EVALUATION OF THE ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY, AND CYTOTOXICITY OF EXTRACTS OF Mikania laevigata Schultz bip (Asteraceae)

Cristina E. Okuyama¹, Jose J. G. Bitencourt¹, Daniel A. F. Oliveira¹, Margarete S. Oliveira¹, Rebecca S. S. Barbosa¹, Alexandra C. H. F. Sawaya², Marcos N. Eberlin³

¹Institute of Health Sciences, Bandeirante University of São Paulo, 02071-013, São Paulo, SP, Brazil.
²Department of Plant Biology, Institute of Biology; ³Thomson Mass Spectrometry Laboratory, Chemistry Institute - State University of Campinas, 13083-970, Campinas, SP, Brazil.

RESUMO
Duas espécies do gênero Mikania (Mikania glomerata e Mikania laevigata), conhecidas no Brasil como guaco são bastante similares em aparência e popularmente usadas para tosse, bronquite e inflamações. Como M. glomerata é encontrada em compêndios oficiais, M. laevigata foi menos estudada. Embora a maioria dos estudos seja feita com extratos hidro-alcoólicos, guaco é consumido em forma de infusos e decoctos, e apenas um estudo usando solvente pouco polar foi encontrado. O objetivo deste estudo foi avaliar como o solvente (água, etanol e éter) pode afetar a atividade dos extratos de folhas de M. laevigata. Estes extratos foram analisados e foi determinado o teor de cumarina, atividade antimicrobiana e antioxidante, e citotoxicidade. O extrato etanólico apresentou a maior concentração de cumarina e o melhor efeito contra E. coli, mas também foi o mais citotóxico. O extrato etéreo foi menos citotóxico, mas também foi menos ativo contra bactérias, uma concentração baixa de cumarina e baixa atividade antioxidante. O extrato aquoso não apresentou citotoxicidade em concentrações abaixo de 20 mg/mL e apresentou a maior atividade antioxidante, com atividade antibacteriana e teor de cumarina similar aos do estrato etanólico, confirmando o uso popular do extrato aquoso de M. laevigata.


ABSTRACT
Two species of the Mikania genus (Mikania glomerata and Mikania laevigata), known locally as guaco, are quite similar in appearance and used interchangeably by the population used for coughs, bronchitis and inflammations. As M. glomerata is found in official compendiums, M. laevigata has been less studied. Although most studies were carried out with hydro-alcoholic extracts, guaco is popularly consumed in the form of teas and infusions, and only one study evaluating a less polar extract was found. The purpose of this study was to evaluate how the solvent (water, ethanol and ether) could affect the activity of extracts of leaves of M. laevigata. These extracts were analyzed as to their coumarin content, antimicrobial and antioxidant activity and cytotoxicity. The ethanol extract had the highest concentration of coumarin and the best effect on E. coli, but was also the most cytotoxic. The ether extract was less cytotoxic, but had less activity against bacteria, a low coumarin content and low antioxidant activity. The water extract wasn’t cytotoxic under 20 mg/ml and had the highest antioxidant activity, with antibacterial activity and coumarin contents similar to the ethanol extract, confirming the popular use of water extracts of M. laevigata.

Key-words: Mikania laevigata. Guaco. Coumarin.

Endereço para correspondência
Universidade Bandeirante de São Paulo,
Rua Maria Cândida, 1813, São Paulo, SP, 02071-013, Brasil
E-mail: cris_okuyama@yahoo.com.br
Phone: +55-11-29679147
INTRODUCTION

The Mikania genus contains over 400 species, 150 of them growing in Brazil (Baratto et al., 2008). Two species, known locally as guaco, are popularly used for coughs, bronchitis and inflammations; Mikania glomerata and Mikania laevigata. These two species are quite similar in appearance and are frequently confused, their main difference being in the shape of the leaves and the flowering period (Moraes, 1997). Mikania glomerata is the species considered the official drug and described as far back as the first Brazilian Pharmacopoeia (Silva, 1929) as well as in more recent government publications (Brasil, 2008). However, M. laevigata is more frequently found in the southeast of Brazil and both species present the characteristic coumarin odor in their leaves. Coumarin has been shown to be responsible for the anticholinergic activity of hydro-alcoholic extract of M. laevigata (Bighetti et al., 2005), involved in both antiulcerogenic and bronchodilator effects.

M. glomerata has been more thoroughly studied and its anti-ophidian (Pereira et al., 1994; Maiorano et al., 2005), anti-inflammatory (Oliveira et al., 1985) anti-allergic (Fierro et al., 1999) and antimicrobial activity (Holetz et al., 2002) determined, as well as its mutagenic potential (Sá et al., 2006; Costa et al., 2008). A lesser number of studies have been carried out with M. laevigata. The mechanism of the trachea relaxation induced by the hydro-alcoholic extract of its leaves has been studied (Graça et al., 2007), as well as the safety of this extract. The antimicrobial activity of its ethanolic extract (Baratto et al., 2008; Duarte et al., 2004) and of its essential oil (Duarte et al., 2005) has been determined. The anti-allergic effect of its hydro-alcoholic and water extract was compared (Santos et al., 2006) to those of M. glomerata. The antiulcerogenic effect of a hydro-alcoholic extract of M. laevigata was analyzed, in comparison with the activity of coumarin (Bighetti et al., 2005) and the activity of syringaldehyde, extracted from M. laevigata, was tested for its anti-allergic properties (Pedroso et al., 2008).

Although most studies were carried out with hydro-alcoholic extracts, as described in the first Brazilian Pharmacopoeia (Silva, 1929) this plant is popularly consumed in the form of teas and infusions. Only one paper was found evaluating the antimicrobial activity of a less polar extract; the essential oils (Duarte et al., 2005). Therefore it is important to determine how the polarity of the solvent affects the activity and safety of these extracts. Furthermore, no studies were found evaluating the antioxidant activity of M. laevigata extracts, nor their cytotoxicity.

The purpose of this study was to evaluate how the polarity of the solvent could affect the activity of the extracts of leaves of Mikania laevigata. These extracts were analyzed as to their coumarin content, antimicrobial and antioxidant activity and cytotoxicity. In vitro methods were preferred to evaluate these parameters. Coumarin is the chemical marker adopted by Brazilian legislation (BRASIL, 2008) for the registration of pharmaceutical products containing M. glomerata extracts, therefore this compound was identified and quantified in all three extracts.

MATERIAL AND METHODS

Plant material and extraction

Leaves of Mikania laevigata Schultz Bip. Ex Baker grown in the organic garden of Instituto Candido Ferreira, Souzas, Sao Paulo, Brazil were collected in February 2008, and identified by comparison with voucher specimens deposited at the Botany Department of Instituto de Biologia, Universidade Estadual em Campinas (UNICAMP) and with descriptions found in (Moraes, 1997). The leaves were air dried at room temperature for a period of 10 days and then ground to a particle size of 2 mm or less.

The resulting powder was divided in three lots and extracted by maceration in an ultrasound bath for 30 minutes with three solvents of different polarities (Milli-Q deionized water, analytical grade ethanol and ether) in the proportion of 30 g powdered leaves to 100 mL of solvent, followed by filtration, in a variation of the method proposed by (Celeghini et al., 2001). The solvents of the resulting solutions were removed by freeze drying (water) or by heating under air circulation at 40°C. The dry extracts were kept in air tight containers in a freezer (-16°C) until used.

HPLC-ESI-MS determination of coumarin concentration in samples

HPLC separation was achieved at ambient temperature (approximately 22-24°C) using a Waters Alliance 2695 pump with a 5 μm Inertisil

ODS-2 column (250 x 4.6 mm I.D.) obtained from Varian (Middleburg, The Netherlands). Separations were done in an isocratic mode using acetonitrile : water (40: 60 v/v) at a flow rate of 1 mL/min, based on the method of (Celeghini et al., 2001). A post-column splitter was used to reduce the flow entering the ESI source to approximately 100 µL/min. The ESI-MS spectra were acquired in the positive ion mode on an Applied Biosystems Q-trap mass spectrometer (Foster City, CA, USA). The operational parameters used were: capillary 5000 V, temperature 350ºC, declustering potential 50 V and entrance potential 10V. A calibration curve was prepared using solutions of coumarin with concentrations ranging from 1 to 100 µg/mL. The extracts were diluted in a 50% acetonitrile solution to a final concentration of 100 µg/ml, and 20 µL were injected.

Antimicrobial activity
Antimicrobial activity was evaluated by the serial dilution method against Candida yeast and Gram positive and Gram-negative bacteria. The water, ethanol and ether extracts were tested against Staphylococcus aureus ATCC 6534, Escherichia coli ATCC 25922 and Candida albicans ATCC 90028 by serial dilution in tubes. The microorganisms were obtained from 24-hour cultures and suspended in saline solution to concentrations of approximately 10⁸ CFU/mL by comparison with McFarland tube no. 1. Saboraud broth was used for the yeast and Mueller-Hinton broth was used for the bacteria. The water extract was dissolved in water and the other extracts were dissolved in 50% ethanol, to the initial concentration of 40 mg/mL. Serial dilution was performed using concentrations between 20.0 mg/mL and 2.5 mg/mL of each extract in the broth. All tests were performed in duplicate. The tubes were inoculated with 20 µL of the microorganism suspension, homogenized and incubated for 24 hours at 37ºC. On the following day, 50 µL was taken from each tube and inoculated in a second tube, incubated for another 24 hours. The Minimal Inhibitory Concentration (MIC) was determined as the lowest concentration of extract in which no growth was visible after the first 24 hours, and the Minimal Bactericidal Concentration by the concentration of extract for which no growth was observed in the second set of tubes. A 50% ethanol solution was used as a negative control to test the possible inhibitory effect of the solvent, whereas oxacillin and nistatine were used as positive controls.

Antioxidant activity
The 1,1-diphenyl-2-picrylhydrazyl free radical scavenging method (DPPH) is a well known in vitro method for the evaluation of the antioxidant activity of natural products. The dry extracts were dissolved in a solution of methanol (Tedia, Fairfield, OH, USA), or water at a concentration of 1 µg/mL. For each extract a series of test tubes were prepared containing between 1 and 1000µL of this solution, and the volume completed to 1000 µL with methanol. Finally 1 mL of a 60 µM solution of DPPH was added to each of the tubes. After 30 min of incubation at room temperature in the dark, the absorbance was recorded at 517 nm on a Cary-50 Varian spectrophotometer (USA). Results were expressed as percentage of decrease with respect to control values (absorbance of 1 mL DPPH solution + 1 mL of methanol or water) incubated under the same conditions. Readings at each concentration were made in triplicate. The means and the standard deviation was calculated for each result and plotted on a graph, and the concentration that reduced the absorbance by 50% (ED₅₀) was calculated for each extract. As a positive control a solution of ascorbic acid in methanol was used.

Evaluation of cytotoxicity by brine shrimp (Artemia salina) assay
A method, utilizing brine shrimp (Artemia salina), is a simple bioassay for natural product research. The procedure determines LC₉₀ values of active compounds and extracts in the brine medium. In the present study it is used to compare the possible cytotoxicity of the three extracts of guaco leaves (Meyer et al., 1982; Santos-Pimenta et al., 2003). Brine shrimp eggs (Maramar, Brazil) were hatched in a shallow rectangular dish (15 x 10 cm) filled with artificial seawater, which was prepared with a commercial salt mixture and distilled water. A plastic divider was clamped in the dish at 2 mm from the bottom to make two equal compartments. The eggs (50 mg) were sprinkled into the one compartment, which was darkened, while the other compartment was illuminated. After 48 hours the phototropic nauplii were collected by pipette from the lighted side, having been separated by the divider from their shells.

Ten shrimp were transferred to each
sample well using a disposable pipette and artificial seawater on a multiwell testing plate. The dry extracts were dissolved in a solution of ethanol or water at different concentrations of each *guaco* extract (0.01 - 20 mg/mL), both solvents were used as negative controls and did not affect the shrimp mobility. Each concentration of the tested samples was transferred to three wells and three wells with only seawater were used as control. Then the wells were maintained without illumination for 24 hours at room temperature. At the end of this period the numbers of actively swimming and immobile shrimp were counted and the LC$_{50}$ values calculated for each extract using the probit analysis method (Finney, 1971).

**Statistical evaluation**

Experimental values were expressed as average ± standard deviation. The variance test (ANOVA), followed by the Bonferroni test was applied, using the InStat program (GraphPad Software, 5.0 version). Values of P<0.01 were considered significant.

**RESULTS AND DISCUSSION**

The marker compound for *guaco* leaves, coumarin, was detected and quantified in all three extracts by HPLC-ESI-MS (Figure 1), at concentrations between 10.28 μg/mL in the ethanol extract and 3.33 μg/mL in the ether extract, solutions were prepared at a concentration of 100 μg/mL (Table 1).

**Table 1.** Percentage of coumarin in the dry extracts, and results of the DPPH antioxidant activity assay (ED$_{50}$ in μg/mL) and *Artemia salina* cytotoxicity assay (LC$_{50}$ in μg/mL).

<table>
<thead>
<tr>
<th>Extract</th>
<th>% of coumarin</th>
<th>ED$_{50}$ (μg/mL)</th>
<th>LC$_{50}$ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>9.58±0.15$^b$</td>
<td>10.5±0.5$^{a,b}$</td>
<td>ND</td>
</tr>
<tr>
<td>ethanol</td>
<td>10.28±0.18$^c$</td>
<td>15.2±0.3$^{a,c}$</td>
<td>62.6</td>
</tr>
<tr>
<td>ether</td>
<td>3.33±0.21$^{b,c}$</td>
<td>28.4±0.8$^{a,b,c}$</td>
<td>78.1</td>
</tr>
</tbody>
</table>

ND = not determined. $^a$Significant difference between water and ethanol groups (P<0.01). $^b$Significant difference between water and ether groups (P<0.01). $^c$Significant difference between ethanol and ether groups (P<0.01).

Popularly, guaco leaves are used in infusions and decoctions, whereas in the Brazilian Pharmacopoeia 1st edition (Silva, 1929) only the ethanolic extracts are described. As coumarin is the chemical marker for *M. glomerata* extracts, the fact that its concentration in both water and ethanol extracts is similar, shows that both these extracts should be biologically actives. The daily recommended dose of coumarin is between 525 and 4,890 μg (Brasil, 2008), which represents a consumption of between 50 to 500 mL of such a solution, demonstrates its wide therapeutic margin. Ether extracted compounds are of less polar nature and had a lower concentration of coumarin.
microorganisms tested. The Minimal Fungicidal Concentration could not be determined for the strain of *Candida albicans* used, as the results showed that it was higher than the maximum concentration used in this test (20 mg/ml). Duarte et al. (2005) also reported that the MIC for *C. albicans* was higher than 2 mg/mL of the ethanolic extract, the highest concentration they tested, but the essential oil of *M. laevigata* showed a MIC of 0.25 mg/mL. Baratto et al. (2008), using hydroalcoholic extracts of *M. laevigata*, did not observe inhibition halos against *S. aureus* or *E. coli*. Duarte et al. (2004) report a MIC of 0.04 mg/mL for a hydroalcoholic extract of *M. laevigata* against *S. aureus*. Holetz, et al. (2002) reported that the MIC for a hydroalcoholic extract of *M. laevigata* was higher than 1 mg/mL against *C. albicans* and of 0.5 mg/mL against *S. aureus* and *E. coli*. No other data were found in literature for the MIC of water extracts or ether extracts of *M. laevigata* leaves. No data on the antimicrobial activity of ether extracts was found in literature. Our results showed that all the extracts have bactericidal properties, but that their effect against yeast is not clear. These results are in line with those of Duarte et al. (2004 and 2005), although the MIC values found in our studies were higher than those reported. This may have been influenced by the time of year when the leaves were collected and the solvent evaporation procedure. Santos et al. (2006) concluded that samples of *M. laevigata* collected in different states varied in their coumarin contents and proposed that the freeze drying process also affected the composition of the extracts.

### Table 2. Minimal Inhibitory Concentrations (MIC) and Minimal Bactericidal Concentrations (MBC) of water, ethanol and ether extracts of *guaco* against *Staphylococcus aureus*, *Eschericia coli* and *Candida albicans* in mg/mL.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>water MIC</th>
<th>water MBC</th>
<th>ethanol MIC</th>
<th>ethanol MBC</th>
<th>ether MIC</th>
<th>ether MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>10</td>
<td>20</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>10</td>
<td>20</td>
<td>10</td>
<td>20</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

The antioxidant activity of the three extracts was evaluated by DPPH (Table I). The ED$_{50}$ is the concentration that inhibits half of the DPPH present; therefore the lower this concentration, the higher the activity of the extract. Although all extracts showed some antioxidant activity, the ether extract showed the lowest activity and the water extract showed the highest antioxidant activity (which was not significantly different from the ethanol extract). The ED$_{50}$ of the positive control, ascorbic acid, was 4 μg/mL. Surprisingly, no other studies were found analyzing the antioxidant activity of *guaco* extracts. This activity is important for patients who are suffering from inflammatory and infectious diseases (such as colds and bronchitis) where endogenous free radicals are released and could be accessory to the recovery from such illnesses (Bianchi and Antunes, 1999). Therefore this antioxidant activity could possibly play an important role in the anti-inflammatory, anti-ophidian and anti-allergic effects of *guaco*.

The *Artemia salina* assay determined the cytotoxicity of the three extracts of *M. laevigata* using the immobility parameter (Figure 2). The water extract, even at the maximum concentration of 20 mg/mL, showed no toxic effect on the shrimp, and therefore the LC$_{50}$ could not be determined. The results are presented in LC$_{50}$, which is the concentration of the extract that causes immobility or death of 50% of the shrimp (Table 1), therefore the higher this concentration, the less toxic the extract. The ether extract was less toxic than the ethanol extract, although both of them were conspicuously more toxic than the water extract.

![Figure 2. Immobility in percent of *Artemia salina* incubated during 24h in three different extracts of *M. laevigata*:ethanolic extract (○),water extract (▲) and ether extract (■) in mg/mL.](image-url)
CONCLUSION

The solvent used affected the concentration of coumarin in the extracts of guaco and their activity. Both the ethanol and water extracts contained the highest concentration of coumarin, confirming their use as bronchodilators. The ethanol extract was also slightly more active against E. coli than the other extracts, but it was the most cytotoxic and had intermediate antioxidant activity. The water extract also had the highest antioxidant activity and the lowest cytotoxicity, suggesting that it is both safe and healthy to use. However further studies of the toxicity of all the extracts should be made. The antimicrobial effect of the water extract was similar to the ether extract. Ether extracted the less polar components of the leaves and a lower concentration of coumarin. It is also the least antioxidant but slightly less cytotoxic than the ethanol extract. These results indicate that the less polar components of M. laevigata, are apparently less active for the activities assayed herein. The strong antioxidant activity of the water extract and its low cytotoxicity indicate that its components should be further studied.

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REFERENCES

Duarte MCT, Figueira GM, Pereira BA, Magalhães PM, Delamelina C. Atividade antimicrobiana de extratos hidro-alcôlicos de espécies da coleção de plantas medicinais CPQBA/UNICAMP. Rev. Bras. Farmacogn. 2004;14:6-8.
Moraes MD. A família Asteraceae na planície litorânea de Picinguaba - Municipio de Ubatuba


