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Artigo

Chromatographic evaluation and antimicrobial activity of Neem (*Azadirachta indica* A. Juss., Meliaceae) leaves hydroalcoholic extracts

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RESUMO: "Avaliação cromatográfica e atividade antimicrobiana de extratos hidroalcoólicos de folhas de Nim (Azadirachta indica A. Juss., Meliaceae)". O Nim (*Azadirachta indica* A. Juss., Meliaceae) é uma árvore indiana conhecida por suas várias atividades farmacológicas, entre elas, a ação antimicrobiana. Dentre mais de 300 compostos já isolados, a azadiractina (AZA) é seu principal componente ativo. No presente trabalho, foram preparados extratos hidroalcoólicos de folhas de Nim em diferentes concentrações de etanol 96% V/V (50%, 60%, 70%, 80% e 90% (V/V)) por meio de percolação estática. A presença de AZA foi verificada por CCD, com eluição dos extratos e da solução padrão de trabalho AZA em cromatoplaca e revelação por solução de anisaldeído/ácido sulfúrico, seguida de aquecimento. Por CLAE, os extratos e da solução padrão de trabalho AZA foram eluídos em coluna C18, fase móvel água:acetonitrila (60:40), fluxo 1,0 mL/min e detecção em λ_{217} nm. Não foi verificada a presença de manchas ou picos correspondentes a AZA nos extratos. Entretanto, a sua atividade foi investigada contra bactérias Gram-positivas, Gram-negativas, leveduras e um fungo filamentoso. Os extratos foram testados em diferentes concentrações para avaliar a relação dose-resposta. Apesar da ausência de AZA, os extratos hidroalcoólicos a 70% e 80% (V/V) de etanol 96% apresentaram atividade contra *Staphylococcus aureus*. Porém, não houve relação dose-efeito, de acordo com o teste de Tukey ($q_{0,05;3;7}$).

Unitermos: *Azadirachta indica***,** nim, CLAE, atividade antimicrobiana, extrato hidroalcoólico.

ABSTRACT: "Chromatographic evaluation and antimicrobial activity of Neem (*Azadirachta indica* A. Juss., Meliaceae) leaves hydroalcoholic extracts". Neem (*Azadirachta indica*) is an Indian tree well known for its several pharmacological activities, including antimicrobial activity. More than 300 composites have already been isolated and azadirachtin (AZA) is its main active component. In the present work, Neem leaves hydroalcoholic extracts were prepared by percolation in 96% ethanol different concentrations (50%, 60%, 70%, 80% and 90% (v/v)). The presence of AZA was tested by TLC by eluting the extracts and a standard solution of AZA through a chromatographic plate developed with anisaldehyde/sulfuric acid solution followed by heating. By HPLC, extracts elution took place on a C18 column, water:acetonitrile (60:40) as mobile phase, 1.0 mL/min flow rate and detection at λ_{217} nm The extracts did not display AZA spots or peaks, however, they were tested against Gram-positive and Gram-negative bacteria, yeasts and a mold fungus. The extracts were tested in different increasing concentrations, in order to detect a dose-dependent relationship of the activity. Despite the absence of AZA, the 70% and 80% (v/v) ethanol extracts showed activity against *Staphylococcus aureus*. However, this activity was not dose-dependent according to Tukey's test ($q_{0,05;3;7}$).

Keywords: *Azadirachta indica*, neem, HPLC, antimicrobial activity, hydroalcoholic extract.

INTRODUCTION

Neem (*Azadirachta indica* A. Juss.) is a tree from the Meliaceae family originated from India. It is known for its pesticide activity against more than 400 insect pests (Siddiqui et al., 2003) and pharmacological activities, such as anti-inflammatory, anti-malaria, anti-fertility, antimicrobial (Subapirya & Nagini, 2005; Dai et al., 1999), anti-acne (Jain & Basal, 2003), acaricidal (Abdel-Shafy & Zayed, 2002), and nematicidal (Sharma et al., 2003). The plant is traditionally used in the Indian region and many of its activities have been studied.

Azadirachtin (AZA, **1**), the main active component of this plant, is a tetranortriterpenoid abundant in the seeds and present in a smaller concentration in the leaves. Other active substances are salanin, 14-

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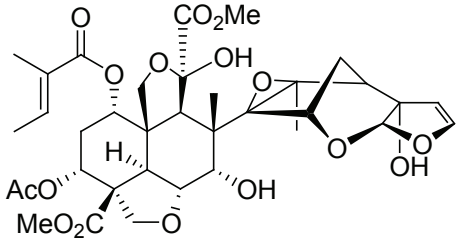
epoxiazadiradione, meliantrol, melianone, gedunin, nimboline, nimbin, deacetilasalanin, azadiractol, azadirone, vilosinin, meliacarpine, over 300 isolated and characterized components (Dai et al., 1999; Sharma et al., 2003; Silva, 2005). In their terpene common chemical structure functional groups such as, acetate, hydroxyl and esters can be linked. The main active component, AZA, is commonly used as the biological marker for this plant.



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There are several studies on the antimicrobial activity of Neem. Some of them have demonstrated activities of extracts from seeds and leaves against *Staphylococcus aureus, Escherichia coli,* as well as, negative results against *Bacillus subtillis, S. paratyphi, S. desynteriae* and *Candida albicans* (Alhmad & Beg, 2001). Neem leaves are efficient against pathogenic fungi, such as *Trichophyton, Epidermophyton, Microsporum, Trichosporon* and *Geotricum* (Khan & Wassilew, 1987). The activity in inhibiting the protease of *Trichophyton* (Iyer & Williamson, 1991), the production of aflatoxin of *A. parasiticus* (Allamed et al., 2001), antifeedant activity (Silva et al., 2007) and the antifungal activity against *Penicillium expansum* (Mossini et al., 2004) have been confirmed. The Neem seed oil also showed a wide spectrum activity against Gram-negative and Gram-positive microorganisms, including *M. tuberculosis* and streptomicine resistant strains (Chopra et al., 1952). The leaves also inhibited the growth of *Vibrio cholerae, Klebsiella pneumoniae, M. tuberculosis* and *M. pyogenes* in vitro (Satyavati et al., 1976).

In this work, we described the antimicrobial activity of Neem hydroalcoholic leaves extracts against Gram-positive and Gram-negative bacteria, yeasts and a mold fungus.

MATERIAL AND METHODS

Plant material and extracts

Neem leaves were collected from a commercial crop in the town of Pompéu (Latitud -18°56′24′′South, Longitud -45°6′0′′West), Minas Gerais state (Brazil). The extracts were prepared by percolation of 500 g of crushed Neem leaves in 500 mL of 96% ethanol at 50%, 60%, 70%, 80% e 90% (v/v), in triplicate. The leaves, previously moistened with adequate solvent, were transferred to a percolator, wherein were completely covered by solvent. After standing for 48 h the first fraction was collected. Additional solvent was poured, standing for further 24 h, when the second fraction was collected. This process was repeated until 500 mL of the extract for each of the different hydroalcoholic concentration were obtained. An aliquot of 125 mL of each extract was dried in a rotatory evaporator to obtain a residue, which was dried in a vacuum oven at 40 ºC for 6 h and left in a desiccator for 50 h. An amount of 100 mg of the residue was accurately weighed and reconstituted in 1.0 mL of ethanol 50% (v/v). The latter solvent was used as blank. The same procedure was repeatedly performed with different residue amounts to prepare extract concentrations at 150, 200, 250 or 300 mg/mL.

Chromatographic evaluation

Thin layer chromatography

The extracts were tested by thin-layer chromatography (TLC). The elution system was a mixture of methylene chloride:methanol (99:1). Samples of the hydroalcoholic extracts and 1.0 mg/mL AZA standard (Chemservice, West Chester, PA, USA, technical grade) in methylene chloride were applied to the chromatographic plate (silica gel 60 F_{254}, Merck, Darmstadt, Germany), using glass capillary tubes and eluted. The plate was developed using anysaldehyde/sulfuric acid solution followed by heating.

High performance liquid chromatography

The neem hydroalcoholic extracts at 70% and 80% (v/v) were tested by reverse phase-high performance liquid chromatography (HPLC). The analyses were performed on an HP1100 series chromatograph (Agilent, Palo Alto, CA, USA) equipped with a quaternary pump, automatic injector and an ultraviolet diode array detector (UV/DAD) module. Separation was accomplished on a C18 column (Agilent, 250 x 4.6 mm) oven set at 30 ºC (CH-500, Eppendorf, Madison, WI, USA). Internal ChemStation software version 07.01 was used for data acquisition. Mobile phase water:acetonitrile (60:40) at 1.0 mL/min flow rate, 20 µL injection volume, and UV detection at λ_{217} nm were used.

Antimicrobial evaluation

Microorganisms and media

The standard microorganisms cultures *Bacillus subtilis* (ATCC 6633), *Micrococcus luteus* (ATCC 9341), *Staphylococcus aureus* (ATCC 6538p), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella thiphymurium* (ATCC14028), *Saccharomyces cerevisae* (ATCC 2601), *Candida albicans* (ATCC

10231), *Candida tropicalis* (ATCC 1641) were used. The microorganisms were grown in nutrient agar (Biobras, Montes Claros, MG, Brazil) and Sabouraud-dextrose agar (Difco, Franklin Lakes, NJ, USA), for bacteria and fungi, respectively.

Antibiotic standards

Chloramphenicol, reference standard (Sigma, Saint Louis, MO, USA), was used as positive control, for bacteria tests, except for *Pseudomonas aeruginosa*, for which gentamicin reference standard (Sigma, Saint Louis, MO, USA) was used. Amphotericin B (Fungison®, Bristol, New York, NY, USA) was used in the tests for yeasts and mold fungus.

In vitro *antimicrobial activity evaluation*

The antimicrobial activity of the hydroalcoholic extracts was determined by means of the disc diffusion method (Nunan et al., 1985; NCCLS, 1993). The inoculum was standardized by transferring colonies from the nutrient agar or Saboraud-dextrose agar to sterile saline up to 10^8 cfu/ml, equivalent to 60% ± 1% transmittance at λ_{580} nm (Junior II 6/20 Coleman spectrophotometer, Maywood, IL, USA). This suspension was ten-fold diluted in saline (1 mL of suspension and 9 ml of saline). An aliquot of 250 µL was withdrawn from the diluted suspension and transferred to an Erlenmeyer flask with 50 mL of the appropriate agar for each microorganism to obtain a 0.05% (v/v) inocula. For *Bacillus subtilis* and *Aspergillus niger* cultures, a 0.1% (v/v) Tween 80 (Synth, São Paulo, SP, Brazil) solution was added.

The agar was composed of a bilayer, either casein soy agar or Sabouraud-dextrose agar (20 mL), was poured into Petri dishes (100 × 20 mm) to form the base layer. After solidification of this layer, portions of 5 mL of inoculated casein soy agar or Sabouraud-dextrose agar were poured over the base layer.

The extracts were initially tested at 100 mg/mL, in triplicate. Six discs (6.0 mm i.d.) were impregnated with 20 µL of the extracts and placed on the surface of the agar containing each microorganism, which was incubated at 36.0 ± 1.0 °C for 24 h and at 22-25 ºC for 48 h for bacteria and fungus, respectively. The inhibition zones were measured with a caliper considering the total diameters. Similarly, each plate carried a blank disc containing 20 µL of ethanol 50% (v/v) and a control antibiotic disc containing 20 µg of chloramphenicol (1.0 mg/mL) or gentamicine (1.0 mg/mL) and 40 µg amphotericin B (2.0 mg/mL), for bacteria and fungi, respectively.

The assay was repeated (n = 3) for those extract solution impregnated disks which showed any microorganism growth inhibition. Concentrations of 100 mg/mL, 150 mg/mL, 200 mg/mL, 250 mg/mL and 300 mg/mL were used. The results were submitted to analysis of variance and the means were compared by Tukey test ($q_{0,05;3;7}$), using Microsoft Excel® 2002.

RESULTS AND DISCUSSION

The extracts evaluated by TLC, presented several spots developed. It is noteworthy that no AZA correspondent spot was present in the Neem extracts. Other spots present possibly correspond to other active substances. The hydroalcoholic extracts have also been tested by HPLC and compared to AZA reference solution (0.4 mg/mL). The AZA reference solution chromatogram is represented in Figure 1. Two elution peaks can be observed, at 7.933 min and 8.780 min with the same spectrophotometric profile, represented in Figure 1. In order to verify whether peak splitting was dependent on pH, 1% (v/v) triethanolamine or 1% (v/v) trifluoroacetic acid in water solutions were prepared and used in the mobile phase. However, there were no differences in the chromatograms by this pH alteration, indicating that the peaks corresponded to distinct substances. Therefore, the peaks at 7.933 min and 8.780 min were considered to be AZA peaks. A representative chromatogram for the extracts eluted by HPLC is represented in Figure 2. As it is evidenced in the chromatogram, there were no AZA peaks at circa of 7.9 and 8.7 min. It is possible to assert that the extracts do not have AZA in a quantifiable amount, indicating its absence in the hydroalcoholic extracts tested. The other peaks present may correspond to other substances in Neem (Subapriya & Nagini, 2005; Martinez, 2002; Siddiqui et al., 2003; Silva, 2005).

The extracts prepared with different ethanolic concentrations were tested against pathogenic microorganisms for determining any antimicrobial activity by the diffusion disk method. The growth inhibition zones measured (in mm) for the positive antibiotic control used were: chloramphenicol (1.0 mg/mL), 29.5 ± 1.8 against *B. subtilis*, 31.8 ± 0.3 against *M. luteus*, 26.0 ± 0.8 against *S. aureus*, 19.5 ± 0.3 against *E. coli* and 19.3 ± 0.3 against *S. thiphymurium*; gentamicin (1.0 mg/mL), 10.0 ± 0.1 against *P. aeruginosa*. Amphotericin B, 12.7 ± 0.3 against *S. cerevisae*, 28.3 ± 0.5 against *C. albicans* and 24.0 ± 0.8 against *C. tropicalis*. Blank control did not show any inhibition zone. The hydroalcoholic extracts at 50%, 60% and 90% (v/v) did not show any antimicrobial activity at the tested concentration (100 mg/mL). The extracts obtained at 70% and 80% (v/v) ethanol, however, did show activity against *Staphylococcus aureus*. However, it was not possible to measure the diameter of the growth inhibition zone, precisely, which featured a local inhibition. The active extracts were tested in different increasing concentrations, in order to determine a possible dose-dependent activity against *S. aureus*. As shown in Table 1, the extracts still showed little activity, verified by measuring the local growth inhibition zone. In Table 2, the results presented show that the variance in the growth

inhibition zones were not statistically significant (Tukey test, $q_{0.05;3;7}$), therefore, there was no relationship between the applied dosage and the extract antimicrobial activity.

Although the antimicrobial activity of the Neem leaves extract against several microorganisms has been described by several authors (Almad & Beg, 2001; Chopra et al., 1952; Subapirya & Nagini, 2005), the same was not verified for the hydroalcoholic extracts tested from the neem grown in Minas Gerais state, Brazil. The absence of AZA, as shown by HPLC chromatogram (Figure 3) and observed by TLC, may justify the little antimicrobial activity of the Neem leaves hydroalcoholic extracts. A possible cause for the absence of those substances may be the crops environmental conditions (climate, soil), in case the tree may not have adjusted well to the climate and soil in Minas Gerais. It is known that there is a great difference in the extracts composition of plants cultivated in different locations. The adaptation to the field and soil composition is an important factor for the proper active ingredient production in the plants, according to Sidhu et al. (2003).

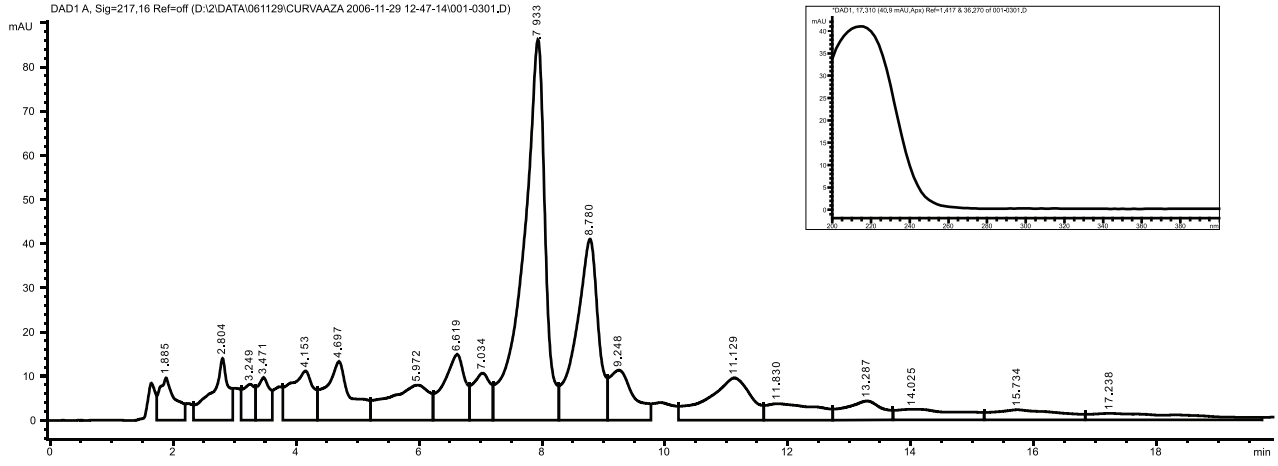

Figure 1. Representative chromatogram of AZA reference solution (0.4 mg/mL) tested by HPLC-UV/DAD, mobile phase Figure 2. Representative chromatogram of AZA reference solution (0.4 mg/mL) tested by HPLC-UV/DAD, mobile phase water:acetonitrile (60:40), C18 column, 20 µL injection volume, λ_{217} nm detection, peaks at 7.933 and 8.780 min. Insert: UV/DAD spectrum for AZA reference solution peaks.

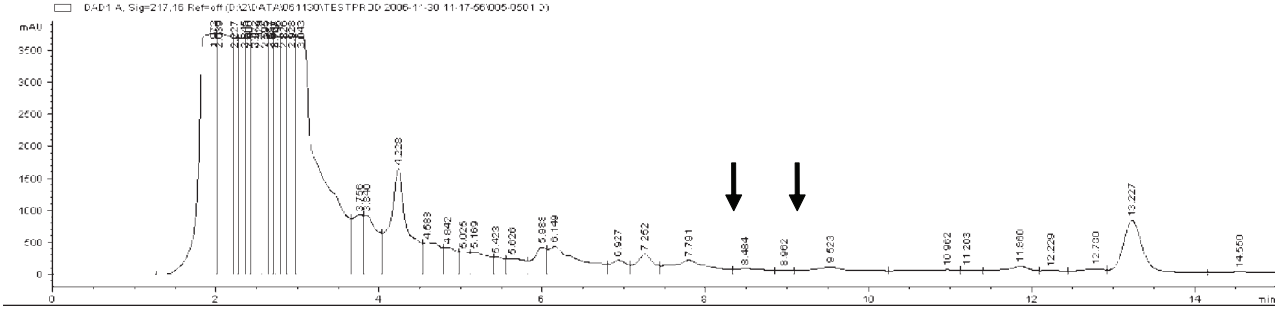

Figura 2. Representative chromatogram of the hydroalcoholic extract at 70% v/v ethanol tested by HPLC, mobile phase water:acetonitrile (60:40), C18 column, 20 µL injection volume, λ_{217} nm detection. Arrows show the expected retention times for AZA peaks at circa of 7.9 and 8.7 min.

Table 1. Influence of different concentrations of neem leaves hydroalcoholic extracts at 70% and 80% (v/v) ethanol on *S. aureus* growth by disc plate dilution method.

	Concentration (mg/mL)		Inhibition zone ± SD[a] (mm)			Mean zone ± SD[a] (mm)
Positive control[b] (chloramphenicol)	1.0		23.35 ± 0.3	24.20 ± 0.3	24.00 ± 0.1	23.80[b] ± 0.4
			22.65 ± 0.8	24.20 ± 0.3	24.40 ± 0.4	
Hydroalcoholic Neem leaves extracts[c]	100	70%	7.00 ± 0.1	6.95 ± 0.1	6.85 ± 0.1	6.93[c] ± 0.1
		80%	6.60 ± 0.0	6.50 ± 0.1	6.70 ± 0.1	6.60 ± 0.1
	150	70%	6.70 ± 0.2	7.35 ± 0.2	7.05 ± 0,1	7.03 ± 0.2
		80%	7.10 ± 0.2	6.60 ± 0.2	6.75 ± 0.1	6.82 ± 0.1
	200	70%	7.30 ± 0.1	7.50 ± 0.1	7.55 ± 0.1	7.45 ± 0.1
		80%	6.10 ± 0.1	6.35 ± 0.1	6.20 ± 0.1	6.22 ± 0.1
	250	70%	7.20 ± 0.3	7.45 ± 0.1	8.10 ± 0.4	7.58 ± 0.2
		80%	6.65 ± 0.2	7.15 ± 0.3	7.10 ± 0.1	6.97 ± 0.2
	300	70%	7.50 ± 0.3	7.25 ± 0.1	7.20 ± 0.1	7.32 ± 0.1
		80%	6.45 ± 0.4	8.00 ± 0.5	6.35 ± 0.4	6.93 ± 0.5

a: standard deviation, b: n = 6 for standard, c: n = 3 for extracts.

Table 2. Statistic results for Tukey's test[a] in order to verify the dose-response relationship for the hydroalcoholic Neem leaves extracts at 70% and 80% (v/v) ethanol against *S. aureus*.

Hydroalcoholic extract at 70% (v/v)[b]						
Concentration (mg/mL)	Mean	250	200	300	150	Discrimination
250	7.58	-	-	-	-	a
200	7.45	0.13	-	-	-	a
300	7.32	0.27	0.13	-	-	a
150	7.03	0.55	0.42	0.28	-	a
100	6.93	0.65	0.52	0.38	0.10	a
Hydroalcoholic extract at 80% (v/v)[c]						
Concentration (mg/mL)	Mean	250	300	150	100	Discrimination
250	6.97	-	-	-	-	a
300	6.93	0.03	-	-	-	a
150	6.82	0.15	0.12	-	-	a
100	6.60	0.37	0.33	0.22	-	a
200	6.22	0.75	0.72	0.60	0.38	a

a: critical $q_{0.05;3;7}$ = 4.17, obtained from Tukey's table, considering 3 different treatments (replicates) and 7 degrees of freedom, b: minimum significative difference (MSD) = 1.39; c: MSD = 11.08.

CONCLUSION

The tests performed on the Neem leaves hydroalcoholic extracts originated from such crop in Minas Gerais indicate that those leaves did not yield enough AZA for detection by TLC or HPLC-UV/DAD, hence the extracts activity against microorganisms was limited. The reasons for this may be due to crop location, plants young age or soil condition. Although AZA could not be detected in the Neem leaves extracts, antimicrobial activity detected against *S. aureus* may be due to the presence of several substances, other than AZA, indicating that the leaves extract can be used against this bacterium, a very important pathogenic microorganism.

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