academicJournals

Vol. 10(27), pp. 1056-1060, 21 July, 2016
DOI: 10.5897/AJMR2015.7829
Article Number: F3E34BC59477
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antibiofilm effect of Tucumã (*Astrocaryum* sp.) endosperm against *Candida albicans*

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Received 28 October, 2015; Accepted 28 June, 2016

Candida albicans **is one of the most relevant human opportunistic pathogens and highly competent to build biofilms on vital and non-vital surfaces. Facing the escalating resistance of microorganisms to current antimicrobials and fungicides, the Amazonian biodiversity may bring raw material to the development of new antimicrobial drugs. *Astrocaryum* sp. is a regional fruit, consumed *in natura* or as ice creams and other local specialties. The seeds, however, are discarded and accumulate in the environment. The present study aimed to characterize the phytochemical composition of the endosperm and to evaluate the effect of its extracts on biofilm formation and eradication by *C. albicans*. The seeds were processed to obtain extracts in hexane and ethanol. Color chromatography procedure and thin layer chromatography were used, followed by a colorimetric phytochemical prospection to identify the major secondary metabolites. Microtiter plates were inoculated with *C. albicans* and incubated for 24 h in contact with the extracts or EDTA (positive control) to test the ability of preventing biofilm formation. To evaluate the biofilm eradication effect, the target strain was inoculated in the plates and incubated for 24 h previously to the addition of the extracts or EDTA. Both hexane and ethanol extracts demonstrated significantly higher effect than EDTA on *Candida* biofilm inhibition, highlighting hexane extract that achieved the lowest percentage of adhesion (9.46±0.9%). The chemical composition indicated mainly terpenes, phenols and antioxidant compounds. These results demonstrate a pharmaceutical potential of *Astrocaryum* sp. endosperm for future developments of antifungal drugs, thus contributing to reduce the environmental impact of this biological waste of Amazonia.**

Key words: Tucumã, endosperm, secondary metabolites, antifungal, antibiofilm.

INTRODUCTION

A complex biomass of different microbial prokaryotes and eukaryotes embedded in a polymeric matrix turned out to be one of the major concerns at hospital facilities, playing a relevant role at nosocomial infections (Cos et al., 2010; Kang et al., 2012). Defined as biofilm, it has been studied in the last forty years and, in that context, *Candida albicans*, opportunistic pathogen associated with the human mucous membranes, has proven to be a relevant

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and challenging organism (Pfaller et al., 2011; Mathé and Van Dijck, 2013). Although an effort to comprehend the mechanisms of attachment and inter-species interactions as well as to avoid *C. albicans* pathogenicity by using a range of fungicides and new techniques such as surface coatings and quorum sensing inhibitors (Tournu and Van Dijck, 2012), the search of new molecules is still an open field of research.

A vast number of bioactive molecules derive from plants (Bérdy, 2005) and the Amazon rainforest is known for its biodiversity, thus enhancing the chances of drug discovery from natural sources (Myers et al., 2000). In Amazonia, many native plants are used by the local population as food, artisanal medicines, cosmetic and others. Some of them may be of economic interest and yet are not well described or were not properly studied concerning their biological properties. The *Astrocaryum* sp. genus includes 24 Amazonian species (Bacelar-Lima et al., 2006). The most common species *Astrocaryum vulgare* and *Astrocaryum aculeatum*, popularly called "tucumã", are well known for their fruits, which are consumed *in natura* or in sandwiches, as filling of "tapioca" (a sort of pancake made of manioc flour), or ice cream. Rich in ß-carotene, the fruit is indicated as alternative in the combat to hypovitaminosis A (Brasil, 2002; Lorenzi et al., 2006) and pursues high content in lipids (32.3%), carbohydrates (14.5%) and proteins (3.51%) (Yuyama et al., 2008). The seed is also edible and yields 30 to 50% of white oil (Lorenzi et al., 2006).

The main way of obtaining the fruits is not by cultivation, but by collecting them from natural occurring trees. Traditionally, whole families work together on harvesting and peeling the fruits, separating the flesh from the seeds and selling them at the local markets. Despite of the potential industrial uses in cosmetics (Lorenzi et al., 2006), biofuels (Lira, 2012) or fish feeding (Brandão, 2011), the *Astrocaryum* sp. hard shells (epicarp) and seeds (endosperm) are mostly left in great amounts in the environment after the manual removal of the fruit flesh.

Recently, hydro-alcoholic extracts obtained from the pulp and peel of *A. aculeatum* were described to pursue an inhibitory effect against Gram positive bacteria and *Candida albicans* (Jobim et al. 2014). In the present work, the aim was to evaluate *in vitro* the ability of *Astrocaryum* sp. endosperm extracts to inhibit or eradicate *Candida albicans* biofilm as a contribution to enhance the economic value of this residues, thus indicating their potential application in the pharmacology.

MATERIALS AND METHODS

Plant material

The *Astrocaryum* sp. seeds were obtained at a local Market in the urban central zone in Manaus, state of Amazonas, Brazil. Observation of families working on site peeling and removing the fruit flesh of *Astrocaryum* sp., revealed that the final destination of the seeds was in the trash. So the authors obtained circa 30 kg of seeds by donation.

Figure 1. Open *Astrocaryum* sp. seed, showing the white and thick endosperm.

The obtained seeds of *Astrocaryum* sp. were immediately transferred to the Laboratory of Biology of Natural Products at the Federal Institute of Amazonas (Manaus, Brazil). They were visually inspected and separated from dirt and other materials. After washing them under tap water, they were dried in a circulating air oven at 40°C for 48 h. The hard epicarp was cracked in a microwave oven after 20 s (Figure 1). The endosperm was removed and processed to obtain the extracts.

Phytochemical analysis

The dry endosperm samples were pulverized to enhance the contact with the solvents, hexane (HE) and ethanol (EE). They were left for extraction by maceration for a period of nine days with sequential changes of the solvent every three days. The obtained extracts (HE and EE) were filtered and concentrated in a rotary evaporator (model 801, FISATOM, Perdizes – São Paulo, Brazil) and stored at -24°C in a freezer.

Each extract was submitted to column chromatography procedure (CCP) using Silica Gel 60 (0.063 to 0.200 mm, Merck, Germany) and the eluents hexane, dichloromethane and ethanol. Subsequently, the obtained fractions were analyzed through thin layer chromatography (TLC) to identify the major compounds. The fractions were developed by TLC plates (Silica Gel 60 F254, Macherey-Nagel, Germany). The presence of secondary metabolites was indicated by anisaldehyde and cerium sulphate; antioxidant, phenols or steroids by 2,2-diphenyl-1-picrylhydrazyl (DPPH); alkaloids by Dragendorff; flavonoids, catechins and tannins by ferric chloride III; terpenes, steroids and aromatic compounds by sulfur vanillin and flavonoids by aluminum chloride. All TLC plates were submitted to UV light at 254-365 nm. A partition using hexane:methanol (1:1) was used to treat the ethanolic extract. The different parts were used in a phytochemical prospection, with a sequence of colorimetric methods (Santos et al., 2014) to determine the most relevant groups of secondary metabolites obtained within the extracts. Briefly, the methods should indicate the presence of 1. steroids and triterpenes, 2. phenols and tannins, 3. alkaloids, 4. coumarins, 5. anthocyanins, anthocyanidins, chalcones, aurones and flavonoids, and 6. flavonols, flavanons, flavanonols and xhantones.

Anti-candida assay

A standard strain of *C.albicans* (ATCC10231) was kindly provided by Oswaldo Cruz Foundation (FIOCRUZ – Rio de Janeiro, Brazil). After cultivation on Sabouraud Dextrose agar (SDA), an overnight culture in brain heart infusion broth (BHI) was used in the tests. The inoculum concentration was determined using a Neubauer chamber and standardized to a concentration of 10^6 cells/ml. Each extract was diluted in a 10% dimetilsulphoxyde (DMSO) solution resulting in a 20 mg/ml standard HE or EE solution to be used as test compounds.

The assay was prepared to evaluate the ability of inhibition and eradication of *C. albicans* biofilm. To evaluate the biofilm inhibition (BI), each 96-microtiter plate was inoculated as follows: 100 µl tripticase soy broth enriched with 1% dextrose (TSB-D) was added to each well followed by 100 µl of each extract (HE or EE), and last of all, 100 µl of microbial suspension (10^6 cells/ml) was inoculated. Three experimental controls were used: TSB-D + inoculum as negative control, where the target strain should grow without interference; ethylenediaminetetracetic acid (EDTA 17%) – as positive control of biofilm inhibition/eradication, and TSB-D solely as a sterility control. All plates were incubated at 35±2°C for 24 h. Each extract and control was tested in triplicate. To access the ability of the extracts and EDTA to eradicate the biofilm (BE), they were added to the wells 24 h later than *C. albicans*, so that the yeast could build a biofilm.

After the incubation period (24 h BI; 48 h BE), each microplate had the results recorded by a microplate reader. The supernatant was carefully washed out (3x with sterile saline 0.9%) and the plates were left at 60°C for 1 h in a Pasteur oven. After this, 120 µl of a crystal violet solution (0.06%) was added to each well and kept at room temperature for 5 min. The plates were washed gently and 40 µl of Dimetilsulfoxyde (DMSO) was added to each well in order to perform the last screening by the microplate reader. The effect of DMSO against *C. albicans* was also evaluated following the same procedure for both BI and BE.

Data analysis

For each microtiter plate, three readings were performed on a microplate reader (TP Reader Plus ELx808, BioTek Instruments Inc., Winooski, USA) at 630 nm. Plates to test BI were read immediately after inoculation (T_0), after 24 h incubation (T_{24}), and after staining with crystal violet solution (T_{24b}). Plates to test BE were read after inoculation (T_0), after 24 h incubation (T_{24}), and after staining with crystal violet solution (T_{48b}). For each well a difference was calculated ($T_{24b} – T_0$ or T_{48b}-T_0). To relate the results of the potential inhibitors to the negative (untreated) control, the latter was considered as 100% biofilm formation and a percentage value for each extract and EDTA was calculated (Trentin et al. 2011). Any value lower than 100% was considered inhibition or eradication of biofilm. A comparison between means gave the significance between each extract and EDTA using a Student's *t*-test (GraphPad® Software) considering $p \leq 0.001$.

RESULTS

Phytochemical composition

The hexane extract (HE) yielded 41.49% against 31.8% of the ethanol extract (EE). Classic CC and TLC methods indicated the presence of terpenes, flavonoids and phenols in the HE. By the EE terpenes and antioxidant compounds were detected mainly. The partition of EE resulted in 3 phases:

1. Soluble in methanol
2. Soluble in hexane and
3. A hydrophilic orange precipitate.

When submitted to the phytochemical prospection, the phases 2 and 3 reacted positively for flavonols, flavanons, flavanonols, xanthones, phenols and tannins. No alkaloids or coumarins were detected by any of the extracts tested.

Antibiofilm effect and data analysis

As summarized in Table 1, the scores below 100% indicate that the formation of *C. albicans* biofilm (BI) was inhibited by all concentrations tested either by the *Astrocaryum* sp. extracts or EDTA. Comparing the mean values by the Students *t*-test, however, the HE was significantly ($p \leq 0.001$) more effective than EDTA at four different concentrations. The strongest inhibitory effect over the cells attachment was achieved by HE at the concentration of 1.5 µg/ml. Concerning the eradication of the adhered cells (BE) all tested compounds were less effective than by the BI-assay at the same concentrations. Also EDTA showed values over 100% at five different concentrations. A dose-dependent effect was demonstrated by the %BI values obtained either by the extracts or EDTA. A gradual increase in biofilm formation was observed along with the concentration reduction. As expected, DMSO did not show any interference with *C. albicans* attachment or biofilm removal during the entire experiment.

DISCUSSION

The endosperm of Arecaceae representatives presents a high content of lipid compounds. Previous works dedicated to the nutritional composition of *Astrocaryum* mesocarp (fruit flesh) found mainly hydrophobic components. Yuyama et al. (2008) demonstrated the lipid content of 32.29 and 61.60%, respectively in the *in natura* and dehydrated mesocarp of *A. aculeatum*. The presence of β-carotene and other antioxidant compounds was previously described for *A. aculeatum* fruits. No literature described until now, the presence of secondary metabolites in the endosperm of *Astrocaryum* sp. The present study gives information about the phytochemical groups of compounds found in the endosperm of *Astrocaryum* sp., and the ability of the seed extracts to interfere with *Candida albicans* adhesion under *in vitro* conditions.

A similar protocol used for bacterial biofilms (Kwasny and Opperman, 2010; Trentin et al. 2011) was applied here to induce *C. albicans* biofilm formation. Jin et al.

Table 1. *Candida albicans* biofilm inhibition (%BI) and eradication (%BE) in response to *Astrocaryum* sp. endosperm extracts.

Concentration	%BI			%BE		
(µg/ml)	HE	EE	EDTA	HE	EE	EDTA
3.000	11.41±1.6*	14.24±6.5	23.94±0.1	46.28±22.1	46.12±17.8	43.57±0.0
1.500	9.46±0.9*	13.35±5.4	26.41±0.5	71.11±20.7*	67.64±11.7*	162.98±0.3
0.750	12.92±5.8	18.66±3.8	25.42±0.2	120.62±12.5	125.73±26.6	139.73±0.5
0.375	20.54±3.5	24.50±5.1	28.98±0.0	164.41±12.1	121.37±28.1	135.21±0.6
0.188	28.59±4.9	28.59±1.6*	36.60±0.1	240.18±67.2	220.84±25.5	183.52±0.0
0.094	32.48±3.8	29.31±5.0	37.69±0.2	183.82±16.9	173.06±45.8	197.52±0.2
0.047	39.80±5.3	39.53±7.0	53.51±0.0	110.46±11.1	136.27±39.0	80.14±0.1
0.023	33.14±1.3*	39.00±6.2	47.08±0.1	53.42±16.3	38.37±4.1	46.50±0.0
0.012	38.58±0.9*	39.07±1.7	43.13±0.1	59.07±43.1	27.92±2.7	19.64±0.3
0.006	39.53±4.7	48.57±1.6*	39.96±0.0	29.12±1.5	28.67±6.1	26.41±0.7
0.003	43.19±3.2	52.26±1.0	50.94±0.2	34.84±5.3	29.80±7.6	23.25±1.2
0.001	53.71±7.3	52.29±9.1	48.57±0.3	69.53±35.7	31.83±8.3	31.15±0.4

Percentage values are given as mean ± standard deviation and were calculated in relation to the untreated controls: biofilm inhibition = 1.011_{OD} and biofilm eradication = 0.443_{OD}. HE: extract obtained in hexane; EE: extract obtained in ethanol. EDTA: ethylene-diamin-tetracetic-acid as control of inhibition/eradication of biofilm; *Statiscally significant compared to EDTA values ($p \leq 0.001$).

(2004) described that the adhesion of *C. albicans* cultures treated with glucose was higher than those cultivated with galactose. In this study, a TSB-broth enriched with dextrose (1%) was used with an average of 1.011_{OD} biofilm formation on polystyrene microtiter plates after 24 hours incubation.

The *Astrocaryum* sp. endosperm extracts as well as EDTA have demonstrated the ability to avoid *C. albicans* adhesion at some extension. The inhibition of biofilm (%BI) was significantly stronger by HE than by EDTA at four different concentrations. At the concentration of 1.5 µg/ml, the HE induced the lowest percentage of *C. albicans* adhesion (9.46%) while the lowest value by EDTA (23.94%) was registered by doubling the concentration. The EE showed a significant inhibitory value compared to EDTA by only two concentrations, scoring the lowest percentage of biofilm formation (28.59%) at 0.188 µg/ml. The tested extracts and controls showed a similar increase in the cell adhesion along with the reduction of concentration.

EDTA is a well-known di-valent cation chelator with the ability of reducing biofilm formation and contributes to remove mature biofilms (Dunne, 2002; Zehnder, 2006). However, it was demonstrated by Ramage et al. (2007) that EDTA inhibits the hyphal formation by *C. albicans*, thus reducing its adhesion ability to polystyrene microplates and interfering only minimally on removing mature biofilms. The present study corroborates those findings indicating a higher effectivity of EDTA on biofilm inhibition than on its removal.

Overall values of %BE were higher than %BI by the *Astrocaryum* sp. extracts and EDTA at the same concentrations. This could be due to the complexity achieved by *Candida albicans* biofilms in which a variety of genes not expressed by planktonic cells lead to resistance enhancement in the attached ones (Mathé and Van Dijck, 2013). Increased values of persistent biofilm (%BE above 100%) were detected at the intermediary dilutions by EDTA (from 1.5 to 0.094 µg/ml) as well as by the extracts (from 0.75 to 0.047 µg/ml). The reason of promoting cell detachment at the highest concentration and again at low concentrations cannot be fully understood and should be further investigated. Both *Astrocaryum* extracts and EDTA showed better results on preventing biofilm formation than on eradication, but HE and EE were significantly ($p \leq 0.001$) more effective than EDTA.

The phytochemical tests indicated the group of terpenes as the major constituents in HE. Terpenes were described as Candida biofilm inhibitors by enhancing the cell wall permeability of planktonic cells and altering the cell surface in such a way that it could avoid their attachment (Braga and Dal Sasso, 2005; Dalleau et al., 2008). Polyphenols, mainly flavonoids, detected in the hydro-alcoholic extracts of *A.aculeatum* fruits and peel were effective against *Enterococcus faecalis*, *Bacillus cereus*, *Listeria monocytogenes* and *C. albicans*. The authors found rutin and the combination of rutin and gallic acid isolated from *A. aculeatum* ethanolic extract to be possibly associated to redox mechanisms that led to inhibition of growth and biofilm formation. In this study, the chemical composition indicated the major presence of terpenes and phenols with antioxidant effect (HE reacted positively to DPPH) that could suggest that these compounds could be the responsible for the better results achieved by HE on biofilm inhibition, thus corroborating

with previous studies.

CONCLUSIONS

This is the first study on the chemical composition and antibiofilm activity of extracts obtained from *Astrocaryum* sp. endosperm. Biofilm formation by *C. albicans* was significantly inhibited by hexane extracts compared to EDTA in lower concentrations. The phytochemical prospection revealed a major composition of terpenes and phenols, thus indicating the need of isolation and characterization of the bioactive compounds. These results indicate the potential use of these usually discarded seeds in the future developments of drugs, especially related to *Candida albicans* biofilm prevention.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This publication was carried out with financial support from the State of Amazonas Research Foundation (FAPEAM – edital 020/2013/PAPAC). The authors thank the National Council for Scientific and Technological Development (CNPq) for the license to study samples of Brazilian genetic heritage (CNPq/CGEN Proc. 010279/2013-0), and the IFAM and FAPEAM for the students research scholarships (PIBIC/IFAM and PAIC/FAPEAM).

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