Journal of Medicinal Plant Research

Volume 11 Number 13, 3 April, 2017

ISSN 1996-0875

Academic Journals

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Journal of Medicinal Plants Research

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Vol. 11(13), pp. 253-259, 3 April, 2017
DOI: 10.5897/JMPR2017.6333
Article Number: 0C14A0263567
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

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

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Received 10 January, 2017; Accepted 10 March, 2017

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).

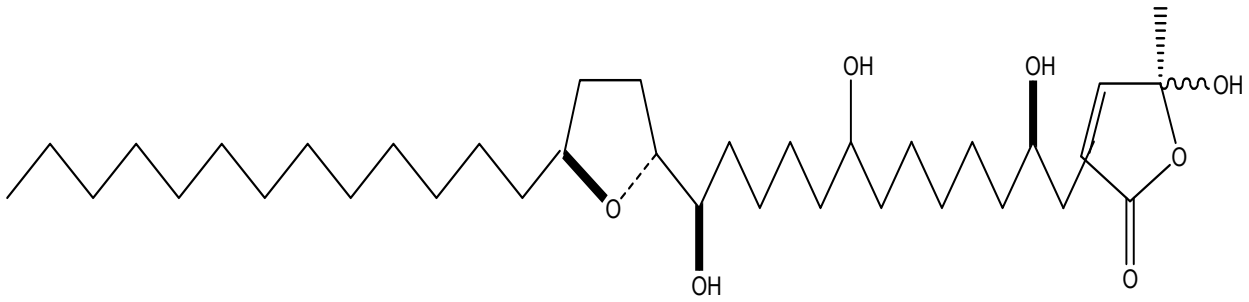

Figure 1. Trans(16,19)-Goniodonina (Trans-epi (16,19,34)-Goniodonina.

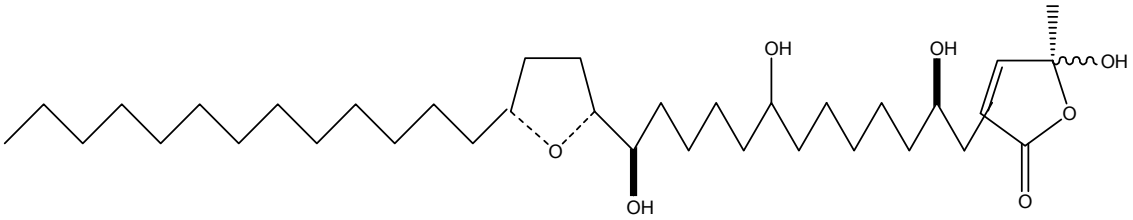

Figure 2. Cis (16,19)-Goniodonina (Cis-epi(16,19.34)-Goniodonina).

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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Vol. 11(13), pp. 260-263, 3 April, 2017
DOI: 10.5897/JMPR2016.6279
Article Number: 8C830E563569
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Insecticidal activity of *Azadirachta indica* A. Juss. extracts on *Aleurocanthus woglumi* Ashby (Hemiptera: Aleyrodidae)

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Received 21 October, 2016; Accepted 20 March, 2017

The use of natural products with insecticidal action is a control method compatible with integrated pest management, since it reduces the impacts caused to human health and the environment. This study aimed to assess the effect of *Azadirachta indica* A. Juss. (Meliaceae) leaf and seed aqueous extracts, on citrus blackfly (*Aleurocanthus woglumi* Ashby). *Citrus* x *latifolia* Tanaka ex Q. Jiménez leaves infested with blackfly nymphs were immersed for five seconds in different *A. indica* leaf and seed extract concentrations (1.0, 2.5, 5.0 and 10 w/v). Distillate water was used as control. The assessments were performed at 3, 6 and 9 days after leaf immersion, by counting dead insect number. The *A. indica* leaf and seed aqueous extracts were efficient in the mortality of *A. woglumi* nymphs, representing an alternative method to control this pest.

Key words: Insecticidal activity, *Azadirachta indica* extracts, *Aleurocanthus woglumi* Ashby

INTRODUCTION

The citrus blackfly (*Aleurocanthus woglumi* Ashby) is an important citrus pest. It is an insect whose feeding activity occurs through mouthparts of the piercing-sucking type that causes damage when feeding on phloem parts from citrus plants either in immature or adult phases (Oliveira et al., 2001). Direct damages are caused by nymphs and adults by means of continuous leaf nutrient suction which results in plant weakness, wilting, and in some cases, plant death. Indirect damages arise from sooty mold which is a symptom resulting from fungus growth on *A. woglumi* nymph exudates over leaves and fruits which impair leaf respiration and photosynthesis (Raga et al., 2013). Citrus black fly may cause 20-80% citrus yield losses, thus affecting fruit production and exportations. About 5 to 10 nymphs per square centimeter are enough to reduce the nitrogen level below the 2.2% needed for

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orange fruit growth. Research carried out in Mexico showed that more than 90% citrus fruit yield reduction occurs when infestations exceed 5 to 7 nymphs/cm²/leaf. When severe attack occurs in young plants or in plants at seedling stage, may result in plant death (Summy et al., 1983; Nguyen and Hamon, 1993; Oliveira et al., 2001).

Because of its widespread geographical presence in Brazil, citrus black fly is no longer considered a quarantine pest for internal market. Despite the economic and social importance for Brazilian citrus production, research for alternative control methods to control *A. woglumi* is rare.

In order to minimize the problems caused by pesticides, such as environmental pollution, effects on non-target organisms and emergence of resistant organisms, alternatives are being investigated, including the use of bioactive plants by extracts and oils (Lovatto et al., 2012). The ovicidal effect of eucalyptus (*Eucalyptus globulus* Labill.), garlic (*Allium sativum* L.), sesame (*Sesamum indicum* L.), castor bean (*Ricinus communis* L.) and carnation (*Dianthus caryophyllus* L.) oils for the control of *A. woglumi* was studied by Vieira et al. (2013). These authors observed that the concentrations of the tested oils decreased the egg viability. They also noted that commercial eucalyptus and garlic oils reduced the hatch *A. woglumi* nymphs.

Azadirachta indica A. Juss.(Meliaceae), popularly known as neem, is the most studied and used plant species due to its high efficacy and very low toxicity to humans (Martinez, 2002). In recent decades, *A. indica* has been extensively studied because it contains terpenoids with powerful insecticidal activity (Schumutterer, 1990). One of the most active terpenoids in neem seeds is azadirachtin, which is used in commercial formulations and its effects include repellency, feeding deterrence, power repellant, growth disruption, interference with metamorphosis, sterility and anatomical abnormalities (Simmonds et al., 1992).

Despite the derivatives from *A. indica* potential on pest management, little is known regarding their action against the citrus black fly. Thus, this study aimed to evaluate the activity of *A. indica* leaf and seed aqueous extracts on the *A. woglumi* nymph mortality, in laboratory conditions.

MATERIALS AND METHODS

This research was carried out at the Entomology Laboratory of the Agronomic Biotechnology Unity of the Maranhão State University in São Luís, state of Maranhão, northeastern Brazil.

Neem leaf and seeds were collected in the Maranhão State University campus, an *A. indica* plant 10 years age and the exsiccate was deposited in the Rosa Mochel Herbarium at the Maranhão State University. After collection the leaves and seeds were immediately dried in a chamber with forced air circulation at 45 °C for 72 h and subsequently ground in a knife mill to obtain a fine powder for the aqueous extract preparation. The leaf and seed extracts were prepared by adding respectively 1.0, 2.5, 5.0 and 10.0 g of dried powder in 100 mL of distillate water. They were manually stirred until reaching complete homogenization. Suspensions were kept at rest for 24 h and thereafter they were filtered to eliminate solid particles and sprayed over the *A. woglumi* nymphs.

Due to difficulties in the establishment of citrus black fly colony in the laboratory, we used leaves of Tahiti lime (*Citrus latifolia* T.) infested by *A. woglumi*. The leaves were collected, from an orchard located in the municipality of São Luís (02º54'30" S and 44º12'51" W).

Leaves de *C. latifolia* infested by *A. woglumi* nymphs were collected at random in the middle part of the trees. Abscission was performed by means of a pruning shears, close to the branch insertion. Thirty nymphs the second and third instars were selected at random in each leaf. The excess was removed with the aid of a stylus. Nymphs were identified by means of visual analysis with a stereo microscope. The nymphs of 2nd and 3rd stages are oval, with dark Brown or Black color with thorns all over the body. The third instar differs from the second only by the size of the nymphs, which measure 0.87 mm in length and 0.74 mm in width with more visible thorns.

Two bioassays were performed: (1) direct immersion of the leaves in the neem leaf extracts; (2) direct immersion of the leaves in neem seed extracts. In both experiments the concentrations were: 1.0, 2.5, 5.0 and 10.0% (w / v). Distillated water was used as control treatment. The experiments were conducted in completely randomized design with five treatments and ten replications. The experimental plot consisted of an infested leaf of Tahiti lime and an average of 30 nymphs.

After immersion, the leaves were disposed on outdoor paper towels to remove excess surface moisture. Thereafter, the leaves were transferred to Petri dishes containing filter paper, which were periodically moistened to maintain leaf turgor and to avoid nymphs death by lack of food and dryness. The petiole was involved in cotton moistened with distillated water, which was humidified daily. The plates were covered with plastic film, perforated and incubated in an acclimatized-controlled chamber maintained at temperature (26 ± 1°C, relative humidity of 70 ± 10% and photophase of 12 h).

The insect mortality was evaluated by counting of dry nymphs at 3, 6 and 9 days after the immersion of leaves in the extracts.

Data were subjected to analysis of variance (ANOVA) and the values compared by the Nemenyi nonparametric test at 5% probability. Analyses were performed using SAS System software, version 8.2 (SAS, 2001).

RESULTS

All tested concentrations of *A. indica* leaf and seed aqueous extracts were toxic to *A. woglumi* nymphs at 6 and 9 days after leaf immersion (DAI) (Table 1).

Nymphs treated with the *A. indica* leaf extracts at 3 (DAI), in the concentrations of 5 and 10% (w/v) caused different mortality when compared to control.

In the bioassay with the *A. indica* seed extract (Table 2) at 3 DAI treatments 5 and 10% concentrations caused higher nymph mortality, differing from the other treatments. At 6 and 9 DAI, the concentrations of 2.5, 5.0 and 10.0% caused significant nymph mortality (higher than 98%).

DISCUSSION

The *A. indica* leaf and seed aqueous extracts at 5.0 and 10% (w/v) concentrations caused nymph mortality above

Table 1. Nymph mortality (%) of *Aleurocanthus woglumi* treated with *Azadirachta indica* leaf extracts (temperature = 26ºC; relative humidity = 80%; fotophase of 12 h).

Concentration (%)	Nymph mortality (%) (EP[1])		
	3 DAI [2]	6 DAI	9 DAI
1.0	42.20 (11.55)[ab]	82.63 (8.12)[a]	92.98 (4.92)[a]
2.5	46.63 (8.00)[ab]	86.97 (6.58)[a]	97.58 (1.46)[a]
5.0	67.68 (9.87)[a]	85.73 (9.94)[a]	98.00 (2.00)[a]
10.0	81.20 (6.35)[a]	100.00 (0.00)[a]	100.00 (0.00)[a]
Control	13.87 (3.66)[b]	38.68 (3.89)[b]	55.62 (6.46)[b]
CV (%)	69.14	37.14	23.05

Means in each column followed by the same letter are not significantly different ($p< 0.05$) using the Nemenyi test. [1] EP: standard error; [2] DAI: days after immersion.

Table 2. Nymph mortality (%) of *Aleurocanthus woglumi* treated with *Azadirachta indica* seed extract (temperature = 26ºC; relative humidity = 80%; fotoperíodo of 12 h).

Concentration (%)	Nymph mortality (%) (EP[1])		
	3 DAI [2]	6 DAI	9 DAI
1.0	29.48(3.77)[b]	77.56(5.43)[ab]	94.74(2.85)[ab]
2.5	53.04(7.17)[b]	95.77(2.38)[a]	99.12(0.88)[a]
5.0	83.06(6.07)[a]	95.00(3.56)[a]	98.63(1.37)[a]
10,0	86.64(5.63)[a]	99.00(1.00)[a]	100.00(0.00)[a]
0.00	15.08(3.66)[b]	41.68(3.89)[b]	59.30(8.37)[b]
CV (%)	63.42	31.06	22.08

Means in each column followed by the same letter are not significantly different ($p< 0.05$) using the Nemenyi test. [1] EP: stand; [2] DAI: days after immersion.

80%, and therefore can be recommended for use in the citrus integrated pest management programs.

Although information about the nymph mortality of *A. woglumi* by *A. indica* extracts are scarce, Silva et al. (2012) assessing the effect of soybean (*Glycine max* (L.) Merr.), corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), and sunflower (*Helianthus annuus* L.) commercial oils and *A. indica* root extract on nymphs of black fly, found that higher oil concentrations (0.5, 1.0 and 1.5%) caused 100% nymph mortality.

Research conducted by Bezerra-Silva et al. (2010), to assess the effect of organic extracts of *A. indica*, *Melia azedarach* L., *Toona ciliata* M. Roem. and *Trichilia pallida* Sw. on eggs and nymphs of *Bemisia tabaci* biotype B, found greater nymphicidal activity in treatments with ethanolic extracts of *M. azedarach* branches (97.56%) and *A. indica* branches and leaves (95.45 and 92.94% respectively). Lima et al. (2011) evaluated the effectiveness of ethanolic extracts of *Ipomoea carnea* subsp., *R. communis*, *Mascagnia rigida* Griseb, *Argemone mexicana* L. and neem oil (*A. indica*), for control of *B. tabaci* biotype B and found that the *Ipomoea carnea* subsp. *fistulosa* and neem oil treatments were the most efficient on the nymph control, showing nymph mortality of 72.41 and 67.26%, respectively.

Thus, in both bioassays of this study it was found that the highest nymph mortality occurs with 6 and 9 DAI, corroborating the findings of Martinez (2002) who found that the larger active ingredient peaks derived from the *A. indica* products, which translocate into the plant, take place five days after the treatment.

Bleicher et al. (2007) evaluated the effect of *A. indica* leaf aqueous extracts and an azadirachtin-based formulation on nymphs of *B. tabaci*. The authors confirmed that azadirachtin and *A. indica* leaf aqueous extracts significantly reduced the average number of live nymphs and did not differ from the standard buprofezin insecticide action.

According to Costa et al. (2004), each plant species may have a variation in the content of active ingredients, depending on the plant stage, used plant structure, and the soil and climate conditions under which plants grow. The *A. indica* seeds and leaves contain terpenoids with powerful insecticidal activity. The leaf active terpenoids include nimbin, deacetylnimbin and thionemon (Simmononds et al., 1992). Thus, in both bioassays in this study, it was found that the *A. indica* leaf and seed extracts effect on *A. woglumi* adversely affect insect development, suggesting that the active ingredients are present in different plant structures, although with varying

concentrations.

According to Coudriet et al. (1985), the sensitivity of nymphs may be related to plant extract action on the neuro-endocrine system that regulates the ecdysteroid or anti-ecdysteroid production. These results are similar to those found by Natarajan and Sundaramurthy (1990), in assessing the nymphal mortality caused by *A. indica* oil (0.5 to 1%) in whitefly (*B. tabaci* biotype B). The authors found that only 14.3 and 13.0% of nymphs reached the adult stage, of which 51.3 and 56.8%, respectively, showed abnormalities and disorders characteristics of the insects endocrine system, according to Saxena and Khan (1985), while in the controls (monocotrophos 0.08% and water), 84.3 and 94.0% of nymphs reached adulthood, with abnormality rates of 7.0 and 2.8% respectively.

Given the high availability and ease of growing of *A. indica* plants, the preparation and use of aqueous extracts may be feasible and efficient to control the citrus black fly. However, more research is needed, such as comparative testing of commercial formulations with the plant extracts, assessment of lethal and sublethal effects of these products on natural enemies, and the development of field work, that are of fundamental importance, because the product action may be altered by environmental conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are thankful to the Foundation for Research, Scientific and Technological Development of Maranhão State, Brazil (FAPEMA) for financial support.

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