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In vitro Assessment the Antifungal Activity of *Dissotis multiflora* (*Melastomataceae*) and *Paullinia pinnata* (*Sapindaceae*) Leaves Extracts on *Candida* Species - Experimental Study

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AN, HG and M. Noubom designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AA and SV acquired, analysed, interpreted data. Authors AC, WA, PB and M. Nyegue managed the literature searches and revised article critically. Author M. Nyegue revised article critically. All authors have seen and approved the final version of the manuscript.

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ABSTRACT

Aims: The aim of this study was to evaluate *in vitro* the antifungal activity of *Dissotis multiflora (Melastomataceae)* and *Paullinia pinnata* (*Sapindaceae*) leaves extracts on six species of *Candida*.

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Study Design: This study was an experimental study.
Duration and Place of the Study: Between March to August 2017, Department of Microbiology, Microbiology laboratory, University of Yaoundé I. Bacteriology laboratory, Yaoundé University Teaching Hospital (YUHC).
Methodology: The fungal strains were isolated from vaginal swab women at the sampling unit of the YUHC. The identification test blastosis and gallery method allowed to differentiate *Candida albicans* ATCC37037 to *Candida krusei*, *Candida tropicalis*, *Candida parapsilosis*, *Candida haemolinii* and *Candida lipolytica* in the Bacteriology laboratory of the YUHC. *C. albicans* ATCC 37037 came from the Microbiology laboratory of the University of Yaoundé I. The antifungal activity of extracts was carried out on agar medium using (aromatogram) and microdilution method. The effect of the combination of methanolic fractions were assess by the chessboard method.
Results: Phytochemical analysis of gude extracts of *D. multiflora* and *P. pinnata* revealed the presence of secondary metabolites such as phenols, tannins, anthraquinones, alkaloids, saponins, steroids and flavonoids in both extracts. In general, all of six fungal strains were susceptible to different extracts and fractions with inhibition diameters ranging from 10.33 mm for methanolic fraction of *D. multiflorao C. parapsilosis* to 19 mm for the same fraction on *C. haemolinii).*
Both the MICs and the MFCs of actives extracts ranged respectively from 0,78 to 12,5 mg / ml and 1,56 to 25 mg/ml, the majority being fungicidal. The combinations showed significant antifungal activity compared to those of the fractions taken individually, especially with MICs reductions of the order to 75%.
Conclusion: The antimicrobial activities of the molecules present in our two extracts could justify their use in traditional medicine in the treatment of candidiasis.

Keywords: Candidiasis; extracts of plants; Dissotis multifolra; Paullinia pinnata; antifungal activity; combinations; species.

1. INTRODUCTION

Fungal infections are still showing a strong resurgence despite the range of antifungals available in the market. In fact, 50% of adults and up to 30% of childrens suffer from candidiasis annually [1]. It is important to note that during their lifetime, 70 to 75% of healthy women have at least one episode of vaginal candidiasis as well as being asymptomatic and recurrent [2]. Factors such as old age, taking corticosteroids and antibiotics, immunosuppressors, wearing prostheses and especially HIV AIDS considerably worsen this situation. In addition, the incessant problem of resistance of *Candida* species to antifungals remains topical [3]. It is therefore necessary to find new therapeutic substances as a solution to conventional antifungals. Cameroon is a country with an immense wealth of medicinal plants. This is the case of *Dissotis multiflora* and *Paullinia pinnata*, used in traditional medicine without scientific basis to treat various diseases related to bacterial and fungal infections. The aim of this study was to evaluate the antifungal activity of two extracts, four fractions (2 methanolic and 2 ethyl acetic 70/30%) of *Dissotis multiflora*, *Paullinia* and their combinations on *Candida* strains, responsible for candidiasis.

2. MATERIALS AND METHODS

2.1 Microorganisms

Six fungal species including *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *C. haemolinii*, *C. lipolytica* and *C. albicans* ATCC 3703, commonly associated with candidiasis infection where used. The clinical isolates were obtained from Yaoundé University Teaching Hospital and the reference's strain was obtained from Laboratory of Microbiology of University of Yaoundé I. Fungal strains kept at 4ºC were activated before any test.

2.2 Collection and Identification of Plant Materials

These plants were harvested at Koupa-Matapit (Cameroon) in July 2017 and their respective identification was done at the Cameroon National Herbarium by comparison with specimen N^0 20950/HNC and 20022/SRF.

2.3 Preparation of Ethanolic Extracts

The leaves of *D. multiflora* Triana and *P. pinnata* Linn were dried for one week under the shade at room temperature and weighed.

These plants (200 g) were dried, ground and macerated in ethanol 95% (600 mL) for 48 hours. The mixture was agitated, filtered with Whatman filter paper and concentrated using water bath at 70ºC.

2.4 Phytochemical Screening

The different secondary metabolites such as alkaloids, tannins, saponins, flavonoids, steroids and phenols in the extracts were revealed using standard methods.

2.5 Antimicrobial Assays

2.5.1 Agar-well diffusion method

The solid medium diffusion method was adopted to carry out this test [4,5]. It is usually done in the same way as an antibiogram except that the extracts solutions replace the antibiotics. Fluconazole® has been used as a reference antifungal. we prepared a fungal suspension in 1 ml of saline solution (0.85%) from young (48 H for C. *albicans* ATCC 37037, *C. krusei* and 72 H for *C. tropicalis*, *C. parapsilosis*, *C. lipolitica*, *C. haemolinii*) and pure colonies. After vortexing, we used the counting technique with a cell of Malassez to adjust the suspensions to those of Mac farland for *Candida* in order to obtain for each strains, a fungal concentration equal to 1.5 x 106 CFU / ml. The fungal inoculum thus prepared was seeded by swabbing on Sabouraud Dextrose Agar+ Chloramphenicol. Sterile disks of 6 mm diameter made with Wattman paper were impregnated with 10 µl of mother liquid extracts and fractions (100 mg/ml). After a few minutes, these discs were deposited on the surface of the agar medium and left at room temperature for 10 min. Discs prepared from a solution of Fluconazole® (4 mg / ml) were added to the series. Petri dishes were then incubated at 37ºC for 24 hours and from 48 to 72 hours depending on the strains. At the end of this incubation time, each disc expressed antifungal activity of the corresponding extract solution by the absence of fungal growth around. These diameters (mm) of the zones of inhibition obtained were measured using sliding calipers. The susceptibility of the fungal strains to the extracts was determined using the inhibition diameter (ID) as: non-sensitive (ID ≤ 8 mm); sensitive (9 ≤ ID ≤ 14 mm); more sensitive (15≤ ID ≤ 19 mm) and extremely sensitive (20 mm ≤ ID [5].

2.5.2 Determination of the Minimal Inhibitory Concentration (MIC) by microdilution method preparation of inoculums of microorganisms [6]

In each well of a microplate (96 wells), a volume of 100 µl of Sabouraud Dextrose liquid medium supplemented with chloramphenicol at 0.5 g/l was introduced. 100 µl of each of the stock solutions of extracts and test fractions prepared at 100 mg / ml, ie 100000 µg / ml, were used introduced in triplicate in the wells of line A. From these wells, successive dilutions (in the direction going from A to G) following a geometric progression of reason 2 made it possible to obtain a concentration range of the extracts to be tested varying from 50000 µg / ml (the wells of the line A) at 0.7812 µg / ml (the wells of line G). A volume of 100 µl of concentrated fungal inoculum at 1.5 × 10 7 CFU / ml was introduced into each well to obtain a final concentration range of extract ranging from 25,000 to 39 µg / ml. Thus, the final volume was 200 µl per ml. Wells and all tests were performed in triplicate. Line H was used as positive yeast growth control and contained the culture medium and the inoculum. The reference antifungal used was fluconazole® at concentrations ranging from 250 µg / ml (wells in column 1) to 0.2441 µg / ml (wells in column 11). The microplate was incubated at 37ºC for 48 and 72 hours depending on the strains. At the end of the incubation time, the fungal growth was demonstrated by adding 40 µl of a 2% resazurin solution in each well of the two test lines. The whole was reincubated at 37°C for 45 min. The change in color from blue to reddish pink indicated fungal growth. The MIC was defined as the lowest concentration of extract that inhibited fungal growth.

2.5.3 Determination of Minimal Fungicidal Concentration (FMC) [6]

About the determination of CMF, 50µl of the test wells of concentrations greater than or equal to the MIC were deposited, spread on SDA + Chl 0.5 g / l medium and incubated at 37ºC for 48 to 72 hours. After incubation, CMF was defined as the lowest extract concentration at which no visible growth of the seed was observed [6].

2.5.4 Interaction study

The checkerboard microdilution method was used to determine the type of interaction

between the two methanolic fractions of the plants [6,7]. Fractional inhibitory concentration was determined following the formula:

$$FIC_{Frac\ 1} = \frac{\text{MIC of Frac1 in combination}}{\text{MIC of Frac 1 without combination}}$$

$$FIC_{Frac\ 2} = \frac{\text{MIC of Frac 2 in combination}}{\text{MIC of Frac 2 without combination}}$$

$FICI = FIC_{Frac1} + FIC_{Frac\ 2}$
$FICI \leq 0,5$ indicate the synergistic interaction
$0,5 \leq FICI \leq 1$ indicate the addity effect
$1 \leq FICI \leq 4$ indicate no interaction
$FICI > 4$ indicate the antagonist interaction [6]

2.6 Statistical Analysis

Results were statistically analysed using IBM SPSS STATISTICS.23 software where $p<0.05$ was considered as statistically significant. Minimal Inhibitory Concentration Index were determined by Microsoft Excel for figures.

3. RESULTS AND DISCUSSION

3.1 Extraction Yield

The yields obtained are respectively 08.76% for *P. pinnata* leaves ethanolic extract and 13.32% for *D. multiflora* leaves ethanolic extract. In general, the quantities of extracts present in the samples vary according to the families to which they belong [5,8]. These results are similar to those of Lasisi and Afagnigni, which obtained similar extraction yields.

3.2 Phytochemical Screening

Phytochemical analysis of the crude extracts of D. multiflora and P. pinnata revealed the presence of secondary metabolites such as phenols, tannins, anthraquinones, alkaloids, saponins, steroids and flavonoids in both extracts.

The anthocyanates presents in *P. pinnata* are absent in *D. multifora*. Among the metabolites present in these two plants were also found in accordance with Ateufack's results obtain with aqueous ethanol leaf of *Dissotis thollonii*, a plant of the same genera [9]. These chemical compounds are responsible for antimicrobial activities. These secondary metabolites are recognized as the main classes of plant-derived antimicrobial compounds [10]. Very little work has been done on the antifungal activities of these two plants. nevertheless, several results on antibacterial activities have been identified. Tannins have an anti-diarrhea effect [5]. They inhibit microbial enzymatic secretion, break membranes, neutralize microbial proteins, etc. flavonoids cause a complexation of the cell wall. Alkaloids has an effect against bacterial DNA [10].

3.3 Antifungal Assay

3.3.1 Determination of inhibition diameter zones

The presence of inhibition zones after inhibition revealed the activity of the extracts and fractions on the fungal strains tested. The results are reported in Table 1. It shows that Inhibition zones ranges from 10.33 ± 0.57 against *C. parapsilosis* to 19 ± 0.00 against *C. haemolinii* for the methanolic fraction *of D. multiflora*. As can be observed, *C. krusei*, *C. tropicalis* and *C. parapsilosis* are sensitive; C. albicans ATCC 37037, *C. lipolytica* and *C. haemolinii* were more sensitive. No significant difference was observed between inhibition zones of methanolic fraction of *D. multiflora* and Fluconazole against *C. krusei*, *C. albicans* ATCC 37037, *C. lipolytica* and *C. haemolinii*.

From 10.66 ± 0.57 against *C. albicans* ATCC 37037 and *C. tropicalis* to 14 ±1 against *C. krusei* for the ethanol extract of *D. multiflora*. 11.33 ± 0.57 against *C. haemolinii* to 15.33 ± 0.57 on *C. krusei* for the ethyl acetic fraction of *D. multiflora*. The methanolic fraction of the *P. pinnata* sample inhibition zones ranges from 12.33 ± 0.57 against *C. haemolinii* to 14.33 ± 0.57 against *C. krusei*. On solid medium, the most sensitive strain was *C. krusei*. Also, we observed against *C. tropicalis* high sensitivity of extracts and fractions than Fluconazole®, antifungal of reference. These different sensibilities of microorganism to extract and fractions plants may depend on the chemical composition, but also on the virulence of the microorganism itself and the environment in which the action takes place [10]. The phenolic compounds have high antifungal activity through their mechanism of toxicity, which manifests itself through inhibition of cell wall proteins, metal ion chelation, and sequestration of substances. The ethanolic extract and the ethyl acetic fraction of *P. pinnata* had no activity.

3.3.2 Inhibitory parameters: Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC) of extracts

On the base of first solid medium sensitivity test, we continued the tests in liquid medium. MIC of

extracts, fractions, nystatin and fluconazole are shown in Table 2. It can be notice that ethanolic extract and ethyl acetic fraction of *P. pinnata* had no activity against *Candida* species. This could be explained by the fact the anthocyanates present in the extracts of *P. pinnata* would have an antagonistic effect with the other compounds and thus reduce its antifungal activity. About other four plant samples, they were active against all fungal strains tested with MICs between 0.78 mg/ml and 12.500 mg/ml. Meoh frac of *D. multiflora* is the most active extract with MICs between 0.78 mg/ml against *C. krusei*, *C. lipolytica*, *C. haemolinii* and 3.125 mg/ml against *C. albicans* ATCC 37037, *C parapsilosis* followed by MeOH frac of *P. pinnata* with MICs between 1.560 mg / ml against *C .parapsilosis* and 6.250 mg/ml against *C. krusei*, *C. albicans* ATCC 37037, *C. lipolytica*, *C. haemolinii*. Eth ext of *D. multiflora* with MICs between 1.560 mg / ml against *C. parapsilosis*, *C. lipolytica* and 6.250 µg / ml against *C. krusei* and *C. haemolinii*. Finally, the ET/AC frac of *D. multiflora* was less active with MICs between 1.560 mg / ml against *C. tropicalis*, *C. lipolytica*, *C. haemolinii* and 6.250 mg/ml against *C. krusei*, *C. albicans* ATCC 37037 and *C. haemolinii*. Despite very limited informations available on antifungal activity of *D. multiflora* and *P. pinnata* plants, we were able to obtain results on the work of some plants of the same genus *Dissotis*. In fact, our results are similar to those who obtained similar MICs (1.250 mg / ml) of crude ethanolic extract from *Dissotis perkinsiae* (*Melastomataceae*) against the growth of two strains of *Candida albicans*, one clinical isolate and one reference strain [11]. That same study asserted that *D. perkinsiae* is active on Enterococcus faecalis with MICs ranging from 40 to 80 µg / mL, *E. coli* (MIC 80 µg / mL) and Staphylococcus aureus (MIC 80 µg / mL). Antioxidant activities of *Dissotis* genus had also been demonstrated [12]. Other study obtained inhibitions of the aqueous leaf extract of *Dissotis thollonii* Cogn (*Melastomataceae*) on bacteria, it consisted of 5 reference layers (*Escherichia coli* ATCC 8739, *E. coli ATCC* 10536, *Salmonella typhi* ATCC 6539, *Enterobacter aerogenes* ATCC 13048 and *E. coli* ATCC 1177) and two clinical isolates (*Staphylococcus aureus* and *Shigella flexneri*) with MICs ranging between 32 to 512 µg/ml.

Table 1. Inhibition zone diameters of extracts and fractions of *D. multiflora*, *P. pinnata* and fluconazole

Fungal species	*D. multiflora* (Ext ETH)	*D. multiflora* (Frac. MEOH)	*D. multiflora* (Frac ET/AC)	*P. pinnata* (Frac. MEOH)	Fluconazole
C. krusei	14 ±1^{a}	11.66 ± 0,57^{c}	15.33 ± 0,57^{a}	14.33 ± 0,57^{a}	16.66±0,57^{a}
C. albicans ATTCP37037	10.66 ± 0,57^{c}	14.33 ± 0,57^{ab}	11.66 ± 0,57^{a}	12.33 ± 0,57^{b}	20.33±0,57^{a}
C. tropicalis	10.66 ± 0,57^{a}	13 ± 0,00^{bc}	12.33 ± 0,57^{a}	13 ± 1^{a}	6.3±0,57^{a}
C. parapsilosis	13.33 ± 0,57^{bc}	10.33 ± 0,57^{a}	12.66 ± 0,57^{a}	13 ± 0,00^{a}	24.33±0,57^{a}
C. lipolitica	12 ± 1^{ab}	14.66 ± 0,57^{c}	14.66 ± 0,57^{a}	14 ± 1^{b}	25.66±0,57^{a}
C. haemolinii	11.66 ± 0,57^{ab}	19 ± 0,00^{d}	11.33 ± 0,57^{a}	12.33 ± 0,57^{a}	25±0,57^{a}

^{a,b,c,d} *The different letters corresponding to different activities with P< 0,05 as statistically significant*

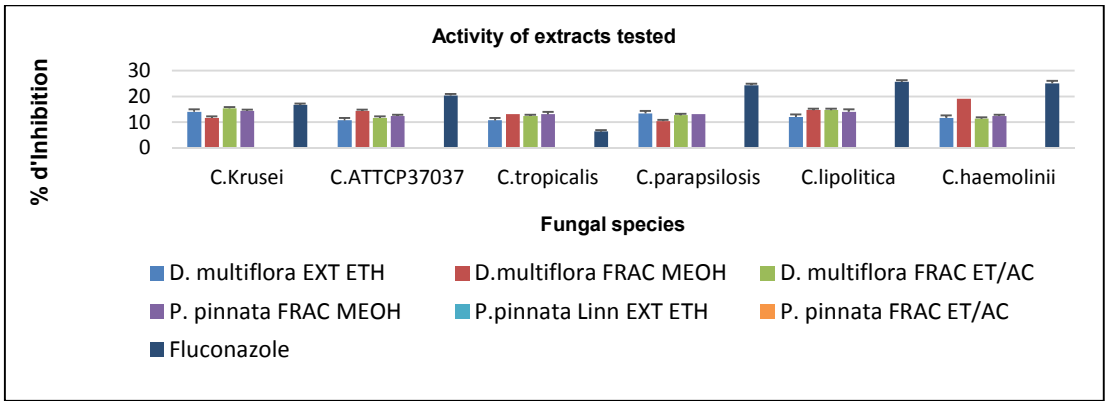

Fig. 1. Inhibition percentage of yeasts to 100 mg/mL and 4 mg/mL respectively concentration of extracts and reference antifungal

Legend: EXT ETH: ethanolic extract; FRAC MEOH: methanolic fraction; FRAC ET/AC: acetic ethyl fraction (70/30)

Table 2. Inhibition parameters of *D. multiflora* Triana, *P. pinnata* linn plants extracts, nystatin and fluconazole

Fungal species		Extract and fractions				Reference's antifungal	
	Parameters	*D. multiflora* (Eth ext)	*D. multiflora* (MeOH frac)	*D. multiflora* (Et/ac frac)	*P. pinnata* (MeOH frac)	Nystatin	Fluconazole
C. krusei	MIC (mg/ml)	6,25	0,78	3,125	6,250	0,062	0,125
	MFC (mg/ml)	25	1,56	12,5	12,5	0,25	0,5
	MFC/MIC	4	2	4	2	4	4
	Interpretation	Fungistatic	Fungicidal	Fungistatic	Fungicidal	Fungistatic	Fungistatic
C. albicans ATCC 37037	MIC (mg/ml)	12,5	3,125	6,25	6,25	0,062	0,25
	MFC (mg/ml)	25	6,25	6,25	6,25	0,062	1
	MFC / MIC	2	2	1	1	1	4
	Interprétation	Fungicidal	Fungicidal	Fungicidal	Fungicidal	Fungicidal	Fungistatic
C. tropicalis	MIC (mg/ml)	3,125	1,56	3,125	3,125	0,125	0,125
	MFC (mg/ml)	12,5	1,56	12,5	6,25	0,5	0,5
	MFC/MIC	4	1	4	2	4	4
	Interprétation	Fungistatic	Fungicidal	Fungistatic	Fungicidal	Fungistatic	Fungistatic
C. parapsilosis	MIC (mg/ml)	1,56	3,125	1,56	1,56	0,25	0,125
	MFC (mg/ml)	12,5	3,125	3,125	3,125	0,5	0,5
	MFC/MIC	8	1	8	2	2	4
	Interprétation	Fungistatic	Fungicidal	Fungistatic	Fungicidal	Fungicidal	Fungistatic
C. lipolytica	MIC (mg/ml)	1,56	0,78	1,56	6,25	0,125	0,25
	MFC (mg/ml)	6,25	1,56	12,5	6,25	0,25	1
	MFC/MIC	4	2	8	1	2	4
	Interprétation	Fungistatic	Fungicidal	Fungistatic	Fungicidal	Fungicidal	Fungistatic
C. haemolinii	MIC (mg/ml)	6,25	0,78	1,56	6,25	0,125	0,125
	CMF (mg/ml)	12,5	1,56	12,5	12,5	0,25	1
	MFC/MIC	2	1	8	2	2	8
	Interprétation	Fungicidal	Fungicidal	Fungistatic	Fungicidal	Fungicidal	Fungistatic

Table 3. Results of interaction study

C. krusei						
Combinaison of fractions	**MIC of individuals fractions (mg/mL)**	**MIC of combination fractions (mg/mL)**	**Individual FIC**	**FICI (∑ FIC)**	**Interprétation**	**% of reduction of MIC**
E2 / E4	0,78 / 6,25	0,195 / 1,562	0,25 / 0,25	0,5	Synergize	75 / 75
C. albicans ATCC 37037						
E2 /E4	3,125/ 6,250	0,781/1,562	0,25 / 0,25	0,5	Synergize	75 / 75

The MFC/MIC ratio showed that the methanolic fractions of *D. multiflora* and *P. pinnata* have a fungicidal action on all 6 species of *Candida* while Fluconazole®, a reference antifungal that had a fungistatic effect on the all of our strains. this resistance to Fluconazole® could be explained by the adaptation of the fungal germ to repeated exposure whereas the efficiency of our plant samples can be explained by the fact that they consist of a complex of bioactive molecules unknown by the fungal germ. These different antifungal activities could justify the use *o*f *D. multifora* and *P. pinnata* plants in herbal medicine against fungal infections.

3.3.3 Interaction study

The combination of methanolic fractions of *D. multifora* and *P. pinnata* showed a synergistic effect against *C. krusei* and *C. albicans* ATCC 37037, showed on Table 3. These synergistic effects would be due to the combined actions of the different active compounds present in each fraction which have very different targets and mechanisms of action on the pathogen. The antifungal activities obtained from this study confirm once again the use of this plants in traditional medicine for the treatment of fungal infections.

4. CONCLUSION

The antimicrobial activities of the molecules present in our two extracts could justify their use in traditional medicine in the treatment of candidiasis.

Like all scientific investigations, this work deserves to be deepened in order to reinforce this approach towards the manufacture of traditional improved medicines (MTA). To achieve this, we plan in the near future is to conduct studies on the toxicity of theses extracts by *in vivo* tests, to evaluate the inhibition parameters of the extracts in combination with some reference antifungals and to make an ovum formulation from theses extracts to cure candidiasis.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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