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ORIGINAL RESEARCH

Antimicrobial and Antiproliferative Activity of *Bauhinia forficata* Link and *Cnidoscolus quercifolius* Extracts commonly Used in Folk Medicine

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ABSTRACT

Background: *Bauhinia forficata* and *Cnidoscolus quercifolius* plants are commonly used in folk medicine. However, few studies have investigated their therapeutic potential.

Aim: Herein, we evaluated the antimicrobial activity of *B. forficata* and *C. quercifolius* extracts against microorganisms of clinical relevance and their antiproliferative potential against tumor cells.

Materials and methods: The following tests were performed: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)/minimum fungicidal concentration (MFC), inhibition of biofilm adhesion, and effects on cell morphology. Antiproliferative tests were carried out with human keratinocytes and six tumor lines.

Results: *Bauhinia forficata* showed antimicrobial activity only against *C. albicans* with MIC of 15.62 µg/mL and MFC higher than 2000 µg/mL. It also inhibited biofilm adhesion and caused alterations in cell morphology. *Cnidoscolus quercifolius* showed no significant activity (MIC > 2.0 mg/mL) against the strains. *Bauhinia forficata* and *C. quercifolius* extracts showed cytostatic activity against the tumor cells.

Conclusion: *Bauhinia forficata* has promising anti-*Candida* activity and should be further investigated for its therapeutic potential.

Clinical significance: The use of medicinal plants in the treatment of infectious processes has an important function nowadays, due to the limitations of the use of synthetic antibiotics available, related specifically to the microbial resistance emergence.

Keywords: Antimicrobial activity, Antiproliferative effect, Biofilm adhesion, *Candida* biofilm, Laboratory research, Medicinal plants.

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INTRODUCTION

At present, drugs derived from natural products are capable of treating 87% of human illnesses, either bacterial, parasitic, immunosuppressant, carcinogenic, or others.[1] In addition, scientific studies have continuously advanced the knowledge of the therapeutic properties of medicinal plants used in folk medicine.

The species *Bauhinia forficata* Link, genus *Bauhinia* L. has been considered as a tonic, expectorant, hypocholesterolemic, hypoglycemic, antimicrobial, antihypertensive, and anti-inflammatory agent.[2] The major presence of flavonoids in its chemical composition has been associated with various pharmacological activities.[3] The traditional use of *B. forficata* as a hypoglycemic is related to its antioxidant activity and the presence of an insulin-like protein in its flavonoid derivatives.[2-5]

Another plant widely used in folk medicine is *Cnidoscolus quercifolius* Pohl, genus *Cnidoscolus*, whose bark and stem are used as anti-inflammatory, diuretic, disinfectant, wound healing, antitumor for the genitourinary

tract, antiseptic and dressing for dermatological and ophthalmic infections, fractures, dysentery, appendicitis, rheumatism, influenza, cough, and also for tooth, ear, and back pain.[6,7] The latex produced in this plant is used in the cauterization and coagulation of wounds and the bark poultice for wound healing.[8] Other parts of the plant are also used for therapeutic purposes, such as the root and shoots.[6,9] Despite the fact that these plants are widely used in traditional medicine, there is still a need for scientific evidence on their biological and therapeutic properties, especially with regard to prevention and/or treatment of oral diseases.

Considered the most common infectious diseases of the oral cavity, tooth decay and candidiasis are associated with the formation of a biofilm structure, composed mainly of streptococci and *Candida* spp. cells respectively, embedded in a polymeric matrix.[10] One of the mechanisms to control this biofilm formation consists in the use of antimicrobials. However, microbial resistance, especially in *Candida* spp., has increased in recent decades, constituting a serious problem for public health.[10,11] Conventional antifungals have limitations related to toxicity, which encourages the discovery of new antifungal compounds that act at different cellular targets with fewer side effects.[12-14] Regarding the control of oral biofilm, chlorhexidine stands out for being a large-spectrum antiseptic acting on Gram-positive and Gram-negative bacteria and fungi. Nevertheless, it has adverse effects, such as taste alteration, teeth staining, and imbalance of the oral microbiota. With this perspective, studies on medicinal plants have been targeting the discovery of new chemical molecules able to overcome the shortcomings of synthetic agents currently used in medicine and dentistry.[15-18]

In this bioprospection study, we evaluated the antimicrobial activity of hydroalcoholic extracts of *B. forficata* and *C. quercifolius* against microorganisms of clinical relevance, particularly in oral candidiasis, and their antiproliferative potential against six tumor cell lines and keratinocytes.

MATERIALS AND METHODS

Plant Material

Bauhinia forficata and *C. quercifolius* leaves were collected in the semiarid region in the Paraiba state, Serra de Bodocongó, municipality of Queimadas (7° 22' 25" S, 35° 59' 32" W), in the mesoregion of Borborema and microregion of Cariri Oriental, PB, Brazil, in September. Voucher specimens were deposited in the collection of the Manuel de Arruda Câmara Herbarium (ACAM), State University of Paraíba (Universidade Estadual da Paraíba – UEPB), Campus I, Campina Grande, Paraíba, under no. 130/ACAM and no. 017/ACAM respectively.

Preparation of Extracts

Bauhinia forficata bark and *C. quercifolius* leaves were dehydrated in an air-circulating oven at 40°C, subsequently ground and immersed in 80% ethyl alcohol (100 g/250 mL) for 48 hours at room temperature. The resulting mixture was filtered, and the filtration residue was immersed twice in the solvent. The final phases were concentrated on a vacuum rotary evaporator at 39°C, and subsequently lyophilized.

Microorganisms

The reference strains of *Candida albicans* ATCC 18804, *Streptococcus mutans* ATCC 25175, *Streptococcus sanguis* ATCC 10557, and *Enterococcus faecalis* ATCC 29212 were used in this study. The strains were maintained as frozen stocks at -80°C until use. For the assays, the strains were subcultured in specific culture media and incubated under specific atmosphere conditions at 35 to 37°C for 24 hours.

Determination of the Minimum Inhibitory Concentration and Fungicidal/Bactericidal Concentrations

The antimicrobial activity of the extracts was identified by determining their MIC and minimum bactericidal concentration (MBC) or MFC, as previously described by the Clinical and Laboratory Standards Institute.[19,20] The tests were performed using 96-well microtiter plates, Brain Heart Infusion (BHI; Difco, Franklin Lakes, NJ, USA) culture medium for bacteria, and RPMI-1640 (Roswell Park Memorial Institute) (Angus Buffers and Biochemicals, Niagara Falls, New York, USA) for yeast. The extracts were diluted in 40% ethyl alcohol (8 mg/mL) and tested at concentrations from 15.62 to 2000 µg/mL. A total of 100 µL of standardized bacterial [1.0×10^6 colony-forming units (CFU)/mL] or fungal (5.0×10^3 CFU/mL) inocula were added into each well.

The positive controls were 0.12% chlorhexidine (Sigma-Aldrich, St. Louis, MO, USA) and nystatin (Sigma-Aldrich, St. Louis, MO, USA) and the negative control was 40% ethyl alcohol. The plates were incubated at 37°C for 24 hours. The MIC was considered as the lowest extract concentration able to inhibit visible microbial growth, subsequently confirmed by 0.01% resazurin staining (Sigma-Aldrich, St. Louis, MO, USA).

To determine the bactericidal or fungicidal activity of the extracts, MBC/MFC tests were carried out by plating aliquots of MIC and higher concentrations onto specific culture media (BHI agar for bacteria and Sabouraud

dextrose agar for yeast). The MBC/MFC was defined as the lowest concentration able to inhibit any microbial growth on the solid culture medium. All tests were performed in triplicate in three independent experiments.

Inhibition of *C. albicans* Biofilm Adhesion

Based on the results of MIC and MBC/MFC, tests of inhibition of biofilm adhesion were performed only with the extract of *B. forficata* against *C. albicans*. Biofilms were grown on 96-well plates (Techno Plastic Products AG, Trasadingen, Switzerland). Initially, 100 µL of a microbial suspension containing 10^5 CFU/mL cultured in RPMI-1640 plus 2% sucrose was transferred to each well of the plate containing the extract at concentrations ranging between 15.62 and 2000 µg/mL. Then the plates were incubated for 48 hours at 35°C with shaking (75 rpm). Untreated biofilms were used as a negative control, and nystatin was used as positive control. The assays were performed in triplicate in three independent experiments. To quantify the adherent cells, the wells were washed with distilled water and dried at room temperature for 45 minutes. Subsequently, 200 µL of 1% crystal violet aqueous solution was added. The wells were washed and destained with 200 µL of 95% ethanol for 45 minutes. One hundred and fifty microliters of the destained solution was transferred to a new 96-well plate, and the amount of crystal violet was measured at 525 nm using a microplate reader (SpectraMax 340 Tunable Microplate Reader; Molecular Devices Inc., California, USA). The absorbance values of the experimental groups were subtracted from the values of the negative control, to assess the amount of adhered microbial cells.[21,22]

Antiproliferative Assay

A human keratinocyte cell line (HaCaT) and six human tumor cell lines were used: NCI-ADR/RES (ovary with phenotype resistance to multiple drugs), 786-O (kidney), NCI-H460 (lung), OVCAR-3 (ovary), HT-29 (colon), and K562 (bone marrow). All lines were cultivated in 25-mL vials with 5 mL of RPMI-1640 medium (Gibco-BRL, Grand Island, New York, USA), supplementing with 5% bovine fetal serum (Gibco-BRL, Grand Island, New York, USA), adding further a mixture of penicillin–streptomycin (1000 U/mL: 1000 µg/mL, 1 mL/L RPMI) at 37°C in a humidified atmosphere with 5% CO_2. The cultures were placed in 96-well plates (100 µL/well) and exposed to extracts diluted in sodium dimethyl sulfoxide at a concentration of 0.1 gm/mL (DMSO, Sigma-Aldrich, St. Louis, MO, USA) standing for 24 hours at 37°C in a humidified atmosphere with 5% CO_2. Before (T_0) and after the addition of the sample (T_1), the cells were fixed with 50% trichloroacetic acid and cell proliferation was determined by spectrophotometric measurement (540 nm) of the protein content, using sulforhodamine B. A concentration–response curve for each cell line was determined by nonlinear regression analysis using Origin 8.0 (OriginLab Corporation) software to establish 50% growth inhibition (GI_{50}), which is the concentration of the hydroalcoholic plant extract required to inhibit 50% cell growth.

RESULTS

MIC and MFC/MBC

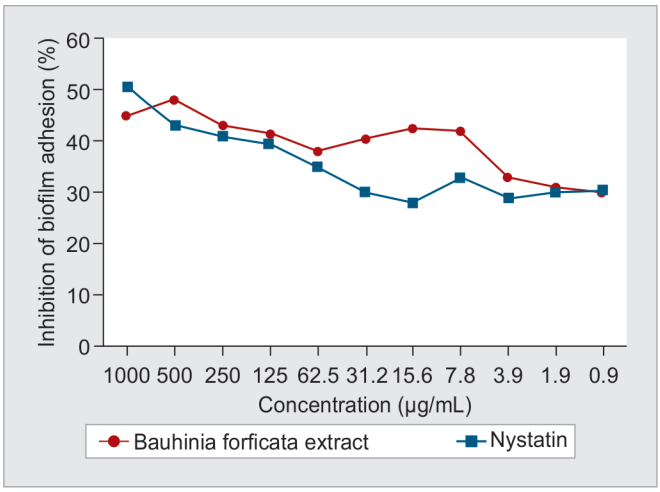
The extract of *B. forficata* showed strong antifungal activity against *C. albicans*, with MIC values of 15.62 µg/mL and MFC higher than 2000 µg/mL, indicating a fungistatic effect. For all other strains (*S. mutans*, *S. sanguis*, and *E. faecalis*), the results of MIC and MBC were higher than 2000 µg/mL. None of the strains tested were susceptible to *C. quercifolius* leaf extract at concentrations up to 2000 µg/mL.

Inhibition of *C. albicans* Biofilm Adhesion and SEM Analysis

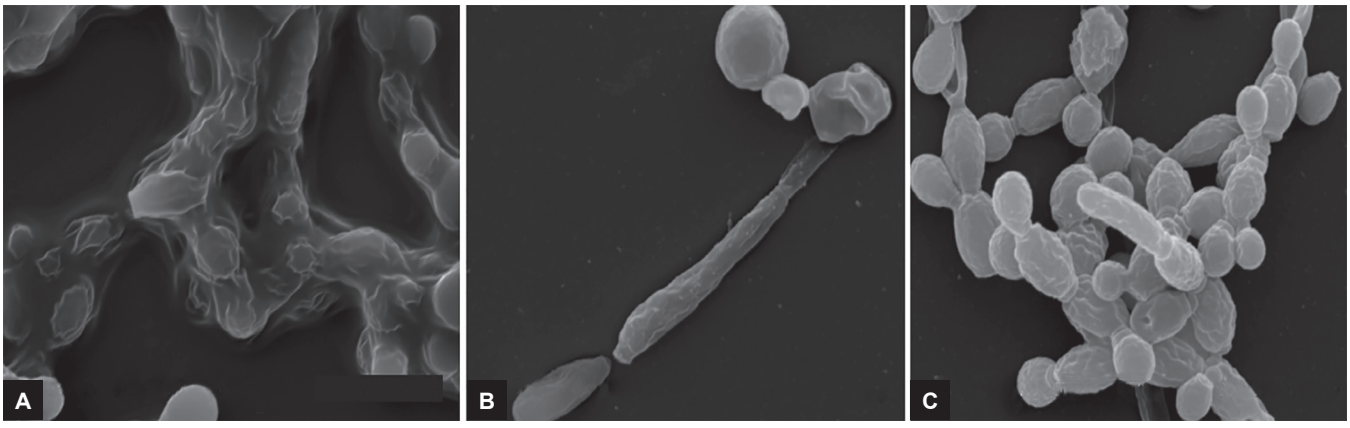
As seen in Graph 1, the extract of *B. forficata* inhibited biofilm adhesion by 33 to 45% at concentrations higher than 7.81 µg/mL, showing a pattern very similar to that of the monodrug nystatin. Scanning electron microscopy (SEM) images revealed alterations in the morphology of *C. albicans* biofilm cells treated with the extract of *B. forficata*, with differences in structure and integrity as compared with the untreated control (Fig. 1).

Antiproliferative Activity

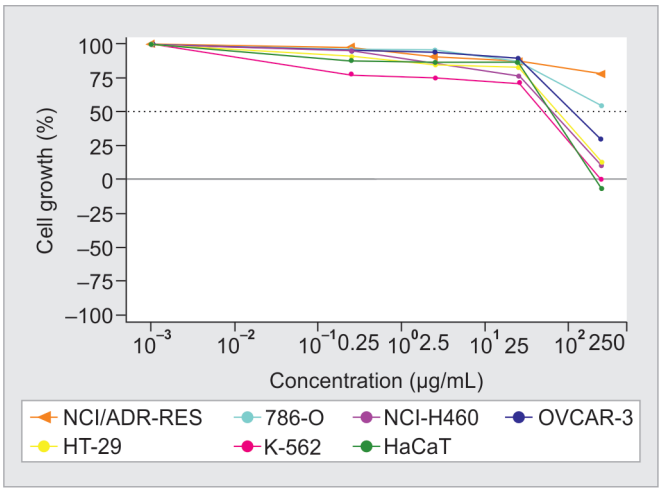
The extracts of *B. forficata* and *C. quercifolius* showed nonspecific cytotoxic activity against tumor cell lines. There was inhibition of tumor cell growth with mean log



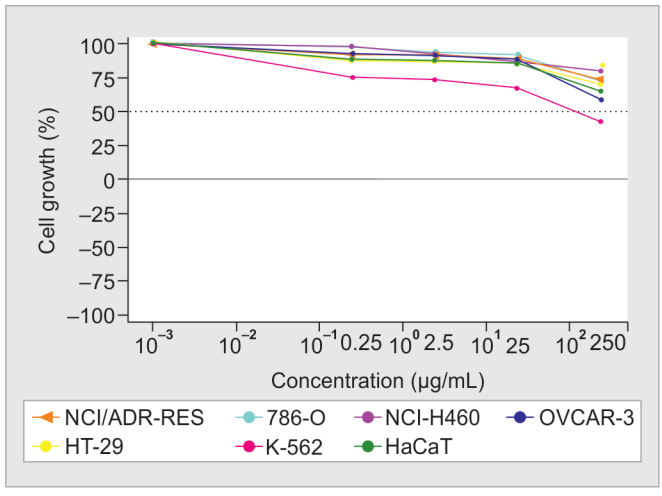
Graph 1: Inhibitory effects of different concentrations of *B. forficata* extract and nystatin (standard antifungal) on biofilm adhesion of *C. albicans* ATCC 18804



Figs 1A to C: Effects on biofilm structure. Scanning electron microscopy photomicrographs (×5000 magnification) of *C. albicans* ATCC 18804 biofilms treated with: (A) *B. forficata* Link extract – 15.62 µg/mL; (B) nystatin (1 mg/mL); and (C) untreated control



Graph 2: Distribution of cell growth inhibition values in human tumor cells treated with *B. forficata* link extract. NCI-ADR/RES (ovary with phenotype resistance to multiple drugs), 786-O (kidney), NCI-H460 (lung), OVCAR-3 (ovary), HT-29 (colon), K562 (bone marrow), and HaCaT (normal keratinocyte cell line). GI_{50}, 50% inhibition of cell growth



Graph 3: Distribution of cell growth inhibition values (GI_{50}) in human tumor cells treated with *C. quercifolius* extract. NCI-ADR/RES (ovary with phenotype resistance to multiple drugs), 786-O (kidney), NCI-H460 (lung), OVCAR-3 (ovary), HT-29 (colon), K562 (bone marrow), and HaCaT (normal keratinocyte cell line)

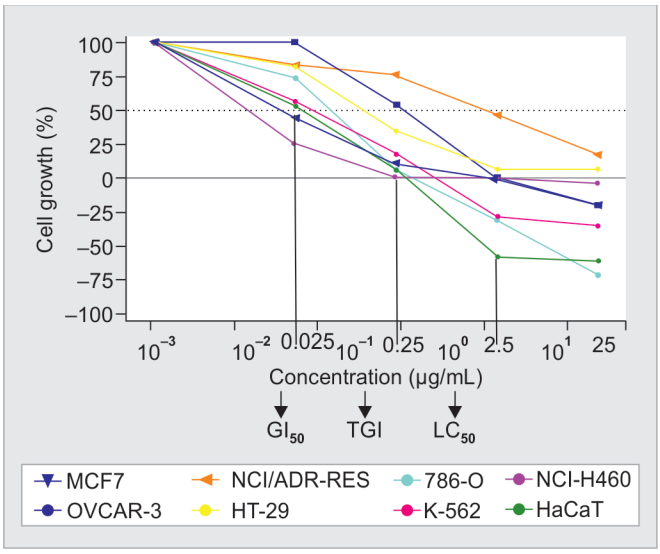
GI_{50} = 2.01 µg/mL (Graph 2) for *B. forficata* and 2.4 µg/mL (Graph 3) for *C. quercifolius*. The results with the positive control (doxorubicin) are shown in Graph 4. For cytostatic effects, curve points are above zero, while for cytocidal effects curve points are below zero; and in the case of total inhibition of growth, T is equal to T_0, indicating that the substance causes 100% cell inhibition (total GI).

DISCUSSION

There has been an increasing interest in bioprospecting natural products as a source of novel molecules for drug development.[1] Herein, we investigated the antimicrobial and antiproliferative potential of extracts of plants commonly used in folk medicine, *B. forficata* and *C. quercifolius*.

The antimicrobial assays carried out in this study showed the strong antifungal potential of *B. forficata* extract against *C. albicans*, but not against streptococci and *E. faecalis*. These findings are in line with those of a previous bioprospection study on medicinal plants of the semiarid region of northeastern Brazil.[23] It has been reported that substances showing MIC up to 500 µg/mL are considered strong antimicrobial agents; moderate if between 500 and 1000 µg/mL; and weak if above 1000 µg/mL.[24,25] With a MIC of 15.62 µg/mL against planktonic *C. albicans*, *B. forficata* extract was further selected to be tested against *C. albicans* biofilm adhesion. On the contrary, the findings of this study do not support the use of *C. quercifolius* as an antimicrobial agent, as its MIC was found to be >2000 µg/mL against all tested strains.

It is known that the ability of a pathogenic microorganism to form biofilm is related to its adhesion potential and the expression of virulence factors.[26] Microbial adhesion to an abiotic or biotic surface is a critical step for yeast surface colonization and further mature biofilm formation. Hence, inhibition of biofilm adhesion is considered an effective target for the development of antimicrobial drugs. In this study, we showed that *B. forficata* extract was able to disrupt biofilm adhesion



Graph 4: Distribution of cell growth inhibition values in human tumor cells treated with doxorubicin (positive control). MCF7 (breast) NCI-ADR/RES (ovary with phenotype resistance to multiple drugs), 786-O (kidney), NCI-H460 (lung), OVCAR-3 (ovary), HT-29 (colon), K562 (bone marrow), and HaCaT (normal keratinocyte cell line). GI_{50}, 50% growth inhibition; TGI, total growth inhibition; LC_{50}, concentration required for 50% cell death

at concentrations as low as 7.8 µg/mL (subinhibitory concentration), which was comparable to the standard drug nystatin. Furthermore, SEM analysis revealed that this extract clearly affected biofilm structure and integrity at MIC. This may be a result of changes in the cell wall structure or altered membrane permeability in the yeast cell. Bioactive molecules present in the extract may act on the cell membrane and/or the intracellular components, with disruption of vital cellular processes, such as the synthesis of deoxyribonucleic acid, ribonucleic acid, or proteins.[22] Studies of mechanism of action are now encouraged to further investigate the antifungal potential of *B. forficata*.

In recent years, an active search for novel antiproliferative agents has also become increasingly necessary. With this purpose, immunological and pharmacological therapies have been tested to find more effective approaches for the treatment of tumors.[27] Therefore, the extracts of *B. forficata* and *C. quercifolius* were also tested for their antiproliferative potential against six tumor cell lines and one normal cell line of keratinocytes. The concentration–response curve is one of the best ways to visualize the antiproliferative activity of a given sample. Agents are considered active if $GI_{50} \leq 30$ µg/mL.[28] The US National Cancer Institute in the anticancer drug screening program considers GI_{50} values ≤ 4.0 µg/mL as having strong antineoplastic activity.[29] The extracts of *B. forficata* and *C. quercifolius* inhibited the growth of tumor cells nonspecifically and were characterized as cytostatic and not as cytocidal agents.[30] Notably, *B. forficata* extract did not affect normal cells (HaCat) even at concentrations higher than its MIC, which indicates a low cytotoxic profile.

It is worth noting that among the various methods described for obtaining extracts, we selected the hydroalcoholic extraction – analogous to the tinctures made in folk medicine – in which the active parts of the plants are mixed with alcoholic beverages.[31] The findings reported herein contribute to the scientific literature on the study of these species and aggregate scientific value to their use in folk medicine, particularly *B. forficata* extract.

CONCLUSION

In summary, the extract of *B. forficata* showed strong antimicrobial activity against *C. albicans* and was able to disrupt biofilm adhesion at low concentrations. Further research is suggested to assess the antifungal mechanism of action by which *B. forficata* extract affects yeast cells. The antiproliferative assays indicated that the extracts of *B. forficata* and *C. quercifolius* have cytostatic potential against tumor cells and should be further investigated for their antitumor activity.

CLINICAL SIGNIFICANCE

The study of medicinal plants as a source of new types of drugs has grown in recent years due to the search for new drugs with greater therapeutic activity, lower toxicity, better biocompatibility, and more accessibility to the population, which, due to cultural aspects, has a good acceptance, reflecting good prospects in the market of therapeutic products made from active natural ingredients. Therefore, studies are needed for the discovery of safe, stable drugs from sources found in nature that are effective against resistant fungi.

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