Full Length Research Paper

In vitro evaluation of antifungal activity and interactive effect of *Anadenanthera colubrina* (Benth)

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Anadenanthera colubrina (Benth) Brenan, a plant known in the Northeastern Region of Brazil as angico, is widely used in traditional folk medicine to treat respiratory and inflammatory diseases. This study aimed to evaluate the antifungal activity, determine the minimum inhibitory concentration (MIC), the minimum fungicidal concentration and the fungal kinetics (death curve) in addition to the interactive effect of the dry extract of angico in association with the antifungals fluconazole and nystatin against yeasts of the genus *Candida*. The dry extract was obtained by rotoevaporation. Tests for evaluation of antifungal activity, determination of the MIC and the MFC as well as the evaluation of the interactive effect with conventional antifungal were done by disk diffusion and microdilution technique. For the evaluation of the angico's effect on fungal growth, death curve was utilized. The results show the angico's antifungal potential in all of the strains tested, having MIC of 1.0 mg/mL. It was observed that the fungal kinetics of 2x MIC, MIC and ½ MIC had similar effects; 6 h was their best time after incubation. There was fungistatic activity reduction (2 log 10 UFC/mL) from the initial inoculum of 1.0 mg/mL. Interactive effect was not observed when used in association with nystatin, but showed synergistic effect when used with fluconazole. In these data, one can see that angico is a species rich in biological activity; being promising species, the isolation and detection of its bioactive compounds is necessary.

Key words: Angico, *Candida albicans*, natural product.

INTRODUCTION

The use of natural products by mankind is as ancient as his own history. During the evolution process that occurred on Earth, the vegetable kingdom always filled an important place, as it is used for food and therapeutic

purposes (Arruda et al., 2006). From individual and community observation of nature, with the utilization of animals and plants with medicinal purposes, the primitive man tried out and used several biologically active species. That followed him since prehistory and evolved throughout the years to compose the primitive man's medicine (Coutinho et al., 2004). Folk medicine is a tradition, where individuals pass information to each other, throughout several generations. This shared knowledge, over time, is the main cause of the diversity in folk medicine (Mahmood et al., 2013). Since 4000 a.C., several historical records were found related to the use of plants for treatment of diseases. The first medical record kept on a Pennsylvania museum dated back to 2100 a.C. and includes a collection of 30 different formulae of medicines with vegetal, animal or mineral components (Duarte, 2006). In 1875, a manuscript found by Georg Ebers in Egypt, named "Ebers Papirus" (1500 a.C.), contains 811 prescriptions and 700 drugs. In China, the first text about medicinal plants (500 a.C.) reports names, doses and guidelines on the use of plants for disease treatments. Some of those plants are still used, such as Ginseng (*Panax*spp), *Ephedra* spp., *Cassia* spp. and *Rheum palmatum L.*, as sources to the pharmaceutical industry (Arruda et al., 2006). Nowadays, approximately 80% of the population in least developed and developing countries rely on plants as a first option to their primary health needs; also, the use of plants as a source of medicines prevails in developing countries as an alternative solution to their health problems (Pilla et al., 2006).

The use of herbal medicines in Brazil is a strong economic alternative in relation to allopathic medicines. It is as a result of the indigenous culture's influence, African traditions and European culture brought by the colonizers (Almeida, 2000). With precarious economic conditions, as most times were allied to the large usage of medicinal plants, a wide commerce of those plants emerged in several brazilian regions. In some of these regions, this commerce is the main financial support to a lot of families, which allows them to continue living in their community and moving to bigger cities (Alves et al., 2007; Almeida et al., 2009).

A great extent of drugs applied in therapeutics come directly or indirectly from natural sources, especially from medicinal plants, that remain an important source to obtain drugs (Carvalho et al., 2007). Angico is a big tree; it has a winding and medial stem and its bark varies from smooth and light to rugged and dark. It is found mainly in high and well drained grounds. It flourishes between September and November with almost no leaves; while the fruit matures from August to September (Lorenzi and Matos, 2002). In folk medicine, angico, when prepared as syrups, is used for cough treatments, pertussis and bronchitis. The maceration of its bark is used for the treatment of inflammation and leukorrhea. When it is prepared with alcohol or cachaça, it is used on external wounds, having hemostatic and healing effects (Matos, 1997; Palmeira et al., 2010). The tear of its bark releases a resin used to treat skin infections (Mors et al., 2000). However, its fruit is considered poisonous, making it impossible to use it for folk medicine (Agra, 1996).

The antimicrobial activity of its plant is, probably, caused by the presence of flavonoids, tannins and terpenes in its leves and fruits. The flavonoids are complex with the bacterial cell wall, resulting in the rupture of the cell wall (Reginatto et al., 2001). The occurrence of fungal infections, dermatomycosis in particular in recent years, has shown a significant increase. In the meantime, this fact may be related to improvements in clinical and laboratory diagnosis, and the increased survival of patients immunocompromised beyond the use of immunosuppressive drugs, which in some cases are misused and may favor installation of microorganisms (Fenner et al., 2006). The fungi that cause these infections, usually, are the dermatophytes (*Epidermophyton, Microsporum, Trichophyton*) and yeasts; among them are *Cryptococcus neoformans* and *Candida albicans*. There are many *Candida* genus species that are able to colonize the skin and human mucosal surfaces (Hassan et al., 2009). This genus is composed of microorganisms, usually opportunistic; however they may cause local or systemic infections on a predisposed person. They affect immunocompromised patients frequently, mainly those going through long antibiotic therapy, chemotheray or even newborns (Samaranayake and Hanes, 2011).

Candida genus is also related to several cases of invasive infections, hence previous colonization of the skin and oral, intestinal and vaginal mucosa by these kind of species is considered an important factor for developing invasive infections; also it is a growing concern in brazilian and worldwide hospitals (Holzheimer and Dralle, 2002). It is relevant to mention that invasive fungal infections are related, among other factors, to high morbidity and mortality rates, difficulties to diagnose some diseases, resistance to antimicrobials and increase of hospitalization time and costs. It is also important to mention that different fungal species may infect humans, animals and plants (Zacchino, 2001). Among the most common antifungals, it is important to emphasize amphotericin B, which is considered as the main drug for treating most fungal infections. However, its use is restricted due to some side effects, like its nephrotoxicity (Zardo and Mezzari, 2004; Lópes-Medrano et al., 2005); nystatin, which presents similar spectrum and mechanisms of action to amphotericin B. However, it is highly toxic when used as injectable formulations (Martinez, 2006). Fluconazole has advantage over amphotericin B for presenting excellent gastrointestinal absorption and distribution in the body (Boucher et al., 2004; Bicanic and Harrison, 2014). The objective of this article was to evaluate the *in vitro* biological activity of angico's dry extract and to determine its fungal activity

through assessing concentrations of minimum inhibitory (MIC), minimum fungicide (MFC), the fungal kinetics (FC) and its interaction with synthetic antifungals like *Candida albicans* ATCC[®] 76485 and ambulatorial lineages of *C. albicans*.

MATERIALS AND METHODS

Anadenanthera colubrina (Benth) Brenan belongs to the Fabaceae family and Mimosoideae subfamily; it is commonly known as angico; black, red, yellow and white angico; bravo, do campo, rajado, fava, jacaré, rosa, do mato, arapiraca, brincos de sagui, cambuí ferro, curupaí, guarapiraca, angico de casca, paricá, cebil and angico de cortume. It is known by the nutritional value of its seeds, proteins, carbohydrates and oils. It is also possible to detect other phytochemicals like lectins, protease and amylase inhibitors, toxins and secondary metabolites (Silva Filho et al., 2013).

Preparation of plant material

This plant was collected in September 2012 in the semi-arid region of Paraíba state, at Serra do Bodocongó located in Queimadas city (7º 22' 25" S, 35º 59' 32"W), at the same region of Borborema and micro region of the West Cariri. *A. colubrina* (Vell.) Brenan specimen, also known as *A. colubrina* (Benth) Brenan, is found at Herbário Manuel de Arruda Câmara (ACAM), located in the Universidade Estadual da Paraíba (UEPB), campus I, Campina Grande, Paraíba (nº 667/ACAM).

An hydroalcoholic extract was obtained from the plant's bark, with 80% alcohol, through maceration technique for 48 h. 10 mg of the plant and 25 ml of solvent proportion were used. Then, it was put in a rotaevaporator and, after, lyophilized.

Microorganisms used and inoculum preparation

For the antimicrobial activity screening, eight clinical strains of *C. albicans* were used (LM 11; LM 94; LM 15; LM 520; LM 14; LM 70; LM 17; LM 410), belonging to the Laboratório de Micologia da Universidade Federal da Paraíba, and a reference strain, *C. albicans* ATCC 76485. The isolated preparations were kept and stored in Ágar Sabouraud Dextrose (Difco®). For testing of the interactive effect the ATCC strain was only used. For the inoculum preparation, isolated colonies of new cultures (24 h) were selected, and with the aid of a inoculation loop, they were transferred to a tube containig 5 ml of NaCl; 0.85%. They were homogenized, comparing its turbidity with a 0.5 tube of the McFarland scale (1.5 x 10^8 UFC/mL).

Antifungal used

The selection of the antifungal discs: fluconazole (25 µg) and nystatin (100 UI) (Cefar®) was based on its use in human clinical medicine.

Determining the antimicrobial activity and the minimum inhibitory concentration (MIC)

Sterile microplates were used containing 96 wells with flat bottoms, where in each well was poured 0.1 ml of Sabouraud Dextrose (Difco®) broth. The plant extract was diluted in 40% alcohol (16 mg/mL-double concentration) and transferred to the first well. Serial

dilutions were then performed to obtain concentrations between 8 and 0.015 mg/mL. Floconazole was used as the positive control and 40% alcohol was used as the negative control. Also sterility controls were performed from the culture medium and angico extract. Cell viability was observed from the inoculum (CLSI, 2008). The plates were incubated at 35/37°C for 24/48h, and the experiments were peformed in triplicate. Fungal viability was detected by adding 20 µL of resazurin (0.01%) in aqueous solution. The plates were reincubated at 35°C for 2 h, and in those wells where fungal growth occurred the resazurin changed to pink. MIC was defined as the lowest concentration of antibacterial agents that inhibited visible growth, as indicated by resazurin staining. The minimum fungicidal concentration was defined as one that prevented the growth of the microorganism, being revealed after sowing.

Fungal kinetic

For this test, the fungal inoculum containing about 10^6 CFU/mL, of Sabouraud Dextrose (Difco®) broth was standardized. The angico's extract was used at three different concentrations: 2x MIC, MIC and 1/2 MIC, 2, 1 mg/mL and 0.5 mg/ml respectively. The loss of cell viability was noticed through the decrease of CFU/ml number, at intervals of 0, 2, 4, 6h, 8h, 10h and 24 h of exposure. An aliquot of 10 uL of test tubes containing the solutions was withdrawn and uniformly seeded on the surface of Petri dishes containing Sabouraud Dextrose Agar. The plates were incubated for 48h at 37°C. The curves were constructed by plotting the mean colony count (log10UFC / mL) versus time of incubation (hours).

Angico's interaction with synthetic antifungals

The analysis of the angico's lyophilized extract interference over effectiveness of antifungal was performed by disk diffusion. The fluconazole discs (25 µg, Cefar®) and nystatin (100 U.I. Cefar®) were soaked with the extract in the following concentrations: 8, 4, 2, 1 and 0.5 mg/mL. It was regarded as interactive effect when there was a change in diameter of the inhibition zones (halo) of microbial growth after this process and, as synergistic interactive effect, if the diameter of the inhibition zones is formed by combining the test product (P). The antifungal (AF) showed an increase of ≥ 2mm when compared to the inhibition zones formed by the AF tested alone. If the inhibition zone formed by the reciprocal activity (AF + P) showed a smaller diameter than the one formed by the AF isolated activity, an antagonistic effect was considered (Cleeland and Squires, 1991; Oliveira et al., 2006). These tests were performed in triplicate and the test's results were obtained by the average of the inhibition zones formed.

RESULTS

All the tested lineages showed sensitivity to nystatin and fluconazole. The inhibition formed opposite nystatin ranged between 23 and 27 mm (mean and SD 24.518 mm ± 0.939) while the inhibition halos in front of fluconazole ranged between 25 and 30 mm (mean and SD 27.592 mm ± 1.494). It was also noticed that the *A. colubrina* extract was active to these tested lineages; it presented halos of 8 mm.

Figure 1 shows the MIC and the minimum fungicide concentration (MFC) of the lineages of tested yeasts, by microdilution. There were the same results in all of them
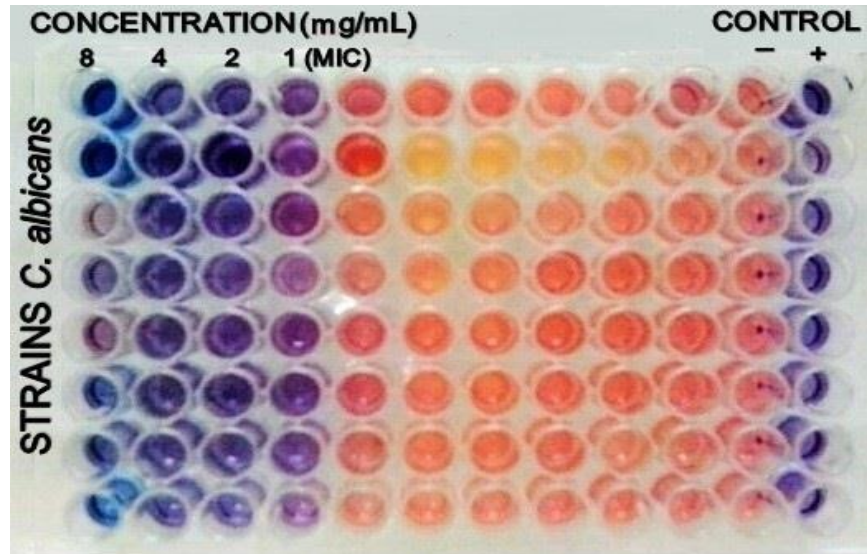
Figure 1. Determining the antifungal activity of the *Candida albicans* strains to the angico's extract.
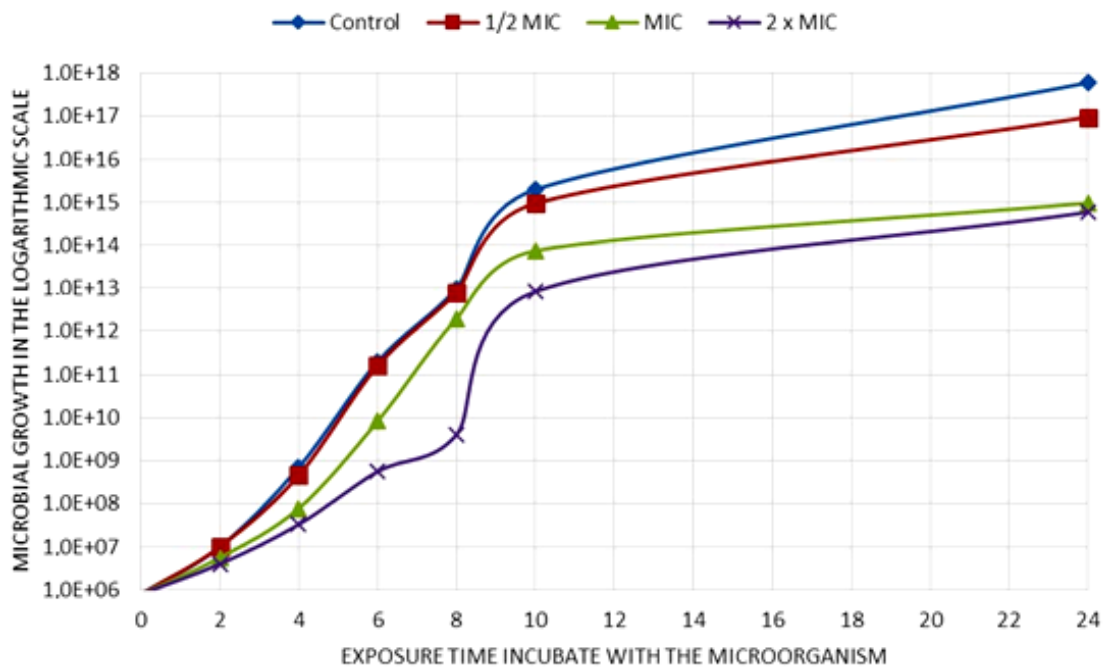

Figure 2. Effect of angico extract on fungal growth kinetics of *Candida albicans* ATCC® 76485. (Control SD± 2.27E+17; ½ MIC SD±3.58E+16; MIC SD±3.59E+14; 2x MIC SD±2.22E+14).

(1.0 and 2.0 mg/ml, respectively). MFC was equivalent to 2x MIC. The change of colours (from blue to pink) in the wells of the microtiter plate, after the addition of resazurin solution shows the decrease of this pigment and indicates the microbial viability. This means that in the wells that changed colours, the concentration of the product was not able to eliminate the yeasts. In the wells with change in colours, the resazurin was not decreased, showing the microbial infeasibility (Rolón et al., 2006). According to the obtained results, it was considered that were similarities in determining the MIC and MFC in the tested lineages.

Figure 2 shows the results of mean values of the angico's solution over *Candida albicans* ATCC® 76485

Table 1. Angico's combination with nystatin and fluconazole antifungals by disk diffusion over the *C. albicans* ATCC[®] 76485 strain.

Antifungals tested	Medium diameters of the growth inhibition zones (mm)					
	Antifungal isolated	Angico's combination with antifungals				
		8 mg/mL	4 mg/mL	2 mg/mL	1 mg/mL	0.5 mg/mL
Nystatin (100 U.I.)	25±1.414	27±1.224	27±1.871	27±1.224	27±1.414	27±1.702
Fluconazole (25 µg/mL)	30±1.632	30±0.707	30±1.414	30±2.121	30±0.707	30±2.449

strain. This shows the number of viable cells through the colony forming units (CFU/ml). When this microorganism was placed in the angico's extract at 2x MIC (2 mg/mL), MIC (1 mg/ml) and ½ MIC (0.5 mg/mL), there was a decrease in the cell multiplication rate in the first hours of its exposure to the growth control without adding the angico. This evidence is the highest rate of reduction of fungal growth 6 h after incubation.

The interactive effects evaluation of the angico and antifungal combination was performed using only *C. albicans* ATCC[®] 76485 strain. There was the presence of a synergistic effect with nystatin, proven by the increase of 2 mm on the diameter of the inhibition zone compared to the halo of the nystatin when tested alone. In relation to the effect of the angico and fluconazole combination, it was considered neutral because no changes in the inhibition zones were observed in none of the added angico concentrations (Table 1).

Analysis of basic descriptive statistics was performed to determine the average, minimum, maximum and standard deviation of each evaluation (antimicrobial activity, interactive effect and fungal kinetics). These parameters were separately evaluated using the Microsoft Excel 2010 and were regarded as the percentage ratio between the standard deviation and arithmetic mean of the tests, under 10%.

DISCUSSION

Medicinal plants have been a rich source for obtaining molecules used therapeutically, since several isolated substances of plants continue to the source of medicines (Foglio et al., 2006; Rocha et al., 2013). The discovery of new drugs that originated from plants led to the isolation of many substances that, still nowadays, are clinicaly used as prototypes for the synthesis of new drugs.

The search for new products and/or drug combinations with antifungal activity is due to the increase of fungal infections worldwide, associated with various stages of immunodeficiency, found mainly in pacients with HIV or immunosuppressive therapies. It increases the number of antifungal prescriptions and favors the appearance of resistant strains, occurring due to the high interaction between the microorganism that causes the infection and antifungal administered (Rivera et al., 2013).

In this study resistant strain to fluconazole was found. The data are compatible with those reported by Castro and Lima (2011). However, there are discrepancies between the accounts of some authors that inform a high number of *Candida* sp. strains resistant to this antifungal (Marr et al., 2000). Other authors indicate that the resistence to fluconazole in *C. albicans* is around 3%, with slight regional variations (Wang et al., 2004; Quintero, 2010).

It is known that the resistant phenomenon is complex and multifactorial. Its mechanisms vary and have several different influences: direct inactivation of the molecule, reduced drug concentration, chemical structure, efflux pumps, as well as physiological changes (Pontón and Quindós, 2006).

The method of microdilution in plates is accurate and practical and it can be used to test microbial sensibility, simultaneously to different drugs. It can easily be used, on a large scale, by laboratories with few technological features. Other techniques such as disk diffusion, E-test, colorimetric methods are used; however, the broth dilution method is considered the standard practice due to its good reproducibility (Koga-Ito et al., 2008).

The results are significant, since the fungistatic activity of the angico's solution over the *C. albicans* was well characterized after 6 h of incubation, with decrease of 3 log 10 CFU/ml when compared to the inoculum of control. Somehow the angico reduced cell multiplication; however, the mode of action needs to be clarified. As Jones et al. (2002) and Shelburne et al. (2004) stated, the fungal kinetics of a product is considered substantially satisfactory if there is decrease in the values of the inoculum tested, compared to the initial inoculum of control; if there are equal or superior numbers of 2 and 3 log 10 CFU/ml, at incubation time of 24 h or less. These lesser degrees of cell death are considered as fungistatic effect. Therefore, according to these definitions, angico has fungistatic effect on the tested strains.

The angico's fungistatic *in vitro* effect observed in this study confirms reports by other authors (Moura et al., 2012), that noted the presence of antioxidant and fungistatic effects on an experimental diet based on angico.

The combined effect of angico extract with antifungals shows the synergistic effect exerted by the extract on interaction with fluconazole in *C. albicans*. According to

Bird et al. (2010), the efficacy of a crude extract may be due to the interplay between the different active constituents that may be present in the extract leading to better activity and/or decrease in potential toxicity of some individual constituents. In spite of that, plant derived antimicrobials are less potent, and plants fight infections successfully. Hence, it becomes apparent that plants adopt a synergistic mechanism between their compounds (Wagner and Ulrich-Merzenich, 2009).

Considering the resistance of yeasts belonging to the genus *Candida* to the main antifungal currently used, it is possible to assert that the search for new chemicals, especially from plants, is very important, as the development of new studies about the effect that these antifungal may cause when used with other products.

The proof of the angico's antibiotic potential *in vitro* and the possibility of this products use on the prevention and treatment of fungal infectious diseases caused by *C. albicans* suggest that toxicological and clinical studies are needed in order to safely determine the possible use of these products as medicine.

Conflict of interests

The authors did not declare any conflict of interest.

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