



Pesquisa Brasileira em Odontopediatria e
Clínica Integrada

ISSN: 1519-0501

apesb@terra.com.br

Universidade Estadual da Paraíba
Brasil

Lira de Souza Sales Rocha, Eveline Angélica; Dantas de Medeiros, Ana Cláudia; Dias de
Castro, Ricardo; Rosalen, Pedro Luiz; Alcântara Saraiva, Karina Lidianne; Pina Godoy,
Gustavo; Rodrigues Apolinário da Silva, Larissa; Sousa Silva Aleixo, Cibelle; Guimarães
Silva, Priscilla; Melo de Brito Costa, Edja Maria

Antifungal Activity, Phytochemical Characterization and Thermal Profile of
Anadenanthera colubrina (Vell.) Brenan

Pesquisa Brasileira em Odontopediatria e Clínica Integrada, vol. 17, núm. 1, 2017, pp. 1-
14

Universidade Estadual da Paraíba
Paraíba, Brasil

Available in: <http://www.redalyc.org/articulo.oa?id=63749543016>

- How to cite
- Complete issue
- More information about this article
- Journal's homepage in redalyc.org

redalyc.org

Scientific Information System

Network of Scientific Journals from Latin America, the Caribbean, Spain and Portugal

Non-profit academic project, developed under the open access initiative



Original Article

Antifungal Activity, Phytochemical Characterization and Thermal Profile of *Anadenanthera colubrina* (Vell.) Brenan

Eveline Angélica Lira de Souza Sales Rocha¹, Ana Cláudia Dantas de Medeiros¹, Ricardo Dias de Castro², Pedro Luiz Rosalen³, Karina Lidianne Alcântara Saraiva⁴, Gustavo Pina Godoy⁵, Larissa Rodrigues Apolinário da Silva¹, Cibelle Sousa Silva Aleixo¹, Priscilla Guimarães Silva¹, Edja Maria Melo de Brito Costa¹

¹Department of Dentistry, State University of Paraíba, Campina Grande, PB, Brazil.

²Post-graduation Program in Dentistry, Federal University of Paraíba, João Pessoa, PB, Brazil.

³Department of Pharmacology and Anesthesiology, Piracicaba School of Dentistry, University of Campinas, Piracicaba, SP, Brazil.

⁴Nucleus of Technology Platforms, Aggeu Magalhães Research Center, Fiocruz, Recife, PE, Brazil.

⁵Department of Pathology, Federal University of Pernambuco, Recife, PE, Brazil.

Author to whom correspondence should be addressed: Eveline A. L. S. S. Rocha, Avenida das Baraunas, S/N, Bodocongo, Campina Grande, PB, Brazil. 58429-500. E-mail: velpb@hotmail.com.

Academic Editors: Alessandro Leite Cavalcanti and Wilton Wilney Nascimento Padilha

Received: 15 November 2016 / Accepted: 30 April 2017 / Published: 21 June 2017

Abstract

Objective: To investigate the antifungal potential of *A. colubrina*, and its phytochemical characteristics, thermal profile and toxicity. **Material and Methods:** To assess potential antifungal activity, the technique of microdilution was used with the determination of the Minimum Inhibitory Concentration and Minimum Fungicidal Concentration, using standard species of *Candida* and recent clinical isolates of *Candida albicans*. Analyses of action of the extract were performed on the wall and cell morphology of *C. albicans*, of the interactive effect between the plant extract and nystatin on *C. albicans* through the checkerboard method, and of growth kinetics. The phytochemical screening was determined by spectrophotometry. The thermal profile was traced with the determination of thermogravimetric curves (TG) and differential scanning calorimetry (DSC). The toxicity was evaluated by the method of hemolysis. **Results:** The extract of *A. colubrina* showed a fungistatic potential against all bacteria tested and it acted by modifying the cellular morphology of *C. albicans*. There was a synergism between nystatin and the plant extract (FIC=0.375), and 53.18% of total polyphenols were determined. The TG curve showed the occurrence of three steps of thermal decomposition. None of the tested concentrations became the effective cytotoxic concentration. **Conclusion:** Further studies should be conducted to understand the efficacy and the mechanisms of action involved in the antifungal activity of the plant extract of *A. colubrina* in order to produce a new drug for the treatment of oral candidiasis.

Keywords: Plants, Medicinal; Plant Extracts; *Candida*; Antifungal Agents.

Introduction

Oral candidiasis is an opportunistic infection that has a high occurrence rate and that is frequently associated with *Candida albicans* [1]. The predisposing factors for its development can be classified into local factors such as use of prosthesis, reduced salivary flow and sugar-rich diet, and systemic factors such as advanced age, endocrine disorders, immunosuppression, use of broad-spectrum antibiotics and nutritional deficiencies [2].

Among the most appropriate drugs used for the treatment of oral candidiasis, miconazole, from the group of azoles, and nystatin, from the group of polyenics, stand out [3]. Despite its efficacy, miconazole can trigger undesired drug interactions, reducing drug absorption and increasing the risk of adverse effects through inhibition of human cytochrome P450 [4,5]. Nystatin, because it binds directly to ergosterol and interacts with a component of the human membrane (cholesterol), may lead to side effects [6,7]. Thus, there is a demand for new antifungals with different structural classes that can act selectively on new cellular targets with fewer side effects [8]. In this perspective, the antifungal activity of medicinal plants, aiming to develop new drugs, has been investigated.

Among plants used by the population for medicinal purposes is the *Anadenanthera colubrina* (Vell.) Brenan, used to treat diarrhea, cough, bronchitis, the flu, inflammation and tissue damage, allergy, rash, constipation, gastritis, among other uses [9-11]. An analgesic action of parts of this plant was identified. It is a result of its antinoceptive effects mediated by central and peripheral mechanisms [12] and with a potential healing and tissue repair action [13]. Regarding antimicrobial activity, activity against *P. aeruginosa* [14], *Staphylococcus aureus* [15] and *Candida albicans* [16] was observed. This study aims to investigate the activity antifungal, phytochemical and thermic profil of the hydroalcoholic extract of the bark of *Anadenanthera colubrina* (Vell.) Brenan.

Material and Methods

Plant Extract Collection

Barks from *Anadenanthera colubrina* (Vell.) Brenan were used. They were collected in the region of the Brazilian semi-arid (7°22'25" S, 35°59'32" W) with the guidance of a botanist. The testimony specimen is deposited in the collection of the Herbarium Manuel de Arruda Câmara (ACAM) of the State University of Paraíba, Campina Grande, Paraíba, under registration n° 667/ACAM. The material was dehydrated in an air circulating oven at 40°C, subsequently ground and then immersed in ethyl alcohol 80% (10g/25mL) for 48 hours at room temperature. The resulting mixture was filtered and the filtration residues were immersed again twice in the solvent. The final three liquid phases were concentrated on a rotary evaporator in vacuum at 39°C, subsequently lyophilized and kept under refrigeration.

Antimicrobial Activity

Microorganisms

The microorganisms used were *Candida albicans* (ATCC 18804), *Candida krusei* (ATCC 34135), *Candida glabrata* (ATCC 15545), *Candida guilliermondii* (ATCC 6260), *Candida parapsilosis* (ATCC 22019), *Candida tropicalis* (ATCC 13803), all provided by the Reference Materials Laboratory of National Institute for Health Quality Control (Oswaldo Cruz Foundation, Rio de Janeiro, RJ, Brazil), and fresh isolates of *Candida albicans* (LM 11, LM 520 LM 70, LM 410) were provided by the Mycology Laboratory of the Federal University of Paraíba.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) was determined by broth microdilution technique using 96-well plates (Alamar Tecno Científica Ltda., Diadema, SP, Brazil) in accordance with the provisions of the document M27A2 [17]. The inoculum with a 24-hour growth was standardized in a spectrophotometer, corresponding to 2.5×10^3 CFU/mL. To each well of the microtiter plate 100 μ L of broth culture medium Sabouraud dextrose, 100 μ L of plant extract, whose concentration ranged from 15.62 to 2,000 μ g/mL, and 100 μ L of the inoculum were placed (final concentration: 5×10^6 UFC/mL). The viability control of the tested yeast strain and the sensitivity control to nystatin of the strain were made. The plates were incubated for 24 hours at 37°C. After the incubation period, 10 μ L of the aqueous solution of resazurin 0.01% were added to each well to perform a MIC reading. Viable microorganisms were reduced from a blue color to a pink color. The experiment was performed in triplicate on three different occasions.

Determination of the Minimum Fungicidal Concentration (MFC)

After determining the MIC, the concentration corresponding to the inhibitory and to higher concentrations, as well as the controls, were subcultured on Sabouraud dextrose agar plates. After 24 hours of incubation at 37°C, MFCs' readings were made, being the MFC the lowest extract concentration in which a visible growth of the subculture was prevented.

Action of the Extract on the Cell Wall of *Candida albicans*

The action of the extract on the cell wall was analyzed by the microdilution in broth technique, with MIC determination, in the presence of sorbitol (0.8 M). Microtiter plates containing 96 wells, with the bottom in a "U" shape, were used (Alamar Tecno Científica Ltda., Diadema, SP, Brazil). In each well of the plate, 100 μ L of broth culture medium Sabouraud Dextrose, previously added to sorbitol with molecular weight of 132,17g (Vetec Fine Chemicals Ltda., Rio de Janeiro, RJ, Brazil), and 100 mL of the extract in concentrations from 32 to 0.25 mg/mL were added. Then, 10 μ L of the microbial inoculum were added at a concentration of 2.5×10^3 CFU/mL in each well. The microplates were seeded and incubated at 37°C for 48 hours, then added to an aqueous resazurin 0.01% solution to perform the MIC reading. The experiment was performed in triplicate.

Cell Morphology Analysis of *Candida albicans*

The cellular morphology of *C. albicans* treated with the plant extract was analyzed by scanning electron microscopy (SEM). Aliquots of fungal strains treated with the plant extract and nystatin, and antifungal free strains in culture medium, were centrifuged at 3,500 rpm for 15 minutes to perform deposition of organic material. The supernatant was removed and the samples were fixed with a solution of 2.5% glutaraldehyde and 0.1 M cacodylate buffer. There was a new centrifugation and a subsequent wash with Caco Buffer 0.1M. After removing the buffer, the fungal material was inserted into a glass coverslip to be processed. The processing consisted of three washes with B. Caco 0.1M for 10 minutes, post-fixation at a 1:1 proportion + Osmium 2% + B. Caco 0.1M for 30 minutes to enable a greater material contrast upon reading, two washes with B. Caco 0.1M for 10 minutes and two washes with distilled water for 10 minutes. Dehydration was also performed with a wash in acetone at concentrations of 30%, 50%, 70%, 90% and 100%. The material was dried in a Critical Point (CPD 030 BAL-TEC) for 2 hours, and then the assembly in stubbs was made (carbon tape and silver ink). The stubbs act as electron conductors. Finally, the gold plating was performed for 30 minutes in a sputtering system (Leica EM SCD 500) and then it was analyzed by scanning electron microscope (Quanta 200 FEG, Fei Company, Oregon, USA).

Analysis of the Interactive Effect

Checkerboard Method

The combined effect of the two substances (nystatin and plant extract of *A. colubrina*) was determined from the microdilution-checkerboard method for derivation of the Fractional Inhibitory Concentration index (FIC index). The microbial inoculum was standardized in a spectrophotometer to obtain a 2.5×10^3 CFU/mL concentration. The extract and the nystatin were tested considering the MIC. Initially, 100µL of the Sabouraud dextrose agar broth culture medium was added to sterilized microplate wells containing 96 wells with a "U" shape (Alamar Tecno Científica Ltda., Diadema, SP, Brazil). Then, 50µL of each substance in concentrations MIC/8, MIC/4, MIC/2, MIC, MICx2, MICx4 and MICx8 were added to the microplate, and finally the inoculum was added. The extract was placed on horizontal lines and the nystatin on vertical lines. The microplates were incubated at 37°C for 48 hours and fungal growth was evidenced by the use of an aqueous 0.01% resazurin solution. The experiment was performed in triplicate.

The FIC index was calculated by the sum of the $FIC^A + FIC^B$, where A represents the plant extract and B the nystatin. The FIC^A is calculated by the MIC^A combined (extract+nystatin)/ MIC^A alone, while the $FIC^B = MIC^B$ combined (extract+nystatin)/ MIC^B alone. This index was interpreted as follows: synergism (<0.5), additivity (0.5-1.0), indifference (>1 and <4) or antagonism (>4) [18].

Growth Kinetics

The study of the interference of the plant extract, associated or not to nystatin, on the growth of *C. albicans* was conducted through the viable cell count method. Initially, 0.5 mL of yeast suspension was inoculated to 4.5 mL of Sabouraud dextrose broth containing different

concentrations of the extract (MIC/8; MIC/4, MIC), nystatin (MIC/8; MIC/4, MIC) and two associations of substances (MIC/8 of extract + MIC/4 of nystatin and MIC/8 of nystatin + MIC/4 of extract). At 0 min and 30 min intervals, 1, 2, 3, 4, 5, 6, 12 and 24 hours after incubation, an aliquot of 10µL of this inoculum was evenly inoculated on Petri plates containing Sabouraud dextrose agar culture medium. In addition, the control experiment was carried out with free antifungal growth. The inoculated plates were incubated at 35°C for 48 hours. After the incubation period, the counting of the number of viable cells was made. It was expressed as CFU/mL and presented in the form of a microbial kill curve. Data were analyzed and processed using SPSS software version 20.0. The Friedman and Kruskal-Wallis tests were made with a 5% significance level.

Phytochemistry Prospecting

To determine the total polyphenol content, the colorimetric method was used. It uses the Folin-Ciocalteu reagent [19]. 1 mL of the aqueous extract solution was added to 1 mL of the Folin-Ciocalteu 1N reagent and, after a 2-minute rest, 2 mL of an aqueous solution of Na₂CO₃ at 20% (p/v) was added, then allowing it to rest for another 10 minutes. Then, the absorbance reading at 757nm was made in a spectrophotometer (Shimadzu® UV mini - 1240), against a blank composed of distilled water, Folin-Ciocalteu reagent and 20% of a solution of Na₂CO₃. The calibration curve was obtained from gallic acid solutions at concentrations of 1, 3, 6, 9, 12, 15, 20, 25, 30, 35, and 40 mg/mL. The concentration of polyphenols was measured in equivalent milligrams of gallic acid.

The determination of the total flavonoid content was performed by adding 5 mL of each extract solution (in methanol) to the same volume of a solution (in methanol) of AlCl₃ at 2% (p/v) [20]. The mixture rested 10 minutes before the absorbance reading at 415 nm against a blank consisting of an AlCl₃ solution was made. For this determination, a calibration curve obtained from quercetin solutions at concentrations of 2, 4, 6, 8, 10, 13, 16, 19, 22, 26, 28 and 30 µg/ml was made. The concentration of flavonoids was expressed in equivalent milligrams of quercetin.

The content of condensed tannins was quantified by adding 0.5 mL of the sample plant extract to 3 mL of a vanillin solution (4% p/v in methanol). Then, 1.5 mL of concentrated HCl (37%) was added [21]. The reaction occurred in test tubes soaked in water at about 22°C. The reading was taken at 500nm against a blank consisting of the vanillin solution, HCl and a solution of ethanol 50% (v/v) in water. The calibration curve for this assay was made using catechin solutions at concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/mL. The concentration of tannins was expressed as equivalent milligrams of catechin. All analyzes were performed in triplicate.

Thermal Profile

Thermogravimetric curves (TG) were obtained with a simultaneous analyzer Model SDT Q600 (TA Instruments) using alumina crucibles containing samples with 8±0.1 mg, in a nitrogen atmosphere, under a flow of 50 mL.min⁻¹. The experiments were conducted between temperature ranges from 25 to 900°C at a heating rate of 10°C min⁻¹. For the calibration of the equipment, a

standard calcium oxalate monohydrate was used. DSC curves were obtained from a calorimeter Model DSC Q20 (TA Instruments) using alumina crucibles containing samples with 2 ± 0.1 mg, in a nitrogen atmosphere, under a flow of 50 mL.min⁻¹. The experiments were conducted between temperature ranges from 25 to 400°C at a heating rate of 10°C min⁻¹. For the calibration of the equipment, an Indium standard (m.p. = 156.6°C) was used.

Toxicity

The toxicity of *A. colubrina* extract was analyzed by the hemolysis method. A red 4% blood cell suspension was prepared in a 0.9% saline solution. Then, 1 mL of this suspension was distributed in test tubes and homogenized with 1 mL of the diluted extract in different concentrations in order to obtain 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg/mL. After 1 hour, the samples were centrifuged at 3,000 rpm for 10 minutes and the reading was visually performed, taking into consideration the amount of erythrocytes lysed. The hemolysis visualization was classified as - (0% hemolysis) + (25% hemolysis), ++ (50% hemolysis), +++ (75% hemolysis), and ++++ (100% hemolysis) [22]. This reading was made in a spectrophotometer with a 540nm wavelength (Shimadzu® UV mini - 1240), using as a blank a 5% saline solution to confirm the results of the visual reading. Two negative controls were used, one being the suspension of 4% red blood cells and the other the diluted plant extract. As a positive control, a Turk Liquid hemolizing solution was used.

Results

The extract of *A. colubrina* inhibited the growth of all strains of tested *Candida* (Table 1) and produced a slight change in the morphology of *C. albicans* ATCC 18804 (Figure 1). The MIC of the extract of this species increased from 1 mg/mL to 8 mg/mL in the presence of sorbitol.

Table 1. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of the hydroalcoholic extract of the bark of *Anadenanthera colubrina* (Vell.) Brenan and of nystatin (Sigma-Aldrich®) against *Candida* species.

	<i>Anadenanthera colubrina</i> (Vell.) Brenan		Nystatin	
	mg/ml		mg/ml	
	(MIC)	(MFC)	(MIC)	(MFC)
<i>C. albicans</i> (ATCC 18804)	1	>2	0.006	0.006
<i>C. glabrata</i> (ATCC 15545)	0,5	>2	0.006	0.006
<i>C. krusei</i> (ATCC 34135)	1	>2	0.006	0.03
<i>C. guilliermond</i> (ATCC 6260)	1	>2	0.006	0.006
<i>C. tropicalis</i> (ATCC 13803)	1	>2	0.006	0.01
<i>C.albicans</i> LM11	0,5	0.5	0.006	0.006
<i>C.albicans</i> LM70	0.25	0.25	0.006	0.006
<i>C. albicans</i> LM 410	0.5	0.5	0.006	0.006

The interactive effect of the extract of *A. colubrina* and nystatin showed a synergistic activity, with a FIC equal to 0.375 (Table 2) and a reduction of the number of CFU/mL ($p < 0.05$) in the first 6 hours (Figures 2, 3 and 4).

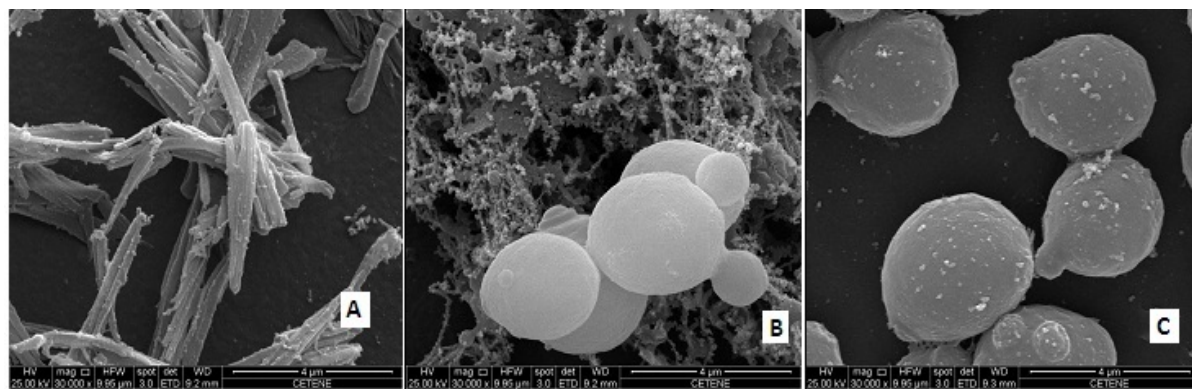


Figure 1. Photomicrography by Scanning Electron Microscopy (SEM) of strains of treated *Candida albicans* (ATCC 18804). A: nystatin (100,000 IU suspension); B: plant extract (1 mg/mL); and C: untreated, after 24 hours of incubation at 37°C.

Table 2. Minimum Inhibitory Concentration (MIC) (mg/mL) of plant extract and nystatin combined, and Fractional Inhibitory Concentration Index (FIC index) against *C. albicans* (ATCC 18804).

Product	MIC (combination) mg/MI	
Vegetal Extract	0.25	0.125
Nystatin	0.00075	0.0015
FIC	0.375	0.375

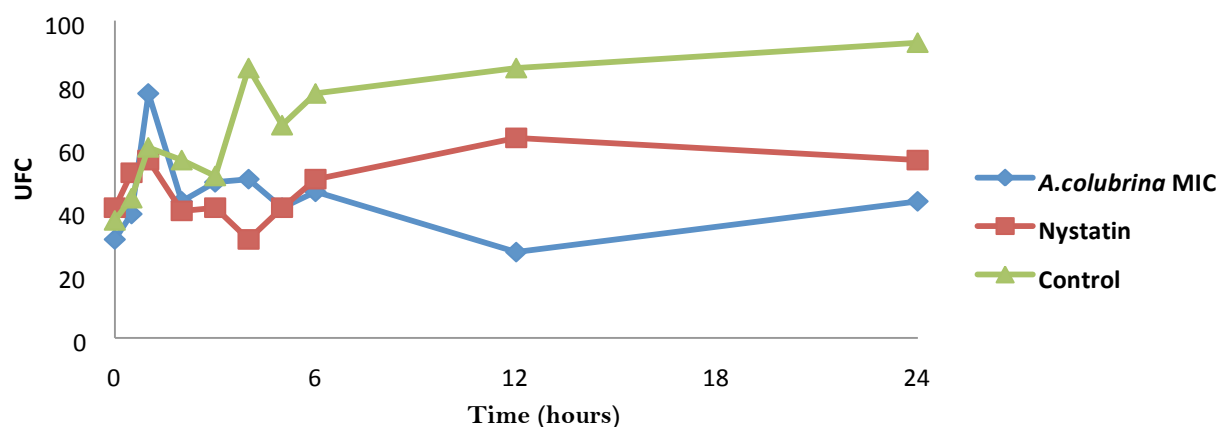


Figure 2. Kinetics of *C. albicans* growth (ATCC 18804) under the activity of the test products in MIC.

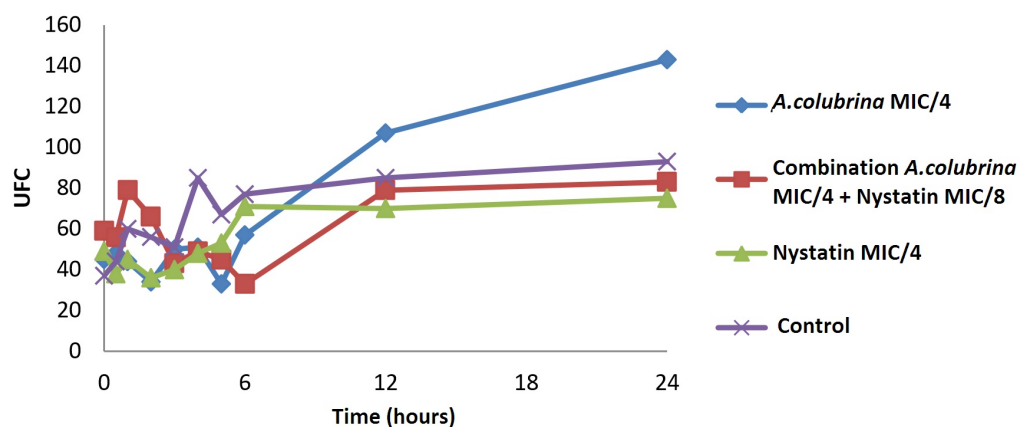


Figure 3. Kinetics of *C. albicans* (ATCC 18804) growth under the activity of the test products in MIC/4.

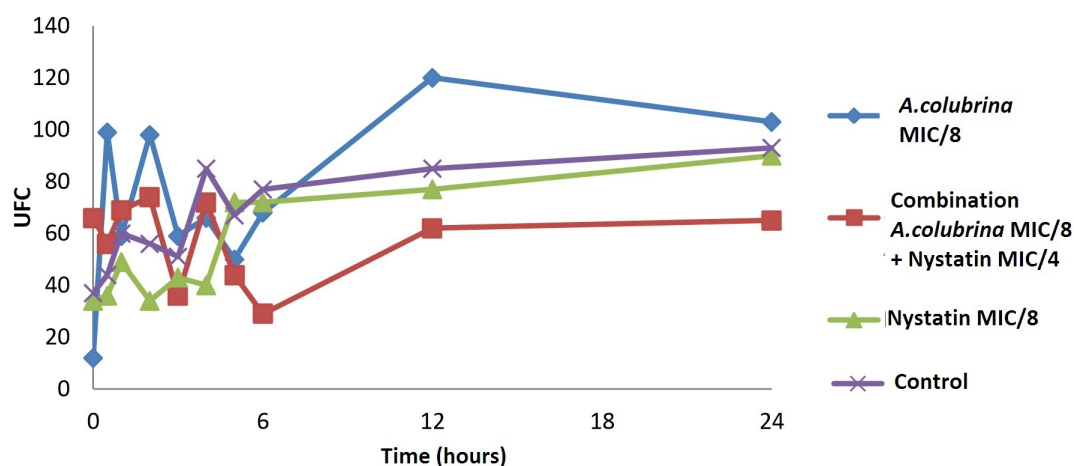


Figure 4. Kinetics of *C. albicans* (ATCC 18804) growth under the activity of the test products in MIC/8.

The TG curve of the extract of *A. colubrina* showed the occurrence of three stages of thermal decomposition and the DSC curves of the extract showed that the thermal processes occurred in temperature ranges from 52.37 to 195,52°C (Figure 5).

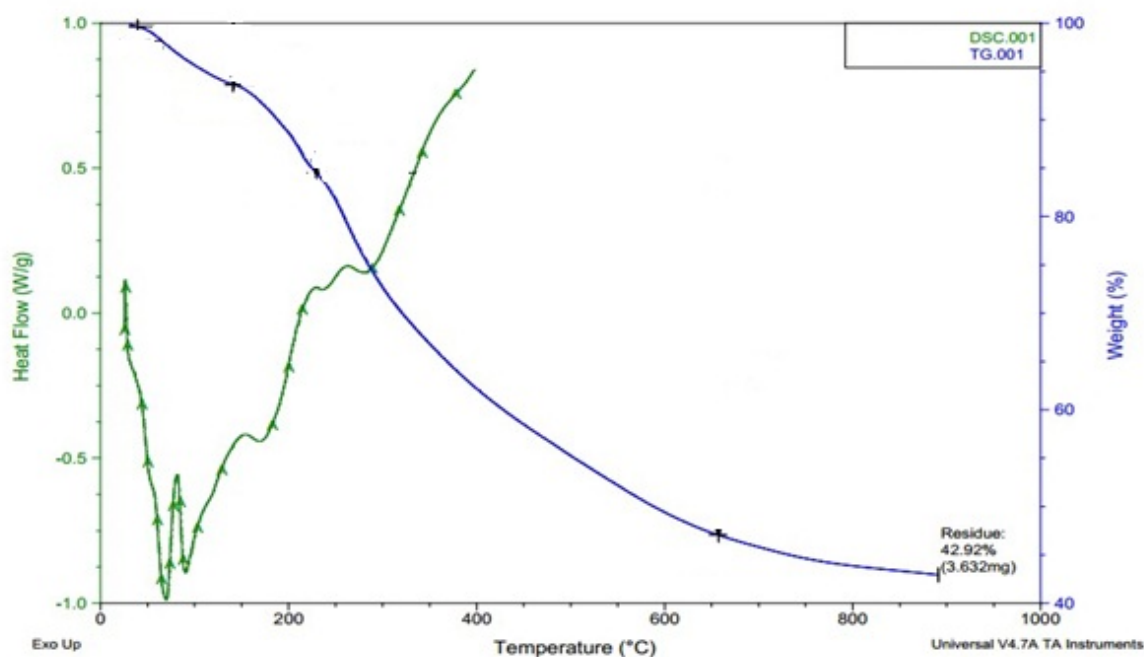


Figure 5. TG and DSC curves. 10°C min⁻¹ heating rate. Temperature ranges from 25 to 900°C for TG, and from 25 to 400°C for DSC.

In the hemolysis assay, the extract of *A. colubrina* showed a toxicity equivalent to 25% when compared to the positive control (Table 3).

Table 3. Visual reading of hemolysis of red blood cell suspensions tested with a lyophilized extract of *Anadenanthera colubrina* Vell. Brenan. Reference: - (0% hemolysis) + (25% hemolysis, or below 25%), ++ (50% hemolysis), +++ (75% hemolysis).

Concentrations (mg/mL)	Intensity Hemolysis
32	+
16	+
8	+
4	+
2	+
1	+
0.5	+
0.25	+
<i>Turk Liquid (Positive control)</i>	++++

Discussion

The hydroalcoholic extract of the bark of *A. colubrina* has an antifungal activity for different species of *Candida*, whose potential ranged from moderate to strong [23]. It is worth noting that other authors consider different parameters to assess the antimicrobial potential of medicinal plants, which is considered moderate or strong when the MIC is less than 500 mg/mL or 100 mg/mL, respectively [24]. These data are relevant especially when discussing sustainability and production of new pharmaceutical compounds. The extract of *A. colubrina* presented a fungistatic profile and was not fungicidal against species of *Candida*, which deserves attention when considering the balance and maintenance of oral microbiota, where the substance controls the growth of the microorganism without removing it completely from the surface where it commonly resides [25].

It is possible that the inhibition of growth of *Candida* species produced by the extract of *A. colubrina* has occurred due to its action on the cell wall, since its MIC increased in the presence of an osmotic shield, sorbitol. Slight alterations in the cell wall were observed in photomicrographs by scanning electron microscopy, especially represented by the loss of the typical rough aspect of the cell without treatment [16]. The action of the extract on the cell wall has a positive clinical significance, for it is a structure that mammalian cells do not have [7,26] which favors selectivity and therefore more security to the host. Although the results suggest the *A. colubrina* as a new source for the development of anti-fungal drugs with greater specificity, there is a great need for more research on its mechanism of action.

Interaction tests of the extract of *A. colubrina* and nystatin indicated a synergistic effect, probably by acting simultaneously on different cellular targets. Nystatin acts on the membrane of the fungal cell [4,5] and *A. colubrina* extract acts possibly on the cell wall. Different mechanisms may be involved in synergistic activity between two agents. Some authors [27,28] described four of them: (1) inhibition of different stages in intracellular fungal biochemical pathways, essential to cell survival; (2) increased penetration of the antifungal agent provided by the action of another antifungal on fungal cell membrane; (3) inhibition of carrier proteinases; and (4) inhibition of different cellular targets simultaneously.

One of the simplest and most known protocols for determination of antimicrobial interaction is the checkerboard test, which provides a two-dimensional array of different concentrations of analyzed substances. This test allows the calculation of the Fractional Inhibitory Concentration Index (FIC index) [18]. The checkerboard test and the microbial kill curve are indicated for this evaluation *in vitro* [29]. The synergistic effect between *A. colubrina* extract and nystatin, even at low concentrations, was maintained over time with a reduction in the number of CFU/mL in relation to the control.

The synergism between antifungal agents may increase the fungal clearance rate, shorten the duration of the therapy, prevent the emergence of drug resistance, broaden the spectrum of activity, and diminish the toxicity associated with the drug, allowing the use of lower doses of antifungal agents [30]. In this study, it was observed that the contact time of the substances with cells positively favored such reduction in the first 6 hours. The time factor is important in the prospecting of antifungals, for the prolonged use of antifungal agents and longer dosing regimens appear to contribute to the development of microbial resistance [31].

In the phytochemical prospecting, total polyphenols, tannins and flavonoids were identified. Their results are in agreement with the findings of other authors [32,33]. The high content of phenolic compounds found in the *A. colubrina* extract may be responsible for its antifungal potential. These compounds are capable of causing metabolic instability in *C. albicans* and they destroy the enzymatic activity of proteasomes, thereby contributing to a reduction in the growth rate of microorganisms, as well as biofilm formation and maturation [34]. The tannins are part of the group of phenolic compounds and have an antimicrobial activity due to the ability to precipitate proteins [35], leading to inactivation of adhesins, enzymes or the transport of proteins in the cell envelope, interfering with solute transportation or decreasing the availability of metal ions essential to the metabolism of microorganisms [36,37]. To a lesser extent, flavonoids have been identified, which also compose the group of phenolic compounds [38], and they may also be associated with the antifungal action of *A. colubrina* extract [38-40].

In thermogravimetric analysis, the first step of TG occurred at temperature ranges from 39.17 to 141.26°C, representing a mass loss of the sample of approximately 5.97%. This step was attributed to water loss and certain volatile products of the sample. This moisture content is in accordance with the criteria of the Brazilian Pharmacopoeia, which establishes for the control of plant material a limit of 14% as the maximum acceptable amount of moisture. The TG is an effective technique to determine the moisture content of the plant and, with these results, it is possible to establish physical parameters for an efficient drying and proper preservation of the material [41]. The second stage occurred within a temperature range from 141.26 to 229.17°C, having a mass loss of 9.13%, corresponding to the first step of extract decomposition. The most significant mass loss of the extract was observed between the temperatures ranging from 229.17°C and 657.39°C, with loss of 37.44%. This event is probably related to the metabolites present in the plant, already identified as tannins and flavonoids.

In the DSC curve, an endothermic peak was observed at 69,96°C, which may be related to the vaporization of the sample, with loss of water and volatile constituents. Decomposition processes start over 195,52°C, which are possibly related to the initial decomposition of secondary metabolites present in the sample as flavonoids, tannins and other [41]. This high temperature for the beginning of compounds decomposition present in the plant suggests a good stability of the plant extract. The data obtained by thermal analysis are directly related to the final quality of a pharmaceutical product, either as to the therapeutic efficacy of the product or as to its stability throughout the period of validity. TGs are used to measure mass variation according to temperature in a controlled atmosphere under a heating program. The DSC is used to measure the heat flow difference between a substance and a reference material in function of a heating or cooling program. However, some difficulties are encountered in obtaining reproducible peaks of plant extracts in DSC curves. This is because these extracts are a mixture of substances that interact in the vegetable matrix. In addition, the degradation products are often formed in different concentrations due to various factors such as the shape and imperfections of the particles, loss of gaseous products and the heating rate the sample. In addition, impurities present in plant extracts have a direct effect on the width of peaks obtained in the endothermic process [41,42].

None of the tested concentrations became an effective 50% cytotoxic concentration (EC50), i.e., able to hemolyze 50% of a 4% suspension of erythrocytes in the hemolysis assay [43]. Thus, it was possible to determine the Selectivity Index (SI) of the plant extract, obtained by a IC50/MIC ratio greater than 32. The higher the value of SI, the more active it is against the microorganism and less toxic to the host [44]. The results indicate that *A. colubrina* can be considered a promising source for the development of new drugs. However, other studies *in vitro* should be conducted to assess biocompatibility and the mechanism of action of the *A. colubrina* extract, mimicking the biological conditions of the oral mucosa, as well as *in vivo* tests to prove its effectiveness aiming to produce a new drug for the treatment of oral candidiasis.

Given the limitations of this study, oral cavity condition differs from *in vitro* status, further studies *in vitro* and *in vivo* should be conducted to understand the efficacy and the mechanisms of action involved in the antifungal activity of plant extract of *A. colubrina* in order to produce a new drug for the treatment of oral candidiasis.

Conclusion

The hydroalcoholic extract from the bark of *Anadenanthera colubrina* (Vell.) Brenan showed a fungistatic activity against *Candida* species and a synergistic interaction when combined with nystatin. It exhibited a high concentration of total polyphenols, a good thermal stability and an absence of toxicity to red blood cells.

Acknowledgements

This study was supported by the State University of Paraíba, the Brazilian Coordination of Higher Education, Ministry of Education (CAPES), National Council for Scientific and Technological Development (CNPQ), Brazil and Centro de Tecnologias Estratégicas do Nordeste (CETENE).

References

1. Li YY, Chen WY, Li X, Li HB, Li HQ, Li W, He L, Yang X, Wang X, Huang Y, Yao Y. Asymptomatic oral yeast carriage and antifungal susceptibility profile of HIV-infected patients in Kunming, Yunnan Province of China. *BMC Infect Dis* 2013; 13: 2-9. doi: 10.1186/1471-2334-13-46.
2. Williams D, Lewis M. Pathogenesis and treatment of oral candidosis. *J Oral Microbiol* 2011; 3(1):1-11. doi: 10.3402/jom.v3i0.5771.
3. Samaranayake LP, Leung K, Jin L. Oral mucosal fungal infection. *Periodontol* 2000 2009; 49(1):39-59. doi: 10.1111/j.1600-0757.2008.00291.x.
4. Lewis RE. Current concepts in antifungal pharmacology. *Mayo Clin Proc* 2011; 86(8):805-17. doi: 10.4065/mcp.2011.0247.
5. Patil S, Rao RS, Majumdar B, Anil S. Clinical appearance of oral candida infection and therapeutic strategies. *Front Microbiol* 2015; 6(1):1-10. doi: 10.3389/fmicb.2015.01391.
6. Shapiro RS, Robbins N, Cowen LE. Regulatory circuitry governing fungal development, drug resistance, and disease. *Microbiol Mol Biol Rev* 2011; 75(2):213-67. doi: 10.1128/MMBR.00045-10.
7. Lyu X, Zhao C, Yan Z, Hua H. Efficacy of nystatin for the treatment of oral candidiasis: a systematic review and meta-analysis. *Drug Des Devel Ther* 2016;10(1):1161-71. doi: 10.2147/DDDT.S100795.
8. Abad MJ, Ansuategui M, Bermejo P. Active antifungal substances from natural sources. *J Organic Chem* 2007; 2(1):116-45.
9. Albuquerque UP, Medeiros PM, Monteiro JM, Lins Neto EMFL, Gomes de Melo J, Santos JP. Medicinal plants of the caatinga (semi-arid) vegetation of NE Brazil: A quantitative approach. *J Ethnopharmacol* 2007; 114(3):325-54. doi: 10.1016/j.jep.2007.08.017.
10. Medeiros PM, Ladio AH, Albuquerque UP. Patterns of medicinal plant use by inhabitants of Brazilian urban and rural areas: A macroscale investigation based on available literature. *J Ethnopharmacol* 2013; 150(2): 729-46. doi: 10.1016/j.jep.2013.09.026.
11. Pedone-Bonfim MV, Lins MA, Coelho IR, Santana AS, Silva FS, Maia LC. Mycorrhizal technology and phosphorus in the production of primary and secondary metabolites in cebil (*Anadenanthera colubrina* (Vell.) Brenan) seedlings. *J Sci Food Agric* 2013; 93(6):1479-84. doi: 10.1002/jsfa.5919.
12. Damascena NP, Souza MT, Almeida AF, Cunha RS, Damascena NP, Curvello RL, et al. Antioxidant and orofacial anti-nociceptive activities of the stem bark aqueous extract of *Anadenanthera colubrina* (Velloso) Brenan (Fabaceae). *Nat Prod Res* 2014; 28(10):753-6.
13. Pessoa WS, Estevão LR, Simões RS, Barros ME, Mendonça FS, Baratella-Evência L, et al. Effects of angico extract (*Anadenanthera colubrina* var. cebil) in cutaneous wound healing in rats. *Acta Cirur Bras* 2012; 27(10):655-70. doi: 10.1590/S0102-86502012001000001.
14. Trentin DS, Silva DB, Amaral MW, Zimmer KR, Silva MV, Lipes NP, et al. Tannins possessing bacteriostatic effect impair pseudomonas aeruginosa adhesion and biofilm formation. *PloS One* 2013; 8(6):1-13. doi: 10.1371/journal.pone.0066257.
15. Silva CR, Oliveira LD, Leão MV, Jorge AO. *Candida* spp. adherence to oral epithelial cells and levels of IgA in children with orthodontic appliances. *Braz Oral Res* 2014; 28(1):28-32. doi: 10.1590/S1806-83242013005000031.
16. Lima RF, Alves EP, Rosalen PL, Ruiz ALCG, Duarte MCT, Góes VFF, et al. Antimicrobial and anti-proliferative potential of *Anadenanthera Colubrina* (Vell.) Brenan. *Evidence-Based Complementary and Alternative Medicine*, 2014, Article ID 802696. doi: 10.1155/2014/802696.
17. Clinical and Laboratory Standards Institute. M27-S3. Reference method for broth dilution antifungal susceptibility testing of yeasts: 3rd. informational supplement. CLSI, Wayne, PA, 2008.
18. Odds FC. Synergy, antagonism and what the checkerboard puts between them. *J Antimicrob Chemother* 2003; 52(1):1. doi: 10.1093/jac/dkg301.

19. Chandra S, De Mejia Gonzalez E. Polyphenolic compounds, antioxidant capacity, and quinone reductase activity of an aqueous extract of *Ardisia compressa* in comparison to mate (*Ilex paraguariensis*) and green (*Camellia sinensis*) teas. *J Agric Food Chem* 2004; 52(11):3583-9. doi: 10.1021/jf0352632.
20. Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasanhoney, as well as their radical scavenging activity. *Food Chem* 2005; 91(3):571-7. doi: 10.1016/j.foodchem.2004.10.006.
21. Makkar HPS, Blümmel M, Borowy NK, Becker K. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *J Sci Agric* 1993; 61(2):161-5. doi: 10.1002/jsfa.2740610205.
22. Pereira VSS, Oliveira CSC, Fumagalli F, Emery FS, Silva NB, Andrade-Neto VF. Cytotoxicity, hemolysis and *in vivo* acute toxicity of 2-hydroxy-3-anilino-1,4-naphthoquinone derivatives. *Toxicology Reports* 2016; 3(1):756-62. doi: 10.1016/j.toxrep.2016.09.007.
23. Aligiannis N, Kalpotzakis E, Mitaku S, Chinou IB. Composition and antimicrobial activity of the essential oils of two *Origanum* species. *J Agr Food Chem* 2001; 49(9):4168-70. doi: 10.1021/jf001494m.
24. Ríos JL, Recio MC. Medicinal plants and antimicrobial activity. *J Ethnopharmacol* 2005; 100(1-2):80-4. doi: 10.1016/j.jep.2005.04.025.
25. Moyes DL, Naglik JR. Mucosal Immunity and *Candida albicans* Infection. *Clin Dev Immunology* 2011; 2011(1):1-9. doi: 10.1155/2011/346307.
26. Ibe C, Walker LA, Gow NAR, Munro CA. Unlocking the therapeutic potential of the fungal cell wall: Clinical implications and drug resistance. *Candida albicans: Cellular and molecular biology*. 2nd. ed. Prasad R (Ed.). Springer International Publishing 2017. pp. 313-46.
27. Johnson MD, MacDougall C, Ostrosky-Zeichner L, Perfect JR, Rex JH. Combination antifungal therapy. *J Antimicrob Chemother* 2004; 48(3):693-715. doi: 10.1128/AAC.48.3.693-715.2004.
28. Castro RD, de Souza TM, Bezerra LM, Ferreira GL, Costa EM, Cavalcanti AL. Antifungal activity and mode of action of thymol and its synergism with nystatin against *Candida* species involved with infections in the oral cavity: an *in vitro* study. *BMC Complement Altern Med* 2015; 24(15):417. doi: 10.1186/s12906-015-0947-2.
29. Estrella-cuenca M. Combinations of antifungal agents in therapy—what value are they? *J Antimicrob Chemother* 2004; 54(5):854-69. doi: 10.1093/jac/dkh434.
30. Rukayadi Y, Lee K, Lee M, Yong D, Hwang JK. Synergistic anticandidal activity of xanthorrhizol in combination with ketoconazole or amphotericin B. *FEMS Yeast Res* 2009; 9(8):1302-11. doi: 10.1111/j.1567-1364.2009.00548.x.
31. Andes D, Forrest A, Lepack A, Nett J, Marchillo K, Lincoln L. Impact of antimicrobial dosing regimen on evolution of drug resistance *in vivo*: fluconazole and *Candida albicans*. *Antimicrob Agents Chemother* 2006; 50(7):2374-83. doi: 10.1128/AAC.01053-05.
32. Gomes de Melo J, Sousa Araújo TA, Almeida TN, Castro V, Lyra de Vasconcelos CD, Desterro RM. Antiproliferative activity, antioxidant capacity and tannin content in plants of semi-arid northeastern Brazil. *Molecules* 2010; 15(12):8534-42. doi: 10.3390/molecules15128534.
33. Campos VAC, Perina FJ, Alves E, Sartorell J, Moura A, Oliveira DF. *Anadenanthera colubrina* (Vell.) Brenan produces steroidal substances that are active against *Alternaria alternata* (Fr.) Keissler and that may bind to oxysterol-binding proteins. *Pest Manag Sci* 2014; 70(12):1815-22. doi: 10.1002/ps.3722.
34. Evensen NA, Braun PC. The effects of tea polyphenols on *Candida albicans*: inhibition of biofilm formation and proteasome inactivation. *Can J Microbiol* 2009; 55(9):1033-9. doi: 10.1139/w09-058.
35. Redondo LM, Chacana PA, Dominguez JE, Fernandez Miyakawa ME. Perspectives in the use of tannins as alternative to antimicrobial growth promoter factors in poultry. *FMICB* 2014; 27(5):1-7. doi: 10.3389/fmicb.2014.00118.
36. Ullah N, Khan FA. An introduction to natural products and phytochemicals with special reference to its antimicrobial activity. *Life Sci J* 2016; 13(10):103-119. doi: 10.7537/marslj.131016.14.
37. Samy RP, Gopalakrishnakone P. Therapeutic potential of plants as anti-microbials for drug discovery. *Evid Based Complement Alternat Med* 2010; 7(3):283-94. doi: 10.1093/ecam/nen036.
38. Orhan DD, Özçelik B, Özgen S, Ergun F. Antibacterial, antifungal, and antiviral activities of some flavonoids. *Microbiol Res* 2009; 165(6):496-504. doi: 10.1016/j.micres.2009.09.002.
39. Lourenço RMDC, Melo PS, Almeida ABA. Flavonoids as antifungal agents. In: *Antifungal metabolites from plants*. Razzaghi-Abyaneh M, Rai M. (Eds.). Springer-Verlag Berlin Heidelberg, 2013. pp. 283-300.

40. Okoth DA, Chenia HY, Koorbanally NA. Antibacterial and antioxidant activities of flavonoids from *Lannea alata* (Engl.) Engl. (Anacardiaceae). *Phytochem Lett* 2013; 6(3):476-81. doi: 10.1016/j.phytol.2013.06.003.
41. Costa RS, Negrão CAB, Camelo SRP, Ribeiro-Costa RM, Barbosa WLR, Costa CEF et al. Investigation of thermal behavior of *Heliotropium indicum* L. lyophilized extract by TG and DSC. *J Thermal Anal Calorim* 2013; 111(3):1959-64. doi: 10.1007/s10973-011-2088-2.
42. Fernandes FHA, Santana CP, Santos RL, Correia LP, Conceição MM, Macêdo RO et al. Thermal characterization of dried extract of medicinal plant by DSC and analytical techniques. *J Thermal Anal Calorim*. 2013; 113(2):443-7. doi: 10.1007/s10973-012-2807-3.
43. Tramer F, Da Ros T, Passamonti S. Screening of fullerene toxicity by hemolysis assay nanotoxicity. In: Reineke J (Ed.). *Nanotoxicity: Methods and protocols method*. 2012; pp. 203-17.
44. Protopopova M, Hanrahan C, Nikonenko B, Samala R, Chen P, Gearhart J, Einck L, Nacy CA. Identification of a new antitubercular drug candidate, SQ 109, from a combinatorial library of 1,2-ethylenediamines. *J Antimicrob Chemother* 2005; 56(5):968-74. doi: 10.1093/jac/dki319.