Antifungal activity of leaf and bark extracts from Brazilian mangrove plants

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The aim of this work was evaluated antifungal potential of extracts from *Avicennia schaueriana* Stapf & Leechm. (Avicenniaceae), *Laguncularia racemosa* (L.) Gaertn. (Combretaceae) and *Rhizophora mangle* L. (Rhizophoraceae) against yeasts and dermatophytes of clinical importance. The leaves and bark from Brazilian mangrove plants were collected, dried and submitted to successive extractions with methanol in order to obtain the organic extracts. Antifungal activity test of the crude extracts was performed by disk diffusion and minimum inhibition concentration (MIC) methods towards the following microorganisms: yeasts (*Candida albicans*, *C. parakrusei*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *Trichosporon beigelii* and *T. pullulans*) and dermatophytes (*Microsporum gypseum*, *Trichophyton mentagrophytes* and *T. rubrum*). The phytochemical profile of the Brazilian mangrove plant extracts showed absence for alkaloids and saponins. However, were detected the presence of flavonoids, tannins and terpenes and steroids. Tannins represented the principal group detected in the bark. The results for the antifungal activity by disk diffusion showed that extracts exhibit a weak activity against yeasts. Only leaf extract from *L. racemosa* was active against *C. glabrata* and *T. pullulans* and the bark extract from *R. mangle* was active against *C. parakrusei*, *T. beigelii* and *T. pullulans*. In contrast, dermatophytes were most sensitive, mainly *T. rubrum* followed by *T. mentagrophytes* and *M. gypseum*. Minimum inhibition concentration (MIC) was determined only for extracts from *L. racemosa* and *R. mangle*. The leaf extract from *L. racemosa* showed MIC values of 115 mg/ml for *C. parakrusei*; 7.13 mg/ml for *T. beigelii* and *T. pullulans*; 45 mg/ml for *M. gypseum*; 22.5 mg/ml for *T. mentagrophytes* and 5.62 mg/ml for *T. rubrum* and the bark extract from *L. racemosa* exhibited MIC of 1.40 mg/ml for *T. rubrum*. The bark extract from *R. mangle* presented MIC values of 230 mg/ml for *C. parapsilosis* and *T. beigelii*; 115 mg/ml for *C. albicans*, *C. glabrata* and *C. parakrusei* and 90 mg/ml for all dermatophytes tested.

Keywords: mangrove; antifungal activity; yeasts; dermatophytes; plant extracts

1. Introduction

Fungal infections on the skin and mucosal surface are common causes of disease in developing countries. These diseases are associated mainly *Candida* species and dermatophytes belong to *Trichophyton* and *Microsporum* genera [1]. Furthermore, the number of cases involving immunocompromised patients developing opportunistic, systemic and superficial mycosis has increased significantly [2]. The yeast *Candida albicans* is the most frequent agent of candidiasis, but some studies indicate that other species non-*C. albicans* such as *C. tropicalis*, *C. krusei* and *C. parapsilosis* are also frequently isolated in patients undergoing prolonged antibiotic therapy [3,4]. Other species of yeast also related to opportunistic infections in patients with serious illnesses belong to the genus *Trichosporon*. The trichosporonose is mainly associated with six species: *T. asahii*, *T. mucoides*, *T. beigelii*, *T. asteroides*, *T. ovoides* and *T. inkin*. Rarely caused by *T. pullulans* and *T. domesticum* [5,6].

The increase in microbial resistance and the high toxicity during prolonged treatment with antifungal drugs have been the reason for the discovery of new biological substances showing promising activity, specific mechanism of action, and low toxicity. Recent research has explored various ecological niches in order to obtain compounds of natural origin with different chemical structures and mechanisms of action.

The marine world offers an extremely rich resource for important compounds of structurally novel and biologically active metabolites [7]. Mangrove is a coastal ecosystem of transition between the terrestrial and aquatic environmental present in tropical and subtropical regions and composed of plants with physiological characteristics and special adaptations that allow long periods of water exposure, high salinity and low oxygen content. Brazilian coastal mangrove forests are basically composed by following species: *Avicennia schaueriana* Stapf & Leechm. (Avicenniaceae), *Laguncularia racemosa* (L.) Gaertn. (Combretaceae) and *Rhizophora mangle* L. (Rhizophoraceae). These plants are used in folk medicine against fever, asthma, angina, bleeding, diarrhea, dysentery and tuberculosis [8-10] as well as astringent, healing, tonic, hemostatic, antimicrobial, antitumor and anti-ulcerogenic properties [11-14].

Due to a few studies "*in vitro*" about antifungal potential of Brazilian mangrove plants, the purpose of this work was to obtain a phytochemical profile and evaluate the antifungal activity of leaves and bark extracts from *Avicennia schaueriana*, *Laguncularia racemosa* and *Rhizophora mangle* towards yeasts and dermatophytes of clinical importance.

2. Material and Methods

2.1 Plant material

Leaves and bark from *Avicennia schaueriana* Stapf & Leechm. (Avicenniaceae), *Laguncularia racemosa* (L.) Gaertn. (Combretaceae) and *Rhizophora mangle* L. (Rhizophoraceae) were collected in the estuary of the Paripe River, Vila Velha, Itamaracá, PE, Brazil, during low tide. Identification of the plants was confirmed by comparison with voucher specimens avaliable in the Herbarium Geraldo Mariz of the Department of Botany, Federal University of Pernambuco, Recife, Brazil.

2.2 Extraction and obtention of the crude extracts

The collected leaves and bark were dried in drying cabinet at ± 60°C for several days until complete removal of water. After this period, plant materials were powdered using mechanic grinder and successively extracted with methanol (300 ml) for 24 hours periods under static conditions. At the end of extraction, each organic extract was filtered and concentrated under reduce pressure using a rotary evaporator at ± 45°C and, finally, stored in a vacuum desiccator until constant weight.

2.3 Phytochemical Screening

Preliminary chemical analysis of the leaves and bark from mangrove plants was performed in order to identify the major chemical groups: alkaloids (Meyer's Reactive), flavonoids (Shinoda's Test), saponins (Froth Test), tannins (FeCl3), terpenes and steroids (Liebermann-Burchard Method) [15].

2.4 Test microorganisms

The following microorganisms were used for the antifungal activity test: *Candida albicans* URM 4385, *Candida parakrusei* URM1134, *Candida glabrata* URM 4264, *Candida tropicalis* URM 4262, *Candida parapsilosis* URM 4607, *Trichosporon beigelii* URM 4437 and *Trichosporon pullulans* URM 4259 as yeasts; *Microsporum gypseum* URM 3645, *Trichophyton mentagrophytes* URM 4156 and *Trichophyton rubrum* URM 4259 as dermatophytes. All the fungal strains were provided by Mycoteca-URM, Department of Micology, Federal University of Pernambuco, Brazil and maintained on Sabouraud Dextrose Agar slants at ± 4°C until further use.

2.5 Antifungal Assay

2.5.1 Preparation of inoculum

The test microorganisms were cultived in Sabouraud Dextrose Agar medium and incubated at 37°C for 24 hours in the case of yeasts and 30°C during 3 days for dermatophytes. After time, cell suspensions were prepared in sterile distilled water and adjusted to 0.5 Mc Farland standard solution for yeasts and microbial suspensions containing mycelial mass fragments were prepared for dermatophytes.

2.5.2 Disk diffusion method

The antifungal activity of the leaves and bark extracts was performed by disk diffusion method [16]. Sterile paper disks were impregnated with 20 µl of crude extracts and deposited on the Petri dishes surface containing Yeast Nitrogen Base Dextrose Agar culture medium previously spreaded with fungal inoculum. The Petri dishes were incubated at 37°C during 24 hours for yeasts and 30°C during 48 hours for dermatophytes. Antifungal activity was confirmed by formation of the inhibition zones around the disks. The experiment was conducted in duplicate and the values of the inhibition diameters were expressed in millimeters.

2.6 Determination of minimum inhibitory concentration (MIC)

2.6.1 Microdilution method for yeasts

The determination of minimum inhibitory concentration of the crude extracts against yeasts was carried out by broth microdilution method [17]. Each yeast was cultived in Sabouraud Dextrose Agar medium and incubated at 37°C for 24 hours. Subsequently, the cell suspensions were prepared and adjusted to 0.5 McFarland standard solution. The crude extracts were solubilized in dimethylsulfoxide (DMSO) and submitted to two-fold serial dilutions (230-3.59 mg/ml concentration range). A volume of 50 µl Yeast Nitrogen Base Dextrose Broth medium, 30 µl of extracts at different concentrations and 50 µl of inoculum were added to each well. The microplates were incubated at 37°C for 24 hours. MIC was defined as the lowest concentration of extract that inhibited visible growth and the absorbance of each well was determined using an automatic ELISA reader at 595 nm. DMSO and antifungal drugs ketoconazole (100 µg/ml),

fluconazole (8 µg/ml) and itraconazole (50 µg/ml) were used as negative and positive controls, respectively. All experiment was conduced in triplicate.

2.6.2 Macrodilution method for dermatophytes

Minimum inhibitory concentration of the crude extracts against dermatophytes was performed by broth macrodilution method [18]. The dermatophyes were cultived in Sabouraud Dextrose Agar medium and incubated at 30°C for 3 days. After time, microbial supensions containing mycelial mass fragments were prepared in tubes with sterile distilled water and glass pearl. In each tube was pippeted 1 ml Yeast Nitrogen Base Dextrose Broth medium, 150 µl of extracts at different concentrations (90-1.40 mg/ml concentration range) and 500 µl of inoculum. Posteriorly, the tubes were incubated at 30°C during 48 hours and the results evaluated by cell growth into them. Organic solvent methanol was used as negative control and antifungal drugs ketoconazole (100 µg/ml), fluconazole (8 µg/ml) and itraconazole (50 µg/ml) as positive control. All experiment was conduced in triplicate.

3. Results and Discussion

The phytochemical screening showed the presence of flavonoids, tannins, terpenes and steroids. However, tannins were the representative group, followed by flavonoids (Table 1). The leaves and the bark of *L. racemosa* had phytochemical profile similar to the tannins and flavonoids, and could be considered a rich source of these chemical groups. According with literature, tannins are between 10-20% and 12-24% in the leaves and bark of *L. racemosa*, respectively [19]. The *R. mangle* bark extract also showed strong intensity to the tannins, corroborating the results obtained by other studies [20-22]. Methanolic extract of mangrove bark contained high proportion of polyphenols, tannins and flavonoids [23].

Table 1 Phytochemical screening from Brazilian mangrove plants.

Mangrove plant	Vernacular name	Part used	Phytochemical group				
			Alkaloids	Flavonoids	Saponins	Tannins	Terpenes and Steroids
Avicennia schaueriana Stapf & Leechm.	Black mangrove	Leaves	-	-	-	+	+
		Bark	-	+	-	++	+
Laguncularia racemosa (L.) Gaertn.	White mangrove	Leaves	-	++	-	+++	+
		Bark	-	++	-	+++	+
Rhizophora mangle L.	Red mangrove	Leaves	-	+	-	++	+
		Bark	-	+	-	+++	+

- = Not detected; + = Weak intensity; ++ = Medium intensity; +++ = Strong intensity

The results of the antifungal activity test using the disk diffusion method, presented in Table 2, show that the mangrove plant extracts have a weak activity for yeasts. Only the extract of *L. racemosa* leaves has been active for *C. glabrata* and *T. pullulans* and extract of *R. mangle* bark has been active for *C. parakrusei*, *T. beigelii* and *T. pullulans*. In contrast, the dermatophytes were more sensitive, particularly to the extracts of *L. racemosa* leaves and *R. mangle* bark.

Extracts of the leaves and bark of *A. schaueriana* were not active for the test microorganisms. This result could be explained by the low intensity to the tannins and flavonoids. The relationship of these phenolic compounds with antifungal activity is well documented in the literature [24-31]. Fungi such as *A. niger*, *Trichoderma viride*, *Botrytis cinerea* and *Penicillium* species were inhibited by tannins in different concentrations.

Table 2 Antifungal activity by disk diffusion method of the plant extracts from Brazilian mangrove.

Test microorganism	Mangrove plant extracts					
	Avicennia schaueriana		*Laguncularia racemosa*		*Rhizophora mangle*	
	Leaves	Bark	Leaves	Bark	Leaves	Bark
Candida albicans	-	-	-	-	-	-
Candida glabrata	-	-	+	-	-	-
Candida parakrusei	-	-	-	-	-	++
Candida parapsilosis	-	-	-	-	-	-
Candida tropicalis	-	-	-	-	-	-
Trichosporon beigelii	-	-	-	-	+	-
Trichosporon pullulans	-	-	+++	-	+	+++
Microsporum gypseum	-	-	+++++	++++	+++	++++
Trichophyton mentagrophytes	-	-	+++++	++++	++++	++++
Trichophyton rubrum	-	-	+++++	++++	++++	+++++

- No activity detected
+ = ≤ 10 mm; ++ = 11 – 14 mm; +++ = 15 – 20 mm; ++++ = 21 – 30 mm; +++++ = > 30 mm

The determination of the minimum inhibitory concentration (MIC) was realized only for the *L. racemosa* and *R. mangle* extracts. The MIC values for yeasts were higher than the MIC values for dermatophytes, and also the yeasts were most sensitive to the extracts by microdilution method employed to determination of MIC than by disk diffusion method. The extract of *L. racemosa* leaves showed MIC 7.13 mg/ml for *T. beigelii* and *T. pullulans*, 115 mg/ml for *C. parakrusei*. The extract of *R. mangle* bark showed MIC of 115 mg/ml for *C. albicans*, *C. glabrata* and *C. parakrusei*, 230 mg/ml for *C. parapsilosis* and T. *beigelii* (Table 3). As recognized, the yeasts are more resistant and MIC values are higher than for other groups of fungi [24]. The MIC values for dermatophytes of the leaves extract from *L. racemosa* were 45 mg/ml for *M. gypseum*, 22.5 mg/ml for *T. mentagrophytes* and 5.62 mg/ml for *T. rubrum*. In contrast, the bark extract of *L. racemosa* showed MIC 90 mg/ml for *M. gypseum* and *T. mentagrophytes* and 1.40 mg/ml for *T. rubrum*. The leaves and bark of *R. mangle* extracts showed MIC values of 90 mg/ml for all dermatophytes tested (Table 4). In similar study with *R. mangle* bark extract, the MIC values were 40 mg/ml for *C. albicans*, *C. parapsilosis* and *C. krusei* and 600 mg/ml for *T. mentagrophytes*, *M. canis* and *M. gypseum* [32]. All yeasts and dermatophytes were sensitive to antifungal drugs tested.

Table 3 Minimum inhibitory concentration by microdilution method of the crude extracts from *Laguncularia racemosa* and *Rhizophora mangle* against yeasts.

Mangrove plant	Extract	Minimum inhibitory concentration (mg/ml)						
		C. albicans	*C. glabrata*	*C. parakrusei*	*C. parapsilosis*	*C. tropicalis*	*T. beigelii*	*T. pullulans*
Laguncularia racemosa	Leaves	> 230	> 230	115	> 230	> 230	7.13	7.13
Rhizophora mangle	Bark	115	115	115	230	> 230	230	> 230

Table 4 Minimum inhibitory concentration by macrodilution method of the crude extracts from *Laguncularia racemosa* and *Rhizophora mangle* against dermatophytes.

Mangrove plant	Extract	Minimum inhibitory concentration (mg/ml)		
		M.gypseum	*T. mentagrophytes*	*T. rubrum*
Laguncularia racemosa	Leaves	45	22.5	5.62
	Bark	90	90	1.40
Rhizophora mangle	Leaves	90	90	90
	Bark	90	90	90

According to our results, the dermatophyte *Trichophyton rubrum* was the most sensitive, followed by *T. mentagrophytes* and *M. gypseum*. This fact is very important because, in Brazil, about 80-93% of infections caused by dermatophytes are by *T. rubrum* [33]. In similar study, the dermatophytes were more sensitive to plant extracts than the other fungi with MIC 32, 64 and 128 mg/ml for *T. rubrum*, *T. mentagrophytes* and *M. gypseum*, respectively [34].

As conclusion, our results represent a significant contribution for characterization of the Brazilian mangrove plants, specially *A. schaueriana* and *L. racemosa*. However, further research is necessary for purification and characterization of biologically active compounds and establish the mechanism of action of the isolate compounds.

Acknowledgements The authors would like to thank CAPES for financial support.

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