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Antimicrobial activity of *Annona crassiflora* Mart. against *Candida albicans*

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This study evaluated the antimicrobial activity of the ethanol extract of *Annona crassiflora* Mart., against the fungus, *Candida albicans* present in the oral microbiota. The tests showed that the fractions of ethanol extract of the root bark and wood root of *A. crassiflora* showed a positive result. Of the strains studied, three showed sensitivity to 12 fractions and sub-fractions of *A. crassiflora* (66%). In the strains studied, strain 05 was the one that proved the most sensitive statistically (p <0.05). Their structures were determined using spectral techniques (NMR 1H and 13C) and based on literature data.

Key words: *Annona classiflora*, *Candida albicans*, antimicrobial activity.

INTRODUCTION

Studies show that pH, aeration, moisture, exudates, squamous cells, food debris and proper condition of temperature favour the growth of a wide and varied microbiota in the oral cavity (Biral et al., 1974). Furthermore, researchers have observed root canals with necrotic pulp (Sen et al., 1995) and endodontic root canal with persistent infections (Waltimo et al., 1993) caused by different kinds of yeast and among them, the most prevalent causative agents belong to the genus *Candida*.

It is has been described in literature that *Candida albicans* may endure exposition to acid media, suggesting that this yeast can be of great importance in pathologies resistant to endodontic conventional treatment (Menim, 1993). So, one of the first goals of endodontic treatment of a tooth presenting pulp necrosis is the elimination of microorganisms from the root canal system with effectiveness, especially in cases of periapical lesions (Estrela et al., 1998).

As a result, calcium hydroxide is the most common endodontic compound used. Its efficacy and use over time has been proven through several papers various (Carrotte, 2004; Anjaneyulu and Nivedhitha, 2014; Kim

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and Kim, 2014). The mechanism of action of this substance on microorganisms can be explained by the influence of pH on growth, metabolism and cell division (Kim and Kim, 2015).

Fluconazole is an antifungal drug used in fungal infections caused by the pathogenic fungi, including *C. albicans*, which is a major contributory factor for cutaneous candidiasis (Abdel-Mottaleb et al., 2009). It is commercially available as parental and oral dosage forms, which are largely confronted with well-known adverse effects including taste disturbances and GI irritation. Despite the great advances of allopathic medicine in the second half of the twentieth century, new pharmacologically active agents obtained by selection of plant extracts have led to the discovery of many clinically useful drugs in the treatment of human pathologies (Fetih, 2016).

Among the plants studied by our research group at the research laboratory in natural resources of the Institute of Chemistry and Biotechnology of the Federal University of Alagoas, there is the *Annona crassiflora* Mart. popularly known as Araticum cork or Marolo (Annonaceae). This species has important food and literature describes its antimicrobial, antifungal and bactericidal properties (Ribeiro and Pascal, 2005). The constituents are anonaina and liriodenine, alkaloids that have antiparkinson, antitumor, antibacterial and antifungal properties, the Anoglabasina-A and Anoglabasina-B, diterpenes with antitumor activity, the Araticulina and Crassiflorina, acetogenins with cytotoxic activity (Corrêa and Penna, 1984; Le Boeu et al., 1982; Santos and Boaventura, 1994a, b; Araya, 2004).

In the present study, the action of plant extract as an antimicrobial agent against *C. albicans* was evaluated. A previous work has demonstrated its antimicrobial activity against microorganisms, bacteria and yeast (Van, 2008; Lulekal et al., 2014; Teka et al., 2015). The antimicrobial activity of the compounds isolated from the plant extract was also studied aiming to obtain active ingredients to be used in endodontic infections therapy.

MATERIALS AND METHODS

Experimental

Columns were used in various sizes and diameters for the performance of chromatographic separations. Silica gel 60 G (70 - 230 mesh) of Merck was used as stationary phase and the columns were eluted at ambient pressure. Comparative observations of the extracts were obtained by chromatographic analysis of analytical thin-layer plates TLC using GF254 silica gel Merck A.

Source of materials

The collection and identification of the plant were done in May 2003 in itumbiara, state of Goiás (18°23'42.4"S, 49°16'30.4"W). A voucher specimen is deposited in the herbarium of UNB under the JEP 3369 number (UB).

Extraction and isolation of chemical constituents

The plant was collected (wood and root bark), air dried and mildly ground to achieve a fine powder that was extracted in 95% ethanol at a room temperature (26 ± 10°C) and filtered. The residue was extracted twice. The extract was evaporated under reduced pressure using a rotary evaporator and stored in a closed bottle at -20°C until it was required for the test. Five fractions were used from the ethanol extract of *Annona crassiflora* Mart. (Root Wood), seven fractions hydroethanolic extract were compared by thin layer chromatography CCD-six fractions of ethanol extract of the root bark.

After elution with the appropriate solvent, chromatographic plates were observed with light in the ultraviolet region at wavelengths of 254 and 336 nm, they were then left in contact with iodine vapor in a vat followed up spraying with ceric sulfate solution or phosphomolybdic acid followed by heating in an oven at 90°C for 5-8 min. The ethanolic extracts of *A. crassiflora*, previously selected were submitted to filtration on activated charcoal, using the polarity of the solvent gradient for elution. After filtration, coal fractions eluted with EtOH and EtOH : H_2O (1: 1) was chromatographed on silica Normal using a Buchner funnel with a gradient increasing in polarity (GCP) of the eluents.

After the filtration process through Buchner funnel and the chromatographic analysis of TLC ADCC were selected timber from the root sub-fractions (RMS) 01, 02, 03 (3 + 4) originated from EtOH fractions: H_2O 1:1 to 09, 10, 11 and 12 originated from EtOH fractions of crude ethanol extract. Then, an assessment in chromatographic column of silica was carried out, following a gradient of increasing polarity mobile phase, using hexane, $CHCl_3$, EtOAc and MeOH. All the sub-fractions were analyzed by TLC and those that showed similarity were combined in the same pre-weighed glass vial, sterilized, and recorded at this time. It was also the target of this work to study 06 fractions of crude ethanol extract of the root bark (FCR) of *A. crassiflora* (Lima et. al., 2006). After the antimicrobial analysis, the most active sample of *A. crassiflora* extract was selected for isolation of the active principle, observing the quantity of material. For the isolation and identification of chemical compounds, sample selected in accordance with the previously demonstrated activity and the amount of material was available Sample 10 (FMR3 + 4) (Root timber). The isolation of the compounds of selected fractions was performed by chromatography on TLC, silica gel columns and Sephadex gel, identification was performed through espctômetro Bruker Avance 400MHz operating frequency of the NMR Laboratory IQB -UFAL. The data were obtained from the experiments NMR (1H and 13C), using the Spin Works program.

Step microbiological

After obtaining the fractions and sub-fractions by chromatography on silica gel and analysis by TLC samples were selected to study the antimicrobial activity, Fluconazole was also used in the assay of antimicrobial activity allopathic drugs (Zelix Lab. Ativus) (750 µg/disk) and Ca (OH)2 (Lab. VETEC Fine Chemicals Ltd.) (24 µg/disk) (standards) effective for fungi endodontic microflora and microorganisms of *C. albicans* strains used were termed as IC01, IC03, IC07, IC09, IC10, IC11 and IC15. The strains of *C. albicans* were acquired in the Laboratory of Biochemistry and Physiology of microorganisms of the Department of Antibiotics, Federal University of Pernambuco (UFPE). The microbiological analysis method employed was the diffusion filter paper discs in Petri dishes (Bauer et al., 1966). The disk impregnated or soaked with the drug comes in contact with the moist agar surface, water is absorbed by the filter paper, and the drug diffuses into the surrounding medium, inhibiting the growth of the microorganism on the agar surface, forming a halo or zone inhibition. The medium used was the liquid

and solid agar Sabouraud (Lab. Merck) for the cultivation of the microorganism and the culture medium on the plates, respectively, prepared according to the manufacturer's instructions. The each Petri plate and glass tube received the liquid and solid agar all sterilized by autoclaving. Then, microorganisms were inoculated in tubes containing culture medium, which was capped and together with the solid agar plates containing capped were left for 24 h incubated at 37°C in a greenhouse. Then, the strains were seeded by a swab the plates containing the culture media. Then, the paper discs soaked with the respective drugs, fluconazole (375 µg/µl/disk), extract (4.5 µg/µl/disk) and $Ca(OH)_2$ (0.61 µg/uL/disk) were placed in plates within a predetermined point. Immediately afterward, the plates were sealed, wrapped in plastic wrap paper and placed for incubation for 24 h in the oven. The following day, the inhibition zones were read, recorded and analyzed by statistical test.

For determination of minimum inhibitory concentration fraction, all strains were tested. Dilution method was used in liquid medium recommended by Andrews (2001). 07 well microliter plates containing 96 wells was used, among these, 06 plates were used for drugs and plate 12 was used for another drug and drug standards. The culture media employed was the Sabourraud handled according to the manufacturer instructions. The plant extracts were weighed and diluted in a system composed of DMSO/Tween 80/water in a ratio of 1:0.5:8.5 to obtain a concentration of solution equal to 2000 µg/ml. The calcium hydroxide, fluconazole were weighed and diluted in water. All solutions were filtered through Millipore filters with pore of 0.45 and 47 mm diameter.

The drugs were solubilized in boiling water in a laminar flow cabinet and then sterilized by millipore membrane (0.4 mm) and the filtrates were placed in small sterile flasks. All plant extracts were at a concentration of 2000 µg/ml. The first wells of each plate were filled with 200 µl of the drug at a concentration of 2000 µg/ml and the other wells received 100 µl of the broth Sabourraud, which received 10µl suspension of each organism at a concentration of 104 cfu/ml. Then, it was transported, 100 ml (2000 µg/l) of the drug well 10 to 20 and so on to achieve a dilution in a minimum concentration (15 µg/ml). All plates were incubated at 30°C/48 h in an oven and, after the incubation period, 20 µl of a tetrazolium chloride solution (0.5%) was placed in each well and the MIC was read at 24 h (Bulgasem et al., 2016).

RESULTS AND DISCUSSION

Only a few strains of *C. albicans* were susceptible to extracts of *A. crassiflora* and its fractions. The results are shown in Tables 1 and 2.

The results of the inhibition halos were analyzed by the Cochran's test and showed that only strains 01, 05 and 10 of *C. albicans* showed sensitivity to drugs derived from plant extracts. Among these strains, strain 05 was the one that proved the most sensitive statistically ($p < 0,05$), showing inhibition zones ranging from 9 to 12 mm for nine of the tested fractions. However, this strain (05) was not sensitive to Fluconazole and calcium hydroxide (Table 1).

After the antimicrobial tests performed using the purified sub fraction (A10) originated from the plant extract (Samples 1 to 13), the assay was performed to obtain minimum inhibitory concentration (MIC) and the results showed that *C. albicans* had MIC = 2000 µg/ml. Regarding standard drugs, fluconazole (sample 19) showed a MIC <15.6 µg/ml, whereas calcium hydroxide (sample 20) showed a MIC of 2000 µg/ml against *C. albicans*, demonstrating a highly significant difference ($p < 0.01$) (Table 2).

All microorganisms were compared with positive (culture medium microorganisms) and negative controls (culture medium without DMSO and microorganisms Tween + 80 + distilled water) and the results showed total growth of microorganisms in the positive control group and no growth, the negative control groups.

Data from the analysis of 1H NMR spectra, 13C NMR and DEPT 135 and comparing the data obtained by physical methods of sample (F10S20) (A3 + 4), the data of 1H NMR, 13C NMR and physical methods obtained from the literature, shows the identity of substances as the acetogenin Goniodonina, which depending on its stereochemistry, may be trans (16:19) -Goniodonina, trans-epi (16,19,34) -Goniodonina, cis (16 19) -Goniodonina, cis-epi (16,19,34) -Goniodinina (Alali et al., 1999; Jiang et al.,1997) (Figures 1 and 2).

In the current study, the antimicrobial activity of *A. crassiflora* against *C. albicans* (fungus), the microorganism in the microbiota of endodontic infections, was investigated according to several studies in the literature (Moller, 1966; Möller et al., 1981; Molander et al., 1998; Saini et al., 2004). To test the antimicrobial activity, every step was followed, from obtaining and purifying the plant extracts, to testing their bactericidal and fungicidal activities, the same way other authors have done (Padjama et al., 1995; Nascimento et al., 2000; Ferreira et al., 2002; Suffredini et al., 2006; Obey et al., 2016).

The standard drugs used, fluconazole and calcium hydroxide, were selected according to the work described in the literature. These drugs are used for these microorganisms microflora endodontic (Warrilow et al., 2012; Maiolo et al., 2014; Bhandari et al., 2014).

In this work, a positive result as the antimicrobial activity of the ethanol extract of *Annona crassiflora* against *C. albicans* was obtained showing a significant difference between the sensitive strains ($p > 0.05$) (Table 2), however, it does not show significant difference when compared with the results of standard substances ($p < 0.05$). Other studies using 10 different plant extracts evaluated the ethanol extract of propolis, the study of three species of *Miconia*, the hexane extract of *Cyperus giganteus*, the essential oils of *C. cassia* and *C. martinii* and essential oils and extracts plants of the Amazon region also had antimicrobial activity against *C. albicans* (Celotto et al., 2003; Pereira et al., 2005, 2006; Almeida et al., 2012).

However, a study testing the crude extract of *Artichium lappa* leaves against endodontic microorganisms, essential oils of *O. basilicum* and *T. vulgaris*. and *Annona glabra* extracts, *Azadirachta indicates*, *Bryophyllum calycinum* and *American Mammea* showed no antifungal activity indicating that *Candida albicans* was the hardest

Table 1. Antimicrobial activity of ethanolic extract and its fractions against *C. albicans* {zone of inhibition mean ± s.d. (mm)}.

Samples	01	03	05	07	08	09	10	11	12	15
1 Ethanolic Extract	–	–	11	–	–	–	–	–	–	–
2 Ethanolic fraction	–	–	7	–	–	–	–	–	–	–
3 Sub-fraction Ethyl acetate (FMR 12)	10	–	10	–	–	–	–	–	–	–
4 Sub-fraction Ethyl (Hex:$CHCl_3$ 1:1) (FMR 9)	–	–	–	–	–	–	–	–	–	–
5 Sub-fraction Ethyl ($CHCl_3$:AcOEt 1:1) (FMR 11)	–	–	–	–	–	–	–	–	–	–
6 Sub-fraction EtOH ($CHCl_3$) (FMR 10)	–	–	–	–	–	–	9	–	–	–
7 Sub-fraction EtOH:H_2O 1:1	–	–	11	–	–	–	–	–	–	–
8 Sub-fraction EtOH:H_2O 1:1 (Hex:$CHCl_3$ 1:1) (FMR1)	–	–	–	–	–	–	–	–	–	–
9 Sub-fraction EtOH:H_2O 1:1 ($CHCl_3$) (FMR 2)	–	–	9	–	–	–	–	–	–	–
10 Sub-fraction EtOH:H_2O 1:1 ($CHCl_3$ ACOE 1:1) (FMR 3)	–	–	12	–	–	–	–	–	–	–
11 Sub-fraction EtOH:H_2O1:1 (ACOEt:MeOH 10%) (FMR 4)	–	–	–	–	–	–	–	–	–	–
12 Fraction Hex:ACOEt 20% (FCR 2)	–	–	–	–	–	–	–	–	–	–
13 Fraction (Hex:$CHCl_3$ 1:1) (FCR 1)	–	–	–	–	–	–	–	–	–	–
14 Fraction ($CHCl_3$) (CR3)	–	–	–	–	–	–	–	–	–	–
15 Fraction (Hex:ACOEt 1:1) (Hex:MeOH 20%) (FCR 4)	—	–	8	–	–	–	–	–	–	–
16 Fraction (Hex:ACOEt1:1) (Hex:MeOH 30%) (FCR 4)	–	–	9	–	–	–	–	–	–	–
17 Fraction (ACOEt) (FCR 6)	–	–	10	–	–	–	–	–	–	–
18 Fraction (Hex:ACOEt 1:1) (ACOEt:MeOH 5%) (FCR 7)	10	–	–	–	–	–	–	–	–	–
19 Sulfocanazol	–	25	–	–	-	26	–	15	–	-
20 Calcium hydroxide		20				12				15

Table 2. Minimum inhibitory concentrations (MIC), means followed by distinct letters differ from each other by the Kruskal-Wallis test.

Microorganism	Compound	MIC	Kruskal-Wallis
Candida albicans	Sample 10	2000 µg/ml	b
	Sample 20	2000 µg/ml	b
	Sample 19	<15.8 µg/ml	a

Means with the same superscripted alphabets and in the same column are not significantly different (p>0.05).

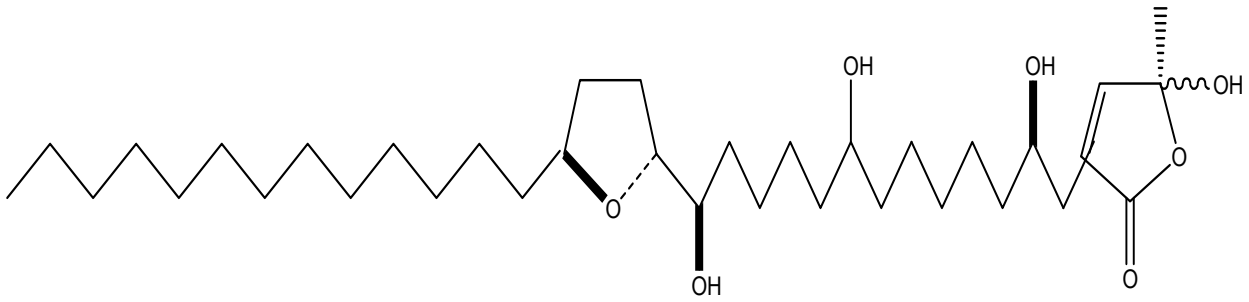

Figure 1. Trans(16,19)-Goniodonina (Trans-epi (16,19,34)-Goniodonina.

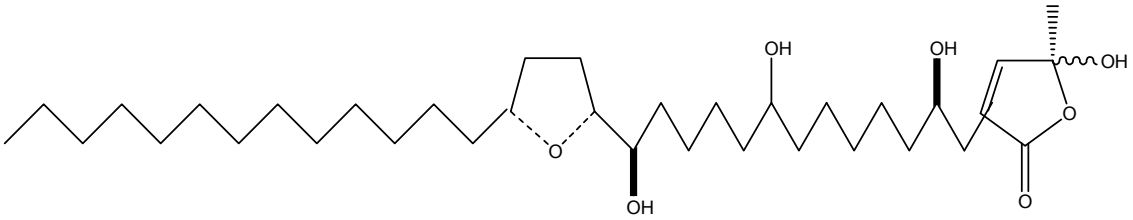

Figure 2. Cis (16,19)-Goniodonina (Cis-epi(16,19.34)-Goniodonina).

species among the ones studied (Menezes et al., 2009; Almeida et al., 2012). In another research, 65 methanol extracts of 56 species of 38 families of plants of Tanzania, traditionally used in the treatment of oral candidiasis were analyzed and showed that *C. albicans* was only sensitive to 4 (15%) of the 26 active plants extracts tested (Pereira et al., 2005). However, evaluating the antifungal activity of hydroalcoholic extract of *Psidium guajava* Linn sheet. (Guava) on the oral cavity yeast, *C. albicans*, *Candida tropicalis*, *Candida krusei* and *Candida stelatoidea,* it was concluded that the *P. guajava* leaf extract (guava) has the ability to inhibit the growth of *Candida* yeasts of the oral cavity, suggesting the use of this extract as alternative means for the treatment of candidosis orais (Alves et al., 2006). Likewise, searching for the antifungal activity against species of oral Candidas, as compared to the *in vitro* activity of anti-candidal *Tulbaghia alliacea*, *Tulbaghia violacea* and *Allium sativum,* it was revealed that the *T. alliacea* extracts exhibited anti-infective activity against species of *Candida* (Lima et al., 2006).

In the present study, the antibiotic controls were constantly high and some extracts induced inhibition which almost reached the antibiotic level when a zone of inhibition wasmeasured. Our results show that between the bacterial strains, there is variation in susceptibility to extracts. The antimicrobial effect of the extract depends on the bacterial strain and the extraction solvent. Also, the calcium hydroxide presented antimicrobial activity *against C. albicans* after 24 h. Similarly, studies claim that the calcium hydroxide in direct contact only carries the full antimicrobial effect after 24 h (Amorim et al., 2006). However, they stated that in direct contact with the bacteria, calcium hydroxide promotes its antimicrobial action after seven days (Estrela and Holland, 2003).

In this study, *C. albicans* showed sensitivity to standard drugs (fluconazole) and MIC of the drugs showed that *C. albicans* has a smaller MIC (<15.8 µg/ml) for the fluconazole than for the plant extract with high MIC = (1000 to 2000 µg/ml), demonstrating that the strains of *C. albicans* used were most resistant to the plant extract tested the same way as in other studies carried out (Schuck et al., 2001; Fonseca et al., 2010; Pavanelli and Garcia, 2013).

In another study Lima et al. (2004), evaluating the potential of antimicrobial action of the aqueous extract of the stem of *Schinus terebenthifolius* Raddi (Aroeira the beach) showed that among the 11 microbial species tested, 8 (73%) were sensitive to aqueous extract of *S. terebenthifolius* in the concentration of 5000 mg/mL. However, the minimum inhibitory concentration (MIC) of the product for some strains was 2500 µg/ml and particularly, *C. albican*s was sensitive to 1250 µg/ml. It should be noted that the effectiveness of the activity is directly linked to MIC. Therefore, a high MIC demonstrates a lower antimicrobial activity.

Following identification of chemical compounds isolated and comparing the data obtained by physical methods, and comparing them with the literature data, it is concluded that it is acetogenins of the same class and the identified 04 samples were A11, A13, A10 and A6, they are similar to acetogenins obtained from the ethanol extract of the root of *Goniothalamus donnaiensis* (*Annonacea*), called Trans (16,19) Goniodonina, Trans-epi(16,19,34) Goniodonina, Cis (16,19) Goniodonina e Cis-epi (16,19,34) Goniodonina that showed cytotoxicity to neoplastic cells (Jiang et al., 1997; Rosa, 2015).

Conclusions

The ethanol extract of the root wood and root bark of *A. crassiflora* Mart. presented antimicrobial activity against *C. albicans*. The calcium hydroxide solution and the standard drug (Fluconazole) in direct contact for 24 h showed antimicrobial activity for *C. albicans* with a MIC = 1000 to 2000 µg/ml (calcium hydroxide) and MIC = 15.8 µg/ml (Fluconazole). The data of 1H and 13C of the sample (A10) when compared with the literature data suggest the presence of Gonodionina and acetogenin mono-tetrahydrofuran.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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