

Full Length Research Paper

***In vitro* antifungal activity of *Dorstenia mannii* leaf extracts (Moraceae)**

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The leaves of *Dorstenia mannii* are used in traditional medicine in Cameroon and other African countries for the treatment of infectious diseases like malaria, skin rashes and stomach disorders. To substantiate this folkloric claim, the crude methanol extract and fractions from the leaves of *D. mannii* were investigated for their antifungal activity. The crude methanol extract was prepared from powdered dried leaves of the *D. mannii*. A portion was subjected to flash liquid chromatography on silica gel to afford test fractions. All test samples were screened for major groups of phytochemicals. Test samples and nystatin (reference) were evaluated for antifungal activity on ten yeasts using agar disc diffusion and broth microdilution methods. The qualitative analysis of crude methanol extract and fractions of *D. mannii* leaves revealed the presence of flavonoids, phenols, steroids and cardiac glycosides. In agar disc diffusion assay, seven of the ten pathogenic fungal strains were sensitive to the crude methanol extract (7/10), *n*-hexane ethyl-acetate (Hex-EA) (75%) (8/10) and ethyl-acetate (100%) (8/10). The minimum inhibitory concentrations (MICs) for the test samples varied from 80 to 1280 µg/ml. The crude extract and ethyl-acetate (100%) were the most active plant samples with both fungistatic and fungicidal effects (MIC/MFC values from 80 to 640 µg/ml) though not as the reference drug. *Candida tropicalis* was the least sensitive to the test samples. Some fractions exerted no fungicidal actions on *Cryptococcus neoformans*, *Candida lusitanae* and *Candida tropicalis*. The present work shows that the crude methanol extract and fractions (*n*-hexane, ethyl acetate and residue) from the leaves of *D. mannii* possess growth inhibitory effect on pathogenic yeast. The active ingredients of this plant could be an addition to the antifungal arsenal to opportunistic fungal yeast pathogens.

Key words: Antifungal activity, *Dorstenia mannii*, yeasts, opportunistic candidiasis.

INTRODUCTION

Nowadays, fungal diseases have emerged and are being increasingly recognized as important public health problems owing to an ever-expanding population of immuno-compromised patients (Miceli et al., 2011).

Fungal infections are usually associated with *Candida*, *Aspergillus* and *Cryptococcus* species but those due to *Candida* species represent the main opportunistic fungal infections worldwide, leading to high morbidity and

mortality in the population (Low and Rotstein, 2011). These changes are linked to the growing population of immuno-compromised patients. During the last three decades, *Candida albicans* has been the most prevalent pathogen in systemic fungal infections (Pfaller and Diekema, 2004). Presently, non albicans species of *Candida* account for more than 50% of fungal infections. The opportunistic yeast mostly reported are *C. albicans*, *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, *Candida kefyr*, *Candida glabrata*, *Candida dubliensis* and *Candida rugosa* and *Cryptococcus neoformans* (Banerje, 2009). Antifungal active principles are diverse and numerous, but few classes of them are currently available against yeast infections because many are toxic (Spampinato and Leonardi, 2013). The high morbidity and mortality rates associated with opportunistic yeast infections indicate that current antifungal therapy to combat candidiasis is still ineffective. The current arsenal of antifungal drugs is exceedingly short and no new antifungal drugs are expected to reach the market any time soon (Pierce and Lopez-Ribot, 2013). Therefore, the discovery of new antimicrobial agents is still relevant. Among the potential sources of new agents, plants have long been investigated because they contain many bioactive compounds that can be of interest in therapy.

Dorstenia mannii Hook f. (Moraceae) is a perennial herb growing in the tropical rain forest of West Africa (Hutchinson and Dalziel, 1954). A decoction of the leaves is used for the treatment of many diseases, but mainly for rheumatism and stomach disorders (Bouquet, 1969). There are few pharmacological studies reported on *D. mannii*. In the genus of *Dorstenia*, there are many plant species that have been proven scientifically to exert antimicrobial properties (Ngadjui et al., 2000). Studies have shown that many species from the *Dorstenia* genus are present in different degree, and have antimicrobial activities (Swain et al., 1991; Abegaz and Ngadjui, 1999). Amongst others, in Cameroon, twigs of *Dorstenia angusticornis* have been tested for their *in vitro* antimicrobial activity (Kuate, 2007). Prenylated flavonoids isolated from *D. mannii* such as 6,8-diprenyleriodictyol (5), dorsmanin C (3) and dorsmanin F (7) were found to be potent scavengers of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (Dufall et al., 2003). Compounds 3, 5 and 7 also inhibited Cu²⁺-mediated oxidation of human low density lipoprotein (Dufall et al., 2003). The twigs of *Dorstenia elliptica* have been tested for their *in vitro* antimicrobial activity against bacteria and fungi (Kuate, 2007). Some compounds from the twigs of *D. mannii* presented growth inhibitory activity mostly on Gram-negative bacteria and Mycobacteria (Mbaveng et

al., 2012).

Outside these activities observed from this genus, traditional pharmacopoeia reveals other particular activities of certain species, such as anti-diabetic, anti-cholesterol, anti-oxidant and anti-malarial properties (Kuate, 2007). The twigs of *Dorstenia barteri* are used in Cameroon for their antimalarial properties (Iwu et al., 1992). In many African countries, the roots of *Dorstenia barnimiana* are used in the treatment of skin diseases (Iwu et al., 1992). The juices of the leaves of *D. elliptica* are used as eye drops in Congo (Bouquet, 1969). The decoction of leaves of *Dorstenia poinsettifolia* is used to treat infected wounds (Tsopmo et al., 1998). *Dorstenia contrajerva* is used in Bolivia like an antidote against snake and insect bites and also to remove worms like tenea (Okunji, 1998). It is equally used in Guatemala to treat diarrhea, in Costa-Rica, Brazil and Mexico to provoke menstruation (Logan, 1973). This same species is used in Argentina like a tonic. The roots of *Dorstenia lanei* are used as infusion against stomach ache, in mouth washing against tooth ache or in fraction mixed with the stems of *Pycnanthus angolensis*, plus small portion of white clay, to calm down head ache and fights rheumatism (Walker and Sillans, 1961). The roots of *Dorstenia drakena* present an anti-secretory effect, a diuretic activity, lachrymal activity, mydratic and spasmolytic activities (Kyerematen et al., 1985). The decoctions of the leaves and roots of *Dorstenia psilurus* are used in the treatment of rheumatism, snake bites, against head ache and stomach ache (Bouquet, 1969).

To the best of our knowledge no study has investigated the effects of *D. mannii* leaf extract on yeast pathogens. Therefore, the present work was designed to assess the *in vitro* antifungal activity of *D. mannii* leaf extracts on some yeast pathogens.

MATERIALS AND METHODS

Plant material

The harvesting of plants was done at the Nkoljobe hill in April 2011, in Yaounde Centre Region of Cameroon Africa; geographic coordinates: 3° 52' 0" North, 11° 31' 0" East. The identification of the plants was developed at the National Herbarium in Yaoundé, Centre Region according to standard procedures, and were conserved under the voucher specimen reference number (No. 2135) at the National Herbarium.

Plant extract preparation

The air dried and powdered leaves of the *D. mannii* (1 kg) were soaked in methanol for 48 h at room temperature with constant

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Abbreviations: DMSO, Dimethylsulfoxide solution; MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration.

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stirring. Then the extract was filtered using a funnel and a filter paper, and then kept in a flask. Removal of the solvent from the obtained extract under reduced pressure yielded 45 g of a dark-green residue that constituted the crude extract. A mass of 40 g of this organic extract was subjected to flash liquid chromatography on silica gel 60 (220 g), and eluted with *n*-hexane, hexane-ethyl acetate gradients (3:1), (1:1), (1:3), ethyl-acetate and finally with methanol to rinse the column, constituting the residue. A total of six fractions were made. The extracts were then kept separately in small bottles and put in the fridge to be used in the antifungal assay.

Phytochemical screening

The crude methanol extract and fractions were subjected to phytochemical screening using standard procedures (Sofowara, 1993).

Fungal strains and growth condition

The microorganisms constituted 10 yeasts (*C. albicans* ATCC 24433, *C. albicans* ATCC 2091, *C. albicans* ATCC 9002, *C. tropicalis* ATCC 750, *Candida lusitanae* ATCC 200950, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *Candida guilliermondi*, *C. glabrata* IP 35 and *C. neoformans* IP 95026). The reference strains (ATCC) were obtained from American Type Culture Collection (Rockville, USA). The two IP fungal strains were obtained from "Institute Pasteur" (Paris, France). The fungal strains were grown at 35°C and maintained on Sabouraud Dextrose Agar (SDA, Conda).

Preparation of paper discs and test solutions

Sterile discs of 6 mm in diameter were prepared from Fisher filter paper P5 (Catalog No. 09-801C). The discs were impregnated with the crude extract or fraction (10 µL) prepared using dimethylsulfoxide solution (DMSO) at the concentration of 5 mg mL⁻¹ (5 µg µL⁻¹).

Antifungal assay (Agar disc diffusion test)

The disc diffusion method (Chattopadhyay et al., 2001) was employed for the determination of antifungal activities of the crude extract and fractions prepared from *D. mannii* leaves. Briefly, 0.1 ml of suspension of yeast containing 1.5 × 10⁶ spores/ml was spread on Sabouraud dextrose agar medium in 90 mm Petri dishes. Filter paper discs, 6 mm in diameter, were impregnated with 10 µl of test solutions (50 µg) and placed on the seeded plates. A negative control was prepared using the solvent (10% DMSO) employed to dissolve the plant extracts. Nystatin trade name Mycostatin® (10 µg/disc) was used as positive reference drug for fungi. The Petri dishes were sealed with parafilm and allowed for 30 min to permit migration of test samples before incubation at 35°C for 48 h. Antifungal activity was evaluated by observing the presence of growth inhibition zones around the paper discs. Each assay in this experiment was repeated three times.

Microdilution assay

The minimum inhibitory concentration (MIC) of the crude methanol extract and fractions were determined through broth microdilution method in 96-well micro-titre plates (Zgoda and Porter, 2001). The 96-well plates were prepared by dispensing into each well 100 µl of

Sabouraud Dextrose broth. The test substances were initially prepared in a volume of 100 µl and each test sample was added into the first wells of the micro-titre plate. Serial two-fold dilutions of these test samples were made and 100 µl of inoculum standardized at 2.5 × 10⁵ CFU mL⁻¹ for yeasts (at 600 nm, Jenway 6105UV/Vis spectrophotometer- 50 Hz/60 Hz) (Tereschuk et al., 1997) was then added into each well. The last wells (N°12) served as sterility controls (contained broth only) or negative control (broth plus inoculum). This gave final concentration range of 1280 to 2.5 µg/ml and 128 to 0.25 µg/ml for the methanol extract or fractions and reference substance, respectively. The plates were sealed with parafilm, then agitated with a plate shaker to mix their contents and incubated at 35°C for 48 h. The minimum inhibitory concentration (MIC) of each test sample was determined by visualizing the turbidity of the wells. The MIC corresponded to the lowest well concentration where no turbidity change was observed, indicating no growth of yeast. The minimum fungicidal concentration (MFC) was determined by adding 50 µl aliquots of the clear wells to 150 µl of freshly prepared broth medium and incubating at 35°C for 48 h. The MFC was regarded as the lowest concentration of test sample which did not produce a turbidity change as above. All tests were performed in triplicates.

Statistical analysis

Where applicable, the data were subjected to one-way analysis of variance, and differences between samples at P ≤ 0.05 were determined by Student-Newmann-Keuls multiple range test using the Statistical Package for the Social Sciences (SPSS) program.

RESULTS

Phytochemical analysis

The qualitative analysis of the crude methanol extract and fractions of *D. mannii* revealed the presence of some classes of compounds with potential antifungal activities (Table 1). Alkaloids, flavonoids, tannins, phenols, steroids and cardiac glycosides were detected. Coumarins and terpenoids were not detected in all the plant test samples. The fractions Hex (100%) and Hex-EA (25%) were poor in major groups of chemical constituents.

Antifungal activity

The crude methanol extract and some fractions from the leaves of *D. mannii* showed antifungal activity. The growth inhibitory effect of these test samples on yeasts is shown in Table 2. Some of the test samples presented fungistatic and fungicidal activities though they were selectively active. Seven of the ten pathogenic fungal strains were sensitive to the crude methanol extract (7/10), Hex-EA (75%) (8/10), EA (100%) (8/10) as indicated with a plus sign "+". The test samples that were sparingly active included Hex-EA (25%), Hex-EA (50%) and the residual fractions. No activity was observed for *n*-hexane (100%) at 5 µg/ml on all the tested yeasts for the discs diffusion assay. The MIC (µg/ml) and MFC (µg/ml) of the crude methanol extract and fractions from the leaves of *D. mannii* on the tested yeast are presented in

Table 1. Phytochemical screening of *D. mannii* methanol extract and fractions.

Groups of chemical constituents	Test substances						
	Crude extract	<i>n</i> -Hexane (100%)	Hex-EA (25%)	Hex-EA (50%)	Hex-EA (75%)	Ethyl-acetate (100%)	Methanol residue
Flavonoids	+	-	-	+	+	+	-
Alkaloids	+	-	-	-	-	-	+
Coumarins	-	-	-	-	-	-	-
Phenols	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+
Saponins	+	-	-	-	-	-	+
Tannins	+	-	+	+	+	+	-
Chalcones	+	-	-	+	+	+	-
Terpenoids	-	-	-	-	-	-	-
Cardiac glycosides	+	-	-	+	+	+	+

+ = Present; - = absent; Hex-EA, *n*-hexane ethyl-acetate.

Table 2. Antifungal activity of *D. mannii* methanol extract and fractions.

Yeast	Test substances							Nystatin
	Crude extract	<i>n</i> -Hexane (100%)	Hex-EA (25%)	Hex-EA (50%)	Hex-EA (75%)	Ethyl-acetate (100%)	Methanol residue	
<i>C. albicans</i> ATCC 2091	+	-	-	-	+	+	+	+
<i>C. albicans</i> ATCC 9002	+	-	+	+	+	+	-	+
<i>C. albicans</i> ATCC 24433	+	-	+	+	+	+	+	+
<i>Candida parapsilosis</i>	+	-	-	+	+	+	+	+
<i>Candida tropicalis</i>	+	-	-	-	-	-	-	+
<i>Candida krusei</i>	-	-	-	-	-	-	-	+
<i>Candida glabrata</i>	-	-	-	-	+	+	-	+
<i>Candida lusitaniae</i>	+	-	-	-	+	+	-	+
<i>Cryptococcus neoformans</i>	+	-	-	-	+	+	-	+
<i>Candida guilliermondii</i>	-	-	-	-	+	+	-	+

+ = Active; - = not active.

Table 3. In this microwell dilution assay, all the test samples demonstrated antifungal activity including the *n*-hexane (100%) fraction was previously not active. The MICs recorded varied from 80 to 1280 µg/ml. The crude methanol extract and EA (100%) were the most active plant test substances as they exerted both fungistatic and fungicidal effects on all the yeasts under study (MIC/MFC values from 80 to 640 µg/ml). *C. tropicalis* was the least sensitive yeast to the test samples from *D. mannii* leaves, followed by *C. krusei* and *C. guilliermondii*. The reference substance (nystatin) inhibited the growth of all the yeasts under study with a MIC range from 1 to 4 µg/ml. Although, *n*-hexane (100%), Hex-EA (25%) and residue fractions inhibited the growth of selected yeast, their activities were not fungicidal on *C. neoformans*, *C. lusitaniae* and *C.*

tropicalis respectively.

DISCUSSION

Many studies have confirmed the role of medicinal plants in health maintenance and promotion, but the major challenge is to provide scientific evidence (Arora and Kaur, 1999). Therefore, the present work was designed to assess the *in vitro* antifungal activity of *D. mannii* leaf extract on some yeast pathogens. The crude methanol extract and fractions from the leaves of *D. mannii* presented variations in antifungal activities across the tested fungal strains. These variations in antifungal activity could be due to the differences in the chemical

Table 3. Minimal inhibitory concentration (MIC)/minimum fungicidal concentration (MFC) of test substances ($\mu\text{g/ml}$).

Yeast	Test substances							
	Crude extract	<i>n</i> -Hexane (100%)	Hex-EA (25%)	Hex-EA (50%)	Hex-EA (75%)	Ethyl-acetate (100%)	Methanol residue	Nystatin
<i>C. albicans</i> ATCC 2091	80/160	320/320	160/320	160/320	160/320	80/160	320/640	1/4
<i>C. albicans</i> ATCC 9002	80/160	320/320	320/320	160/320	160/320	80/80	320/640	1/8
<i>C. albicans</i> ATCC 24433	80/320	640/640	160/640	80/320	80/320	80/160	320/640	1/4
<i>Candida parapsilosis</i>	80/160	640/640	320/640	80/320	80/320	160/320	320/640	4/4
<i>Candida tropicalis</i>	80/320	na/na	na/na	na/na	na/na	160/320	640/na	2/4
<i>Candida krusei</i>	640/640	na/na	160/640	na/na	na/na	80/160	na/na	1/2
<i>Candida glabrata</i>	320/320	na/na	80/320	160/320	160/320	160/320	80/320	1/8
<i>Candida lusitanae</i>	160/640	640/na	640/na	160/320	160/320	160/320	640/640	4/16
<i>Cryptococcus neoformans</i>	320/640	640/na	640/1280	160/320	160/320	80/320	640/640	4/16
<i>Candida guilliermondi</i>	160/320	na/na	na/na	640/640	640/640	160/320	640/640	2/16

na = Not active.

composition of these extracts as the secondary metabolites of plants have many effects including antimicrobial properties (Cowan, 1999). In addition, the activity of these plant extracts could be influenced by the nature of the plant material or its origin as well as the climatic conditions in which plant grows, the plant part used, or the solvent used for extraction, because plants have different constituents depending on those factors (Ncube et al., 2008). Again for the same plant test sample (crude methanol extract and ethyl-acetate (100%) fraction which were most active), the sensitivity of the fungal strains varied considerably. In the agar disc diffusion assay, the *n*-hexane (100%) fraction did not exert growth inhibitory activity on all the tested yeasts. Other fractions like the Hex-EA (25%) and residue were sparingly active. Surprisingly, these test samples demonstrated an improved antifungal activity in the microdilution assay. It could be understood that antifungal molecules from test samples are more mobile and easily available to penetrate or adhere to the fungal cell wall in liquid medium than in solid medium.

Antimicrobial activity of plant extracts are routinely classified on the basis of susceptibility tests that produce MIC in the range of 100 to 1000 $\mu\text{g/ml}$ (Simoes et al., 2009). The activity is considered to be significant if MIC values are below 100 $\mu\text{g mL}^{-1}$ for crude extract and moderate when the MICs vary from 100 to 625 $\mu\text{g/ml}$ (Ncube et al., 2008; Kuete, 2010). Excepting the *n*-hexane (100%) fraction that exhibited weak activities, the other test samples inhibited the growth of most of the studied yeasts from significant to moderated extent. These antifungal results are supported by previous studies reporting on a candidal activity of the twigs of *D. mannii* (Mbaveng et al., 2012); antibacterial activity of the leaves of the same plant (Fokunang et al., 2012). A Keen look at the MFC values indicates that most of them are not more than fourfold their corresponding MICs. This

proves that fungicidal effects of many tested samples could be expected on the sensitive strains (Mims et al., 1993).

Qualitative phytochemical analysis of *D. mannii* leaf extracts revealed the presence of flavonoids, alkaloids, phenols, steroids, tannins, saponins and cardiac glycosides which could contribute to the observed antifungal activities (Teke et al., 2011; Fankam et al., 2011). The antimicrobial activity of flavonoids isolated from the genus *Dorstenia* have been reported (Kuete et al., 2007; 2010b; Mbaveng et al., 2008; Ngameni et al., 2009). Most of the phytochemicals found act as potent preventive agents against microbial diseases. It is generally found that plants containing diverse classes of chemicals are of superior biological activities (Kilkenny et al., 2011). Thus, it may also be deduced that the higher the diversity and the quantity of these chemical classes a plant may contain, the stronger and broader the spectrum of biological activities the plant molecules may exhibit (Wangchuk et al., 2011). Among the ten pathogenic yeasts tested *C. albicans* was the most sensitive to the extracts, while *C. krusei* and *C. guilliermondi* were the most resistant. This is important since *C. albicans* is the most prevalent pathogen in systemic fungal infections (Pfaller and Diekema, 2004).

Conclusion

The overall results obtained revealed that the crude methanol extract and fractions (*n*-hexane, ethyl-acetate and methanol residue) from the leaves of *D. mannii* in this study possess inhibitory effect on the growth of pathogenic yeast. These findings emphasize the evidence that frequent use of *D. mannii* leaf extracts could be an alternative to prevent infections from opportunistic fungal yeast pathogens.

Conflict of interests

The authors have not declare any conflict of interest.

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