of 100mg/ml in distilled water. Fluconazole at 100mg/ml was used as an antifungal agent for the positive control. Indeed, 10 µl of the fungal suspension to be tested was added to the first wells of each line of the microplate. The first two wells of each line of the microplate served respectively as negative (only the fungal suspension) and positive control (fungal suspension mixed with 100 µl of fluconazole solution). Then, 100 µl of the stock extract solution was placed in the 3rd well. Five serial dilutions of reason two were then performed starting from the 3rd well up to well 8 where the additional 100 µl was discarded. For each extract the same procedure was followed for the six different *Candida* strains. The microplates were covered and incubated at 37°C for 24 hours. The MICs correspond to the minimum concentrations where 50% of the yeasts are inhibited. They were estimated by counting the number of yeast using the Malassez cell under the microscope.

Data processing and statistical analysis

The data were entered using Microsoft Excel 2010. Graph Pad Prism version 8.0 was used to perform the graphs and statistical tests. Each mean was presented with standard variations for each fungal strain. With ANOVA, the means of the inhibition diameters of each extract on the fungal strains were compared with each other by Dunnett's ' multiparametric test (ANOVA). The significance level was set at 5%.

Results

Isolation, identification and antibiogram of *Candida* strains

A total of 50 *Candida* strains were isolated and identified from the collected samples (Figure 1). *Candida krusei* (36.00%) was the most identified species followed by *Candida albicans* (18.00%).

Figure 2 presents the results of the sensitivity of the isolated *Candida* strains to antifungal agents. It can be seen from this figure that all the species isolated were sensitive to amphotericin B, an antibiotic of the polyene family widely used against candidiasis. Regarding the other antifungal agents, the strains studied showed variable levels of resistance. In fact, the highest resistance levels were obtained for fluconazole (58%), itraconazole (28%) and nystatin (20%).

Table III presents the resistance profile of each fungal species to the different antifungal agents. Analysis of the data in this table shows that 11% of *Candida albicans* strains were resistant to fluconazole while the majority of *Candida glabrata* strains were resistant to itraconazole (66%). However, 50% of these strains were resistant to nystatin and fluconazole respectively. Concerning *Candida krusei* strains, 100% were resistant to fluconazole. As for *Candida dubiniensis*, 40% of them were resistant to fluconazole. Similarly, the majority of *Candida spp* strains were also resistant to fluconazole.

Table III: Resistance profile of isolated *Candida* strains to the conventional antifungals tested

Species	Numbers	Interpretation of inhibition diameters	Antibiotics					
			AMB	NYS	FLU	KET	CLT	ITR
Candida albicans	09	Sensitive (%)	100	100	89.89	100	100	100
		Intermediary (%)	0.00	0.00	0.00	0.00	0.00	0.00
		Resistant (%)	0.00	0.00	11.11	0.00	0.00	0.00
Candida glabrata	06	Sensitive (%)	100	50.00	50.00	33.33	33.33	33.33
		Intermediary (%)	0.00	0.00	0.00	33.33	50.00	0.00
		Resistant (%)	0.00	50.00	50.00	33.34	1667	66.67
Candida krusei	18	Sensitive (%)	100	77.78	0.00	55.56	72.22	61.11
		Intermediary (%)	0.00	0.00	0.00	33.33	16.67	16.67
		Resistant (%)	0.00	22.22	100	11.11	11.11	22.22
Candida dubliniensis	05	Sensitive (%)	100	80.00	40.00	60.00	80.00	80.00
		Intermediary (%)	0.00	0.00	20.00	20.00	0.00	0.00
		Resistant (%)	0.00	20.00	40.00	20.00	20.00	20.00
Candida spp.	12	Sensitive (%)	100	83.33	50.00	75.00	75.00	66.67
		Intermediary (%)	0.00	0.00	08.33	08.33	08.33	0.00
		Resistant (%)	0.00	16.67	41.67	16.67	16.67	33.33

Legend: AMB: Amphotericin B, NYS: Nystatin, FLU: Fluconazole, KET: Ketoconazole, CLT: Clotrimazole, ITR: Itraconazole

Tables IV and V present the frequencies of *Candida* strains producing virulence factors. From these tables, it appears that all the isolated strains produce about 40% of the virulence factors such as gelatinase, hemolysin, lecithinase, adhesin, hydrophobicity and biofilm.

Table IV: Frequency of Candida strains producing gelatinase, hemolysin and lecithinase

Species	Number	Proportions (%)							
		Gelatinase	Hemolysin			Lecithinase			
			Hemolysis type			Degree of production			
			α-Hemolysis	β-Hemolysis	Total	Low	Medium	Strong	Total
Candida albicans	09	22.22	22.22	66.67	**88.89**	22.22	0.00	66.67	**88.89**
Candida dubliniensis	05	0.00	0.00	80.00	**80.00**	60.00	0.00	20.00	**80.00**
Candida glabrata	06	0.00	66.67	0.00	**66.67**	33.33	0.00	66.67	**100**
Candida krusei	18	16.67	44.44	44.44	**88.88**	50.00	0.00	33.33	**83.33**
Candida spp.	12	08.34	33.33	33.33	**66.66**	58.33	8.33	8.34	**75**
Total	**50**	**47.23**	**36.00**	**44.00**	**78.22**	**52**	**8.33**	**58**	**85.44**

Table V : Frequency of *Candida* strains producing adhesin, hydrophobicity and biofilm

Species	Number	Proportions (%)											
		Adhesin				Hydrophobicity				Biofilm			
		Degree of production				Degree of production				Degree of production			
		Low	Medium	strong	Total	Faible	Medium	Strong	Total	Low	Medium	strong	Total
Candida albicans	09	0.00	55.56	44.44	100	0.00	55.57	33.33	88.90	0.00	55.56	44.44	100
Candida dubliniensis	05	0.00	60.00	40.00	100	20.00	20.00	20.00	60.00	0.00	60.00	40.00	100
Candida glabrata	06	0.00	33.33	66.67	100	0.00	50.00	33.33	83.33	0.00	33.33	66.67	100
Candida krusei	18	0.00	61.11	38.89	100	0.00	22.22	44.44	66.66	0.00	61.11	38.89	100
Candida spp.	12	8.33	66.67	25.00	100	25.00	41.67	8.33	75.00	8.33	66.67	25.00	100
Total	50	8.33	58.00	40.00	100	08.00	36.00	30.00	74.78	8.33	58.00	40.00	100

Phytochemical screening of plants tested on *Candida* strains

Table VI shows the major chemical groups contained in the plant organs studied. Gallic tannins and leuco anthocyanins were identified in all plants studied. In contrast, reducing compounds, sterols and alkaloids were absent in all plant species studied. It should be noted that reducing compounds, terpenes, sterols and alkaloids were present only in the leafy stems of *Senna alata*. Flavonoids were identified only in the leafy stems of *Senna alata* and the whole plant of *Mitracarpus hirtus*.

Table VI : Major chemical groups contained in the plant organs studied

	Acalypha wilkesiana	*Senna alata*	*Mitracarpus hirtus*
Tanins	+	+	+
Catechic tanins	-	-	-
Gallic tanins	+	+	+
Flavonoids	-	+	+
Anthocyanes	-	+	+
Leuco-anthocyanes	+	+	+
Saponosides	-	-	+
Reducing compounds	-	+	-
Sterols/ terpenes	-	+	-
Mucilages	-	-	-
Alkaloids	-	+	-

Legend : (+) = Presence ; (-) = Absence

Total polyphenol contents of the studied plant extracts

Table VII presents the total polyphenol contents of the extracts. Analysis of the data presented in this table shows that *Acalypha wilkesiana* is significantly richer (p 0.05) regardless of the type of extract in total polyphenols than *Senna alata* and *Mitracarpus hirtus*.

Table VII : Polyphenol contents of plant extracts

	Content ± Standard deviation (mgEAG/g dry matter)		
Extract type	***Acalypha wilkesiana***	***Senna alata***	***Mitracarpus hirtus***
Aqueous	$23369.83^{b,c}$ ±41.25	26[36 ± 71.39	2414.56 ± 124.95
Hydro-ethanolic	$10956.43^{b,c}$ ±58.08	2608.25 ± 135.24	2690.29 ± 20.59

For each type of extract, the letters (a,b and c) marked indicate significant differences at the 5% threshold. a: significantly different from *Acalypha wilkesiana*; b: significantly different from *Senna alata* and c: significantly different from *Mitracarpus hirtus*.

Antiradical activity of the studied plant extracts

Table VIII presents the inhibitory concentrations of the DPPH radical (IC_{50}) of the studied extracts. From this table, we observe a variation of the antiradical potential according to the plant and the type of extract. A comparison between the types of extracts of the studied plants reveals that the aqueous extract of *Acalypha wilkesiana* was more active on the inhibition of the DPPH radical compared to the hydro-ethanolic extract which presented the best antioxidant power for *Senna alata* and *Mitracarpus hirtus*.

Table VIII: Antiradical activity of the extracts of the studied plants

Plantes/Molécule de référence	Type d'extrait	IC50 (mg/mL)
Acalypha wilkesiana	Aqueous extract	0.01
	hydro-ethanolic extract	0.06
Senna alata	Aqueous extract	0.06
	hydro-ethanolic extract	0.03
Mitracarpus hirtus	Aqueous extract	0.07
	hydro-ethanolic extract	0.03
Galic acid		0.03

Antifungal activity of the plant extracts studied

From figure 3 presenting the antifungal activity of the plants on the reference strain, we note that only the aqueous and hydro-ethanolic extracts of Mitracarpus hirta had shown inhibitory activity on the growth of the strain of *Candida albicans* ATCC 90028. However, the anti-candidus activity of these extracts remains lower than that of fluconazole used as reference molecule.

From figure 4, it appears that the hydroethanolic extract of Mitracarpus hirta inhibited the growth of all the clinical strains tested with variable diameters of inhibition according to the concentrations tested (100, 50 and 25 mg/ml). A better anti-candidosis effect was obtained for this extract at the concentration of 100 mg/ml.

The Minimum Inhibitory Concentrations (MIC) were determined according to each antifungal strain (Table IX). The data obtained showed that the lowest MIC was 12.5 mg/ml. These MICs were obtained for *Candida albicans* ATCC 90028 and *Candida krusei*.

Table IX: Minimum Inhibitory Concentration of the hydroethanol extract

Candida strains	MIC (mg/ml)
Candida albicans ATCC 90028	12.5
Candida albicans 1	50
Candida glabrata 1	25
Candida albicans 2	25
Candida albicans 3	50
Candida glabrata 2	50
Candida albicans 4	50
Candida glabrata 3	50
Candida krusei 1	12.5

Discussion

This study aimed at exploring the anti-candidus properties of aqueous and hydro-ethanolic extracts of *Acalypha wilkesiana*, *Mitracarpus hirtus* and *Senna alata* on candida strains from stool samples collected from PLHIV in hospitals in Southern Benin. From these samples, 50 *Candida* strains were isolated and identified. *Candida krusei* (36.00%) was the most identified species followed by *Candida albicans* (18.00%). The high proportion of *Candida krusei* found in this study could be explained by the emergence in recent years of new *Candida* species responsible for candidiasis in women ([16] ; [18]). The high frequency of *Candida albicans* has also been mentioned in several reports in the scientific literature ([20] ; [13]).

According to these studies, *Candida albicans* is the most virulent *Candida* species responsible for candidiasis in women. Wang et al. [18] pointed out that candidiasis is a major public health problem that is very recurrent in the lives of women complaining of abnormal vaginal discharge.

The study of the susceptibility of isolates to antifungals showed that all species were susceptible to amphotericin B, a polyene antibiotic widely used in treatment. However, regarding the other antifungal agents, the strains studied showed variable levels of resistance. In fact, the highest levels of resistance were obtained for fluconazole (58%), itraconazole (28%) and nystatin (20%). For fluconazole the most affected strains were *Candida krusei* (100%), Candida dubiniensis (40%), *Candida spp* (41,67%) and Candida albicans (11%). The resistance observed to itraconazole was much more related to *Candida glabrata* (66%), *Candida spp* (33%), *Candida krusei* (22%) and *Candida dubliniensis* (20%). These last four strains, with the exception of *Candida spp*, were also the most resistant to nystatin. In the scientific literature, the works of some authors have reported resistance of *Candida* strains especially *Candida glabrata* to fluconazole, ketoconazole, clotrimazole and itroconazole used in our study ([15] ; [19]). The high resistance observed for fluconazole could be explained by acquired resistance due to fluconazole being a fungistatic and not a fungicide [21]. One known resistance mechanism involves the ERG11 gene. Indeed, several mutations associated with this gene promote its hyperactivity thereby increasing ergosterol production. Increased production of this azole decreases the activity of fluconazole and thus promotes resistance ([21] ; [22]).

Furthermore, the *Candida* species isolated in this study exhibit 15 different antifungal resistance patterns including eight resistance patterns observed for *Candida krusei* species, 6 *Candida spp* and 5 for *Candida glabrata*. This wide variability in strain resistance to antifungals could be related to the inactivation of proteins essential for ergosterol biosynthesis leading to ergosterol depletion (target of amphotericin B) and formation of other sterols [23]. In addition, the increasingly frequent use of antifungal molecules in hospitals could promote resistance to antifungal agents and a risk of toxicity [23].

Part of this study was also devoted to the search for virulence factors (hemolysin production, biofilm formation, adhesin, lecithinase, gelatinase and hydrophobicity) of the identified strains to establish their pathogenicity. The results obtained showed that all the isolated strains except *Candida glabrata* and *Candida dubliniensis* are producers of all the investigated factors but in variable proportions. Biofilm, adhesin and lecithinase are the factors most produced by the identified strains. *Candida albicans* biofilm formation reflects a powerful resistance structure that is installed by many pathogens such as *Candida albicans* to resist against antimicrobials ([24] ; [25]). Lecithinase is a hydrolytic enzyme produced by pathogens allowing them to destroy the cell wall structures they adhere to and use it to colonize others by producing biofilms [14]. The results of this study corroborate those of other authors who had also demonstrated a high production of this enzyme useful for the manifestation of virulence in *Candida* strains ([26] ; [26]). Regarding hydrophobicity, most of the isolated *Candida* strains were hydrophobic. The expression of this virulence-indicating trait of *Candida* strains found in the present study may well explain the high involvement of these

species in candidiasis cases. Regarding the production of hemolysin, our results are close to those of other studies that had also shown that *Candida* isolates produced this toxin to express their pathogenicity ([25] ; [26]).

The above mentioned data evoke the need to explore alternative solutions for the discovery of new bioactive molecules with antifungal properties in order to fight effectively against the resistance to classical antifungals. In this perspective, the in vitro antifungal effects of aqueous and hydroethanolic extracts of *Acalypha wilkesiana*, *Mitracarpus hirtus* and *Senna alata* on *Candida* strains from samples collected from hospitals in South Benin were explored. The results obtained showed a variable antifungal activity depending on the plant, the type of extract and the strain tested. Indeed, on *Candida albicans* ATCC 90028, only *Mitracarpus hirtus* was active on the inhibition of the growth of this strain with a better effect for the hydro-ethanolic extract. The effect of this extract was tested on clinical strains. The data obtained showed that the hydroethanolic extract of *Mitracarpus hirta* inhibited the growth of all the clinical strains tested with variable diameters of inhibition according to the concentrations tested (100, 50 and 25 mg/ml). A better anti-candidosis effect was obtained at the concentration of 100 mg/ml. The strains of *Candida krusei* 1, *Candida glabrata* 1, *Candida albicans* 3 are the most sensitive to this extract. The antifungal activity of *Mitracarpus hirta* revealed by the present study would be related to the secondary bioactive metabolites (tannins, flavonoids, anthocyanins, leucoanthocyanins, saponosides) identified in this plant. Indeed, it is documented that the secondary metabolites identified in the plants used in this study are responsible for the biological properties of medicinal plants. According to [28], phenolic compounds including flavonoids, tannins, leucoanthocyanins present in medicinal plants are responsible for the observed antifungal effects [29].

Moreover, considering the pathogenicity of the studied strains expressed by virulence factors such as the production of hemolysins, biofilm formation, adhesin, lecithinase, gelatinase and hydrophobicity, it can be suggested that the anti-candidus activity of the tested extracts also prevents the possible effects of these strains related to their virulence factors. Indeed, according to Pandey et al. [27] , the production of hydrolytic enzymes by *Candida albicans* would enhance yeast adhesion. As lecithinase is a phospholipase, its production by *Candida albicans* could destroy cell lipids, one of the main membrane constituents [30] and promote oxidative stress ([31] ; [32]).

The antioxidant activity of Mitracarpus hirtus extracts demonstrated in the present study would help prevent oxidative stress that these candida strains could induce in their mechanism of action. However, the results of the anti-candiddosic tests concerning the extracts of *Acalypha wilkesiana* and *Senna alata* revealed their inactivity on the reference strain (*Candida albicans* ATCC 900258). These results are not in agreement with some data in the literature that reported the anti-candidosis effect of these two plants ([33] ; [34]). This difference may be explained by the nature of the strain tested or even the condition of the plant used for extraction.

Conclusion

This study explored the antifungal properties of aqueous and hydroethanolic extracts of *Acalypha wilkesiana*, *Mitracarptus hirtus* and *Senna alata* via the inhibition of the growth of candida strains isolated from samples collected in some hospitals in Benin. The results obtained showed that the isolated strains have different resistance profiles and express several virulence factors. The antifungal tests proved the anti-candidus properties of the leafy stem of *Mitracarptus hirtus* with a better for its hydro-ethanolic extract. This anti-candidus potential of the plant is supported by its rich secondary metabolites and antioxidant activities. Further studies will be devoted to the in vivo efficacy of *Mitracarptus hirtus* and the mechanism of action of the anti-candidus effect.

List Of Abbreviations

HIV : Human immunodeficiency virus

AIDS : Acquired Immune Deficiency Syndrome

PLWHIV : People living with HIV

DPPH : 2,2-diphenyl-1-picrylhydrazyl

ATCC : American Type Culture Collection

TRM : Tetrazolium Reduction Medium

CFU : Colony forming unit

Declarations

Ethics approval and consent to participate

The study was conducted under the approval of the ethics committee of the research unit in applied microbiology and pharmacology of natural substances. *Acalypha wilkesiana, Senna alata* and *Mitracarpus hirtus* were collected under the approval of the ethical committee of the Research Unit in Applied Microbiology and Pharmacology of Natural Substances. N°URM-020_2022.

All methods involved in the studies on both plants and humans were carried out in accordance with relevant guidelines and regulations.

Informed consent was obtained from all subjects and/or their legal guardian(s).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

Authors declare no competing interests

Funding

No funding was obtained for this study

Authors' contributions

JRK participated in all stages of the production of this article. FL and DV provided the scientific direction of the works. LF, BAF, ED, EA, FA FT, carried out laboratory manipulations and data processing. All authors have contributed to the manuscript edition.

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Figures

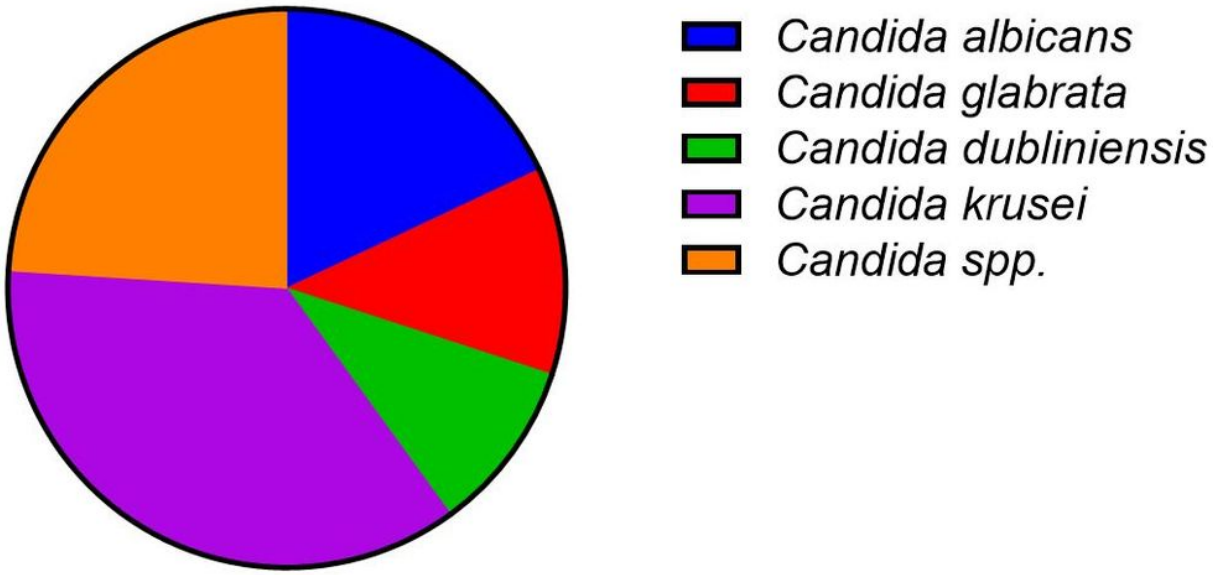

Figure 1

identification frequence of different *Candida* species

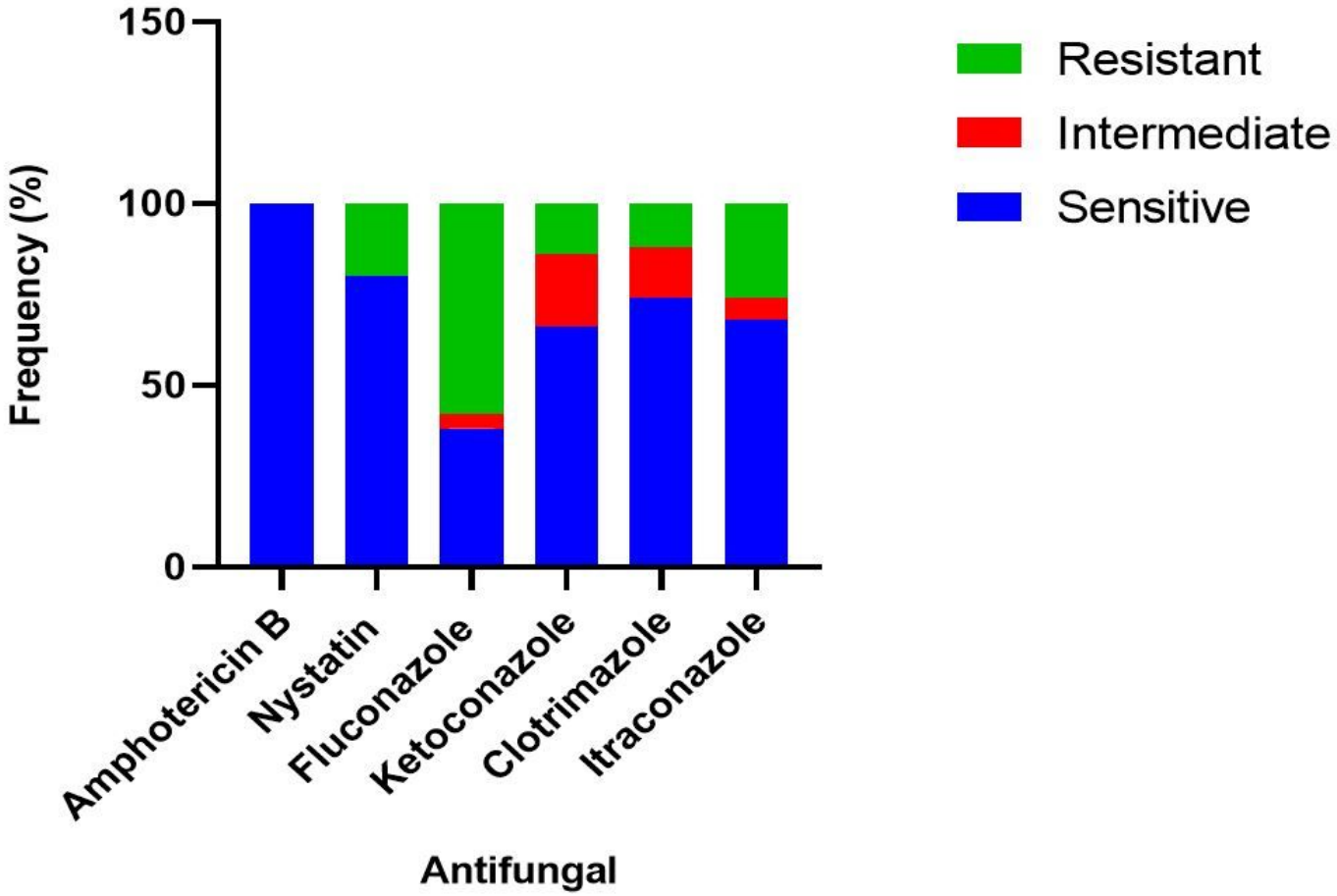

Figure 2

Sensitivity of isolated strains to conventional antifungals

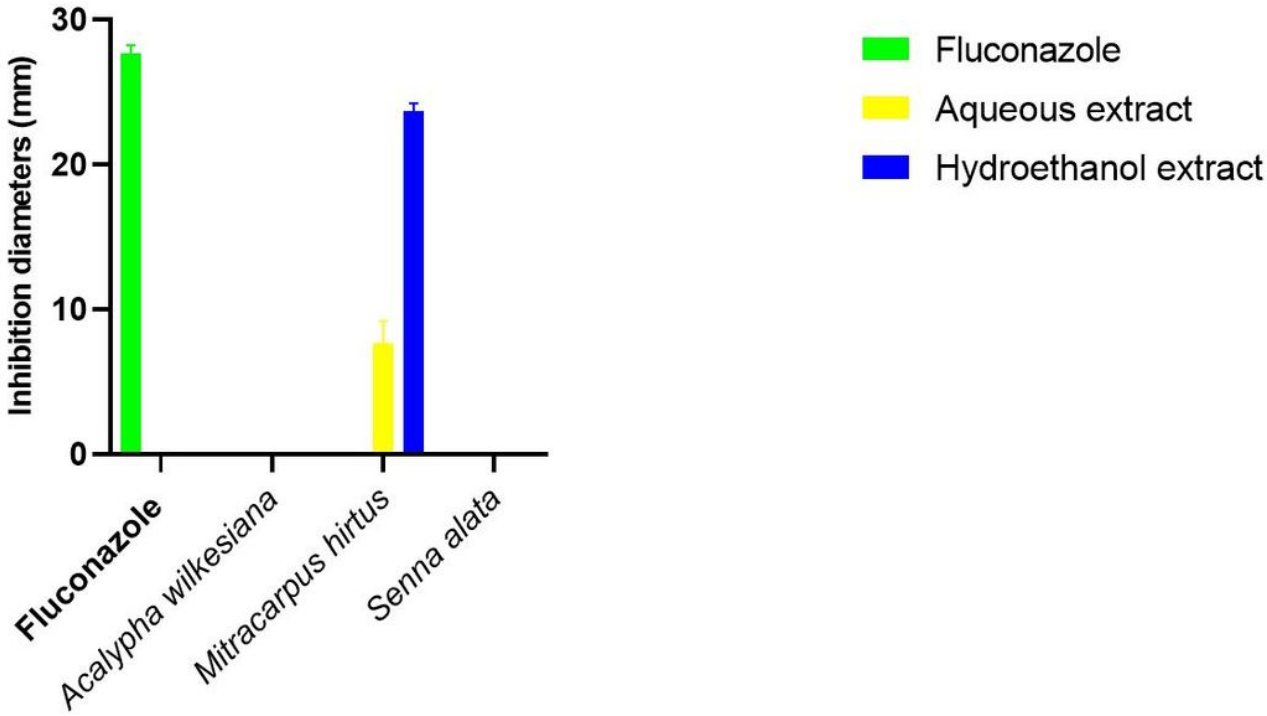

Figure 3

antifungal activity of the plants on the reference strain

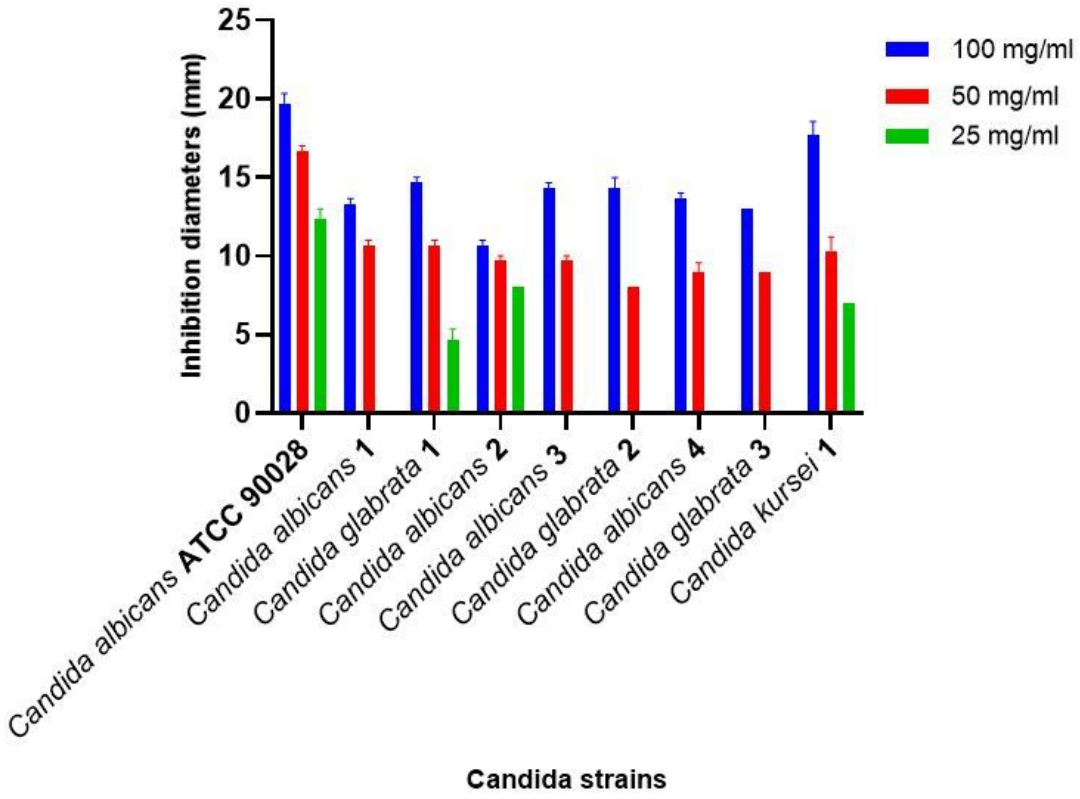

Figure 4

Effect of *Mitracarpus hirta* hydroethanol extract on the strains tested at different concentrations