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

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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, I), the main active component of this plant, is a tetrnortriterpenoid abundant in the seeds and present in a smaller concentration in the leaves. Other active substances are salanin, 14-
epoxiazadiradione, meliantrol, melianone, gedunin, nimborne, 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.

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 °C 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 anisaldehyde/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 °C (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 λ217 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 thipymurium* (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 gentamicine 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 Sabouraud-dextrose agar to sterile saline up to 10⁶ cfu/ml, equivalent to 60% ± 1% transmittance at λ₅₈₀ 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 °C 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 (q₀.₀₅;ₙ), 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<sub>0.05;3;7</sub>), 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 water:acetonitrile (60:40), C18 column, 20 µL injection volume, λ<sub>217</sub> nm detection, peaks at 7.933 and 8.780 min. Insert: UV/DAD spectrum for AZA reference solution peaks.

Figure 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<sub>217</sub> nm detection. Arrows show the expected retention times for AZA peaks at circa of 7.9 and 8.7 min.
Table 2. Statistic results for Tukey’s test in order to verify the dose-response relationship for the hydroalcoholic Neem leaves extracts at 70% and 80% (v/v) ethanol against *S. aureus*.

<table>
<thead>
<tr>
<th>Concentration (mg/mL)</th>
<th>Mean ± SD</th>
<th>Discrimination</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>7.00 ± 0.1</td>
<td>6.93 ± 0.1</td>
</tr>
<tr>
<td>80%</td>
<td>6.60 ± 0.0</td>
<td>6.00 ± 0.1</td>
</tr>
<tr>
<td>150</td>
<td>7.60 ± 0.2</td>
<td>7.05 ± 0.1</td>
</tr>
<tr>
<td>80%</td>
<td>7.10 ± 0.2</td>
<td>6.75 ± 0.1</td>
</tr>
<tr>
<td>200</td>
<td>7.30 ± 0.1</td>
<td>7.55 ± 0.1</td>
</tr>
<tr>
<td>80%</td>
<td>6.10 ± 0.1</td>
<td>6.20 ± 0.1</td>
</tr>
<tr>
<td>250</td>
<td>7.20 ± 0.3</td>
<td>8.10 ± 0.4</td>
</tr>
<tr>
<td>80%</td>
<td>6.65 ± 0.2</td>
<td>7.10 ± 0.1</td>
</tr>
<tr>
<td>300</td>
<td>7.50 ± 0.3</td>
<td>7.20 ± 0.1</td>
</tr>
<tr>
<td>80%</td>
<td>6.45 ± 0.4</td>
<td>6.93 ± 0.5</td>
</tr>
</tbody>
</table>

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.
ACKNOWLEDGEMENTS

The authors thank Memovip Ltda. for financial support and providing the neem leaves.

REFERENCES


