Antimicrobial activity of *Aspilia latissima* (Asteraceae)

Jeana M.E. Souza¹, Marilene R. Chang², Daniela Z. Brito², Katuye S. Farias¹, Geraldo A. Damasceno-Junior³, Izabel C.C. Turatti⁴, Norberto P. Lopes⁴, Edson A. Santos⁵, Carlos A. Carollo¹

¹Laboratório de Produtos Naturais e Espectrometria de Massas, Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brazil.  
²Laboratório de Pesquisa em Microbiologia, Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brazil.  
³Laboratório de Botânica, Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brazil.  
⁴Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil.  
⁵Coordenação do Curso de Licenciatura em Química, Universidade Tecnológica Federal do Paraná, Apucarana, PR, Brazil.

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Abstract

We evaluated the antimicrobial activity of *Aspilia latissima* - an abundant plant from the Brazilian Pantanal region - against *Candida albicans*, *Candida parapsilosis*, *Candida krusei*, *Candida tropicalis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Escherichia coli* and *Staphylococcus aureus*. The crude extracts and fractions showed activity in all tested microorganisms. The chloroform fraction of the leaves and roots showed the most antimicrobial activity against *S. aureus*, with an MIC of 500 μg/mL. This fraction was submitted to bioautographic assays to characterize the activity of the compounds. Two bands from the leaves (L-A and L-B) and three bands from the roots (R-C, R-D and R-E) were bioactive. Within the root-derived bands, the terpene derivatives stigmasteryl, kaurenoic acid and kaura-9(11), 16-dien-18-oic acid were identified. Antibiotic activity of *A. latissima* is reported for the first time.

Key words: *Aspilia latissima*, *Staphylococcus aureus*, bioautographic, MIC, terpenes.

Introduction

Drugs derived from natural products have made and continue to make huge contributions to human health. Indeed, they have been part of folk medicine for thousands of years (Kingston, 2011; Ortholand and Ganesan, 2004).

Natural products provide a diverse source of bioactive compounds, with an estimated 25% to 50% of marketed drugs having been obtained from natural sources (Newman and Cragg, 2012). This proportion increases to about two-thirds for antibacterial treatments, showing the importance of this class to infectious disease drug discovery (Roemer et al., 2011). According to Mishra and Tiwari (2011), the treatment of infectious diseases with natural products is efficient due to the ability of natural products to interact with specific targets within cells. Newman and Cragg (2012) showed that the pharmaceutical industry remains focused on treatments for infectious diseases, including microbial, parasitic and viral infections.

This interest in identifying new antimicrobial agents is due to the emergence of resistant microorganisms, which are a major problem in hospitals, particularly in intensive care units (ICUs). ICU patients are exposed to various antimicrobial agents, and this exposure provides a great opportunity for the co-transmission of resistant bacteria from patient to patient (Rice, 2009).

There are approximately 270,000 species of plants all over the world, and Brazil is considered to have the greatest
plant diversity (14%) (Lewinsohn, 2006; Peixoto and Morin, 2003). Among the local flora, there are a large number of species used in folk medicine as antimicrobial treatments, with some species belonging to the Asteraceae family. The Aspilia genus (Asteraceae) exhibited such biological activities as antibacterial (Ambrosio et al., 2008) and antifungal (Yongabi et al., 2009) effects, attributed to the presence of kaurane-type diterpenoids and sesquiterpene lactones (Bohllmann et al., 1981; Page et al., 1992). Aspilia latissima, one of the species of the Aspilia genus, is a semi-aquatic bush that grows in abundance on the banks of the Paraguay River in the Brazilian Pantanal region. The chemical or biological properties of this plant have not been studied yet. Given the emphasis on discovering new drugs, especially antibiotics, from plants, the present work focused on evaluating the antimicrobial activity of A. latissima and the chemical composition underlying those effects.

Material and Methods

Plant material

Plant material (leaves and roots) was collected in March 2012 from adult A. latissima plants on the banks of the Paraguay River, Corumbá, MS, Brazil (19°34’36”, S 5°7’11” O), with license from the Brazilian CGEN (n° 246/2009). The plant was identified by Prof. Dr. Geraldo Alves Damasceno Jr., and a voucher specimen was deposited at the GCMS Herbarium as number 5173.

Preparation of plant extracts

The dried leaves (430 g) were pulverized and percolated (ethanol:water 7:3) at 20 drops/min for 96 h. The percolate was concentrated to obtain 190.06 g (44.2% yield) of the crude extract.

The dried roots (31 g) were extracted using a pressurized fluid extractor (ASE 150, Dionex) using ethanol:H2O (7:3) as the extraction solvent. The program consisted of 5 min of static time, a temperature of 100 °C, a washing volume of 60% and a purging time of 100 s in two cycles on the 100 mL extraction cell. The extract was concentrated to obtain 7.21 g (yield 23.25%) of the crude extract.

The crude extracts from the leaves and roots were fractionated using the ASE extractor. The plant extract was adsorbed on 21 g of silica gel (70-230 mesh, Sigma, St. Louis, MO, USA), and a chromatographic column was prepared on the 100-mL extraction cell, with silica gel (22 g) in the bottom half and the adsorbed extract in the upper half. The solvent gradient used was hexane, chloroform, ethyl acetate, ethanol and ethanol:H2O (7:3). In the fractionation process, a 5-min static program was used, with a temperature of 100 °C, a wash volume of 60% and a purge time of 100 s in two cycles. The fractions were concentrated and the yields calculated (Table 1).

Table 1 - Fractions obtained from crude extracts (leaves and roots) and yields.

| Fraction                      | Code       | Yield (%) |
|-------------------------------|------------|-----------|
| Leaves – hexane fraction      | L-HX       | 4.72      |
| Leaves – chloroform fraction  | L-CHCl3    | 3.32      |
| Leaves – ethyl acetate fraction | L-EtAc    | 4.72      |
| Leaves – ethanol fraction     | L-EtOH     | 61.2      |
| Leaves – ethanol:water fraction | L-EtOH:H2O | 26.04   |
| Roots – hexane fraction       | R-HX       | 0.22      |
| Roots – chloroform fraction   | R-CHCl3    | 0.43      |
| Roots – ethyl acetate fraction | R-EtAc    | 4.52      |
| Roots – ethanol fraction      | R-EtOH     | 63.87     |
| Roots – ethanol:water fraction | R-EtOH:H2O | 30.96   |

Antimicrobial activity assay

The following strains from the American Type Culture Collection (ATCC) were used for the antifungal and antibacterial evaluations: Candida albicans (ATCC 90028), Candida parapsilosis (ATCC 22019), Candida krusei (ATCC 6258), Candida tropicalis (ATCC 750), Pseudomonas aeruginosa (ATCC 9027), Enterococcus faecalis (ATCC 29212), Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 25923), which were kindly provided by the Adolfo Lutz Institute-SP. The microorganisms were maintained in BH1 (brain heart infusion) broth with 15% glycerol to -20 °C. The tests were conducted from 24-h subcultures at 35 °C (± 2 °C) in Sabouraud Dextrose agar for fungi and Muller Hinton agar for bacteria.

Disc diffusion method

The evaluation of the in vitro antibacterial and antifungal activity was performed using the disk diffusion method and following the guidelines of the Clinical and Laboratory Standards Institute in Doc. M44-A2 of the CLSI (2009) and M2-A8 of the NCCLS (2003). For the crude hydroethanolic extracts, two concentrations were used: 10 mg/mL and 40 mg/mL. For the fractions, only the 40 mg/mL concentration was used. The spherical discs were impregnated with 25 μL, resulting in final concentrations of 250 μg/disc (10 mg/mL) and 1,000 μg/disc (40 mg/mL). As a negative growth control, discs impregnated with dimethylsulfoxide (DMSO) were used. As a reference, discs containing antimycotic (fluconazole, 25 μg) and antibacterial (ceftazidime, 30 μg; oxacillin, 1 μg; and vancomycin, 30 μg) controls were used. The assays were performed in triplicate; the presence of a halo was considered indicative of bioactivity. The diameter of inhibition zones were measured with a caliper.
Broth microdilution assay

The fractions with the highest activity in the disk diffusion were tested for their minimum inhibitory concentration (MIC) using microbroth dilution, following the guidelines of the Clinical and Laboratory Standards Institute in Doc. M27-A3 of the CLSI (2008) for fungi and M7-A6 of the NCCLS (2003) for bacteria.

The plant fractions were tested at final concentrations of 2,000, 1,000, 500, 250, 125, 62.5, 31.25, 15.125 and 7.8125 μg/mL. The interpretation of the tests was performed by visual inspection after 24 h at 35 ± 2 °C for bacteria and 48 h at 35 ± 2 °C for fungi. To better visualize the growth of the micro-organisms, 30 μL of resazurin solution were added to the cultures (100 μg/mL) and incubated for 2 h at the same temperature.

Bioautography

Bioautography was performed using S. aureus for those A. latissima leaf and root fractions that showed low MICs, using the methodology described by Valgas et al. (2007) and Rahalison (1991). The thin layer chromatography (TLC) plates were eluted with CHCl₃:EtAc (8:2) and dried completely before the experiment was conducted. The TLC plates were developed with a solution of p-iodonitrotetrazolium violet (INT) (2 mg/mL) and incubated for 2 h. The experiment was performed in duplicate. After incubation, inhibition zones were made visible (as clear halos).

Isolation and characterization of compounds

The fractions L-CHCl₃ and R-CHCl₃ were submitted to TLC on glass plates (20 cm x 20 cm) prepared with silica 60GF254, with 150 mg divided among three plates for each fraction were applied. The TLC plates were eluted using CHCl₃:EtAc (8:2). The extraction was monitored by UV light at 257/365 nm. The bands corresponding to the compounds were removed from the plates and extracted with EtAc and MeOH. The preparative plates revealed five bands for L-CHCl₃ and six bands for R-CHCl₃. Bioautography showed inhibition zones in two of the bands for L-CHCl₃ and three of the bands for R-CHCl₃. The band R-C and R-E were identified as kaurenoic acid and acid kaura-9(11), 16-dien-18-oic (Figure 1).

Gas chromatography–mass spectrometry (GC-MS)

Compounds from the active bands were subjected to analysis by GC-MS in a gas chromatograph (GC-MS-QP-2010, Shimadzu) with an AOC-20i autoinjector, a DB-5MS column (30 m x 0.25 mm x 0.25 μm) using helium as carrier gas, a temperature injector at 250 °C and an injected sample volume of 1 μL. We used the following temperature program: 200 °C for 10 min and 290 °C for 35 min. The mass spectra were obtained by electron ionization at 70 eV.

Nuclear magnetic resonance (NMR)

One-dimensional NMR ¹H and ¹³C spectra were obtained using a Bruker DPX -300 (300/75 MHz) spectrometer and CDCl₃ as solvent.

Statistical analysis

±-Wallis/Dunn (p < 0.05) test was used as the data were not normally distributed (Gaussian). The GraphPad InStat computer program was used to perform the analyses.

Results

The anti-microbial activity of the crude extracts of A. latissima was studied using two concentrations, 10 and 40 mg/mL. The results summarized in Table 2 show that the growth inhibition for both concentrations is similar when tested against C. albicans, C. parapsilosis, P. aeruginosa, S. aureus and E. faecalis.

We observed from the results shown in Table 3 that all fractions (with the exception of R-EtAc) showed activity against E. faecalis. We also highlight the R-EtOH:H₂O as being the most effective against C. parapsilosis, with its halo of inhibition statistically similar to the positive control.
Table 2 - Antimicrobial activity of *A. latissima* crude extract obtained from leaves and roots (250 μg/disc, 1,000 μg/disc), evaluated by the disc diffusion assay. Inhibition Zone (mm).

| Samples         | Fungi  | Bacteria |
|-----------------|--------|----------|
|                 | Ca     | Cp       | Ct       | Ck       | Pa     | Sa     | Ef     | Ec     |
| DMSO            | NA     | NA       | NA       | NA       | NA     | NA     | NA     | NA     |
| FLU             | 32 ± 3<sup>h</sup> | 22 ± 3<sup>h</sup> | 27 ± 3<sup>h</sup> | 16 ± 2<sup>b</sup> | NA     | NA     | NA     | 29 ± 1<sup>b</sup> |
| CFT             | NA     | NA       | NA       | NA       | 23 ± 3<sup>h</sup> | NA     | NA     | NA     |
| OXA             | NA     | NA       | NA       | NA       | NA     | 25 ± 0<sup>b</sup> | NA     | NA     |
| VAN             | NA     | NA       | NA       | NA       | NA     | NA     | 22 ± 3<sup>b</sup> | NA     |
| Leaves 250 μg   | 8 ± 2<sup>b</sup> | 8 ± 1<sup>b</sup> | -       | -       | 8 ± 1<sup>b</sup> | 7 ± 1<sup>b</sup> | 7 ± 1<sup>b</sup> | -       |
| Leaves 1,000 μg | 11 ± 1<sup>b</sup> | 8 ± 1<sup>b</sup> | 10 ± 2<sup>b</sup> | 8 ± 1<sup>b</sup> | 8 ± 0<sup>b</sup> | 7 ± 0<sup>b</sup> | 8 ± 1<sup>b</sup> | -       |
| Roots 250 μg    | 9 ± 1<sup>b</sup> | 9 ± 1<sup>b</sup> | 10 ± 1<sup>b</sup> | 10 ± 0<sup>b</sup> | 8 ± 1<sup>b</sup> | 8 ± 1<sup>b</sup> | 9 ± 0<sup>b</sup> | 7 ± 1<sup>b</sup> |
| Roots 1,000 μg  | 10 ± 2<sup>b</sup> | 8 ± 0<sup>b</sup> | 11 ± 1<sup>b</sup> | 11 ± 1<sup>b</sup> | 10 ± 1<sup>b</sup> | 11 ± 1<sup>b</sup> | 11 ± 0<sup>b</sup> | 9 ± 1<sup>b</sup> |

NA: Not applicable; Statistical Test: Kruskal-Wallis/Dunn (p < 0.05); Different letters indicate statistically significant differences, with comparisons made in a column-wise fashion. Ca: *C. albicans*; Cp: *C. parapsilosis*; Ct: *C. tropicalis*; Ck: *C. krusei*; Pa: *P. aeruginosa*; Ec: *E. coli*; Sa: *S. aureus*; Ef: *E. faecalis*. FLU: flucloxazole (25 μg), CFT: cefetazidime (30 μg), OXA: oxacillin (1 μg) and VAN: vancomycin (30 μg).

Table 3 - Antimicrobial activity of *A. latissima* fractions (CHCl<sub>3</sub>, EtAc, EtOH and EtOH:H<sub>2</sub>O) obtained from crude extract of leaves and roots (1,000 μg/disc), evaluated by the disc diffusion assay. Inhibition Zone (mm).

| Samples         | Fungi  | Bacteria |
|-----------------|--------|----------|
|                 | Ca     | Cp       | Ct       | Ck       | Pa     | Sa     | Ef     | Ec     |
| DMSO            | NA     | NA       | NA       | NA       | NA     | NA     | NA     | NA     |
| FLU             | 32 ± 3<sup>h</sup> | 25 ± 0<sup>b</sup> | 28 ± 3<sup>h</sup> | 16 ± 2<sup>b</sup> | NA     | NA     | NA     | NA     |
| CFT             | NA     | NA       | NA       | NA       | 24 ± 1<sup>b</sup> | NA     | NA     | 29 ± 1<sup>b</sup> |
| OXA             | NA     | NA       | NA       | NA       | NA     | 25 ± 0<sup>b</sup> | NA     | NA     |
| VAN             | NA     | NA       | NA       | NA       | NA     | NA     | 21 ± 1<sup>b</sup> | NA     |
| L-HX            | 9 ± 1<sup>b</sup> | 10 ± 0<sup>b</sup> | 13 ± 1<sup>b</sup> | 12 ± 0<sup>b</sup> | 10 ± 0<sup>b</sup> | 12 ± 1<sup>b</sup> | 11 ± 1<sup>b</sup> | 9 ± 0<sup>b</sup> |
| L-CHCl<sub>3</sub> | 8 ± 1<sup>b</sup> | 10 ± 0<sup>b</sup> | 11 ± 0<sup>b</sup> | 11 ± 2<sup>b</sup> | 9 ± 1<sup>b</sup> | 15 ± 0<sup>b</sup> | 10 ± 1<sup>b</sup> | 9 ± 1<sup>b</sup> |
| L-EtAc          | -      | -        | 10 ± 0<sup>b</sup> | 10 ± 0<sup>b</sup> | -      | -      | 10 ± 1<sup>b</sup> | -      |
| L-EtOH          | 11 ± 1<sup>b</sup> | 9 ± 1<sup>b</sup> | 10 ± 1<sup>b</sup> | 10 ± 0<sup>b</sup> | -      | -      | 11 ± 0<sup>b</sup> | 8 ± 1<sup>b</sup> |
| L-EtOH:H<sub>2</sub>O | 10 ± 0<sup>b</sup> | -      | 10 ± 1<sup>b</sup> | 8 ± 0<sup>b</sup> | -      | -      | 9 ± 1<sup>b</sup> | -      |
| R-CHCl<sub>3</sub> | 10 ± 0<sup>b</sup> | -      | 8 ± 1<sup>b</sup> | 10 ± 0<sup>b</sup> | 10 ± 1<sup>b</sup> | 20 ± 1<sup>b</sup> | 10 ± 1<sup>b</sup> | 9 ± 1<sup>b</sup> |
| R-EtAc          | -      | -        | 10 ± 0<sup>b</sup> | -      | 9 ± 1<sup>b</sup> | 15 ± 1<sup>b</sup> | 7 ± 1<sup>b</sup> | 10 ± 1<sup>b</sup> |
| R-EtOH          | 9 ± 1<sup>b</sup> | 9 ± 1<sup>b</sup> | -      | 10 ± 2<sup>b</sup> | 10 ± 0<sup>b</sup> | -      | 11 ± 1<sup>b</sup> | -      |
| R-EtOH:H<sub>2</sub>O | 10 ± 1<sup>b</sup> | 11 ± 1<sup>b</sup> | 10 ± 1<sup>b</sup> | 10 ± 2<sup>b</sup> | 10 ± 1<sup>b</sup> | -      | 11 ± 0<sup>b</sup> | -      |

NA: Not applicable; Statistical Test: Kruskal-Wallis/Dunn (p < 0.05); Different letters indicate statistically significant differences, with comparisons made in a column-wise fashion. Ca: *C. albicans*; Cp: *C. parapsilosis*; Ct: *C. tropicalis*; Ck: *C. krusei*; Pa: *P. aeruginosa*; Ec: *E. coli*; Sa: *S. aureus*; Ef: *E. faecalis*. FLU: flucloxazole (25 μg), CFT: cefetazidime (30 μg), OXA: oxacillin (1 μg) and VAN: vancomycin (30 μg).

Inhibition. We noted that the activities of L-CHCl<sub>3</sub> and R-CHCl<sub>3</sub>, which caused halos of 15 ± 0 mm and 20 ± 1 mm, respectively, were closer to the activity of the positive control (oxacillin), which caused a halo of 25 mm. The fractions causing inhibition halos for all fungi (L-HX, L-CHCl<sub>3</sub>, L-EtAc and R-EtOH:H<sub>2</sub>O) and bacteria (L-HX, L-CHCl<sub>3</sub>, R- CHCl<sub>3</sub> and R-EtAc) were chosen for determining MICs. The only fractions that showed a significant MIC were L-CHCl<sub>3</sub> and R-CHCl<sub>3</sub>, with an MIC of 500 μg/mL when tested against *S. aureus*.

Bioautography was used to characterize the compounds within L-CHCl<sub>3</sub> and R-CHCl<sub>3</sub> that contributed to the antimicrobial activity against *S. aureus*. In Table 4 and Figure 2, we summarize and show the antimicrobial activity of the isolated bands.
**Table 4** - Secondary metabolites from L-CHCl₃ and R-CHCl₃ activity against *S. aureus* observed by bioautography test.

| L-CHCl₃ Activity | Compounds                          | R-CHCl₃ Activity | Compounds                          |
|------------------|-----------------------------------|------------------|-----------------------------------|
| L-A +            | n-Hexadecanoic acid               | R-A -            | NI                                |
| L-B +            | Stigmasterol kaur-9(11), 16-dien-18-oic kaurenoic acid | R-B - | NI |
| L-C -            | NI                                | R-C +            | kaurenoic acid and acid kaur-9(11), 16-dien-18-oic |
| L-D -            | NI                                | R-D +            | Stigmasterol kaurenoic acid kaur-9(11), 16-dien-18-oic |
| L-E -            | NI                                | R-E +            | kaurenoic acid kaur-9(11), 16-dien-18-oic acid |
|                  |                                   | R-F -            | NI                                |

+ Positive; - Negative; NI = Not Identified.

**Figure 2** - Bioautography plates of L-CHCl₃ and R-CHCl₃ against *S. aureus*, stained with INT (2 mg/mL). L-A = n-hexadecanoic acid, L-B = kaur-9(11), 16-dien-18-oic, R-C = mixture of kaurenoic acid and kaur-9(11), 16-dien-18-oic acid, R-D = stigmasterol, R-E = mixture of kaurenoic acid and kaur-9(11), 16-dien-18-oic acid.
The bioactive bands isolated by TLC were sent for GC-MS analysis. The compound L-A was characterized as \( n \)-Hexadecanoic acid, with a molecular formula of \( C_{16}H_{32}O_2 \) and an \( m/z \) of 256 [M–].

Another bioactive band from the leaf extract was L-B, the major compound in which, representing 46% of the area on the chromatogram, was the steroidal stigmasterol, having a molecular formula of \( C_{29}H_{48}O \) and an \( m/z \) of 412 [M–]. Other compounds identified in L-B were the acid kaura-9(11), 16-dien-18-oic (\( C_{20}H_{30}O_2 \)) and kaurenoic acid (\( C_{20}H_{30}O_2 \)), with \( m/z \) ratios of 302 [M–] and 300 [M–], respectively.

In the root extracts, three bioactive bands were found. The band R-C and R-E were identified as kaurenoic acid and acid kaura-9(11), 16-dien-18-oic, each representing different areas of the chromatogram: kaurenoic acid from R-C accounted for 45.45% of the area, and kaura-9(11), 16-dien-18-oic acid from R-E accounted for 67.92% of the area; kaura-9(11), 16-dien-18-oic acid from R-C accounted for 43.78% of the area; kaura-9(11), 16-dien-18-oic and kaurenoic acid were characterized by \( ^1H \)-NMR and \( ^13C \)-NMR, and the main classes of antibiotics (Rana et al., 2012; Yagi et al., 2012). Better results of the disk diffusion test were obtained for L-CHCl\(_3\) and R-CHCl\(_3\) against \( S. aureus \) and, in previous work, plants containing this fatty acid contributed to its antibacterial activity against \( S. aureus \) and other Gram-positive bacteria (Keawsa-ard et al., 2012; Pasdaran et al., 2012; Yagi et al., 2012).

### Discussion

We observed in the disk diffusion screening test that the crude extracts were active against all tested micro-organisms and were considered contain potential antimicrobial compounds (Table 2). Those results suggested promising activity against the tested micro-organisms.

All of the fractions from the crude extract (except R-EtAc) showed activity against \( C. krusei \), which we considered an important result because the intrinsic resistance of this fungus to fluconazole is a serious problem in the treatment of candidemias (Scorzoni et al., 2013). Another prominent result was that effectiveness of the R-EtOH:H\(_2\)O against \( C. parapsilosis \), one of pathogens responsible for nosocomial infections, with its activity being statistically similar to that of the positive control.

For \( E. faecalis \), all tested fractions were active. This bacterium resides in the human intestine and is a principal cause of serious nosocomial infections, which are difficult to manage due to the intrinsic and acquired resistance to the main classes of antibiotics (Rana et al., 2013).

Confirming the results of the disk diffusion test, the MICs of L-CHCl\(_3\) and R-CHCl\(_3\) against \( S. aureus \) were the only ones with an interesting activity, given the article of Rios and Recio (2005) that suggests that extracts with MIC values above 1,000 \( \mu g/mL \) should be discarded.

**Table 5**: \( ^1H \)-NMR (δ) data for kaurenoic and kaura-9(11), 16-dien-18-oic, (4.alpha.) acids.

|   | kaurenoic acid | kaura-9(11), 16-dien-18-oic acid |
|---|----------------|---------------------------------|
| H | R-C\(^a\)       | R-D\(^a\)                       | R-E\(^a\)                  | (Batista et al., 2005)\(^b\) |
| 11 | -               | -                               | -                           | -                           |
| 13 | 2.61 (1H, sl)  | 2.61 (1H, sl)                   | 2.61 (1H, sl)               | 2.64 (1H, m)                |
| 17a| 4.72 (1H, s)  | 4.72 (1H, s)                    | 4.72 (1H, s)                | 4.73 (1H, s)                |
| 17b| 4.78 (1H, s)  | 4.78 (1H, s)                    | 4.78 (1H, s)                | 4.79 (1H, s)                |
| 18 | 1.22 (3H, s)  | 1.21 (3H, s)                    | 1.22 (3H, s)                | 1.24 (3H, s)                |
| 20 | 0.95 (3H, s)  | 0.92 (3H, s)                    | 0.95 (3H, s)                | 0.95 (3H, s)                |

\(^a\)300 MHz, CDCl\(_3\); \(^b\)50 MHz, CDCl\(_3\).
In L-CHCl₃, stigmasterol, acid kaura-9(11), 16-dien-18-oic, (4.alpha.) and kaurenoic acid (L-B) were identified. Stigmasterol also contributed to antibacterial activity against *S. aureus* (Guilhon et al., 2012; Yagi et al., 2012). Diterpenes with a kaurene skeleton are known for their antimicrobial activity. In the study by Pereira et al. (2012), kaurenoic acid showed significant activity against *S. aureus*. Indeed, Okoye et al. (2012) demonstrated that the antibacterial activity of *Annona senegalensis* is due to kaurenoic acid. The compounds identified in this work have other studies and demonstrate a wide variety of biological activities, for example kaurenoic acid is found in several medicinal plants such as Copaifera (Paiva et al., 2002). This compound in addition to the antimicrobial activity presented and discussed here has a great potential as a new drug. Among the studies, kaurenoic acid demonstrated strong antileishmanial activity (IC₅₀ values of 4.0 μg/mL) (Santos et al., 2013) antimalarial activity (Batista et al., 2013) anti-inflammatory and antinociceptive effect (Mizokami et al., 2012). The occurrence of active secondary metabolites in this species, allows its application not only as a possible antimicrobial agent, but also with a new source of these compounds.

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The present study demonstrated that crude extracts of the roots and leaves of *A. latissima* showed antimicrobial activity against all tested microorganisms. Through the identification of the active compounds by bioautography, we identified the same compounds in the leaves and roots, with the presence of stigmasterol in L-B and R-D; kaurenoic acid in L-B, R-C, R-D and R-E; and kaura-9(11), 16-dien-18-oic acid in R-C, R-D and R-E. These results show that these compounds accumulate in more than one part of the plant. In addition, we emphasize that bioautography is effective for isolating antimicrobial compounds, but the resolution is not high enough to achieve complete compound separation.

Finally, we conclude that the accumulation of the same compounds in the leaves and roots explains the similar antibacterial activity of leaf and root extracts, and the presence of steroids and kaurene diterpenes are responsible for the bacteriostatic activity of the extracts and fractions. Moreover, this study contributed to the identification of some secondary metabolites in this plant species.

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**Table 6** - $^{13}$C-NMR (δ) data for kaurenoic and kaura-9(11), 16-dien-18-oic, (4.alpha.) acids.

| C  | kaurenoic acid | kaura-9(11), 16-dien-18-oic (4.alpha.) acid |
|----|----------------|------------------------------------------|
|    | R-D⁹ | R-E⁹ | (Batista et al., 2005)⁹ | R-D⁹ | R-E⁹ | (Batista et al., 2005)⁹ |
| 1  | 40.71 | 40.69 | 40.7 | 40.71 | 40.69 | 40.7 |
| 2  | 19.09 | 19.08 | 19.1 | 20.16 | 20.14 | 20.1 |
| 3  | 37.91 | 37.66 | 37.7 | 38.35 | 38.25 | 38.2 |
| 4  | 43.65 | 43.67 | 43.2 | 44.65 | 44.70 | 44.7 |
| 5  | 57.01 | 57.04 | 57.1 | 46.54 | 46.57 | 46.6 |
| 6  | 21.82 | 21.82 | 21.8 | 18.42 | 18.42 | 18.4 |
| 7  | 41.27 | 41.27 | 41.3 | 29.66 | 29.69 | 29.6 |
| 8  | 44.22 | 44.22 | 44.2 | 42.29 | 42.54 | 42.2 |
| 9  | 55.10 | 55.09 | 55.1 | 155.90 | 155.89 | 155.9 |
| 10 | 39.68 | 39.69 | 39.7 | 38.78 | 38.79 | 38.8 |
| 11 | 18.48 | 18.42 | 18.4 | 114.86 | 114.87 | 114.9 |
| 12 | 33.10 | 33.10 | 33.1 | 37.91 | 37.91 | 37.9 |
| 13 | 43.84 | 43.84 | 43.8 | 41.27 | 41.23 | 41.2 |
| 14 | 39.64 | 39.65 | 39.7 | 44.93 | 44.93 | 44.9 |
| 15 | 48.95 | 48.95 | 48.9 | 50.31 | 50.29 | 50.3 |
| 16 | 155.90 | 155.89 | 155.9 | 158.56 | 158.56 | 158.5 |
| 17 | 102.97 | 102.98 | 103.0 | 105.45 | 105.47 | 105.5 |
| 18 | 28.95 | 28.96 | 29.0 | 28.24 | 28.23 | 28.2 |
| 19 | 182.55 | 184.2 | 184.8 | 182.55 | 184.2 | 184.7 |
| 20 | 15.62 | 15.59 | 15.6 | 23.63 | 23.59 | 23.6 |

⁹75 MHz, CDCl₃; ⁵⁰ MHz, CDCl₃.
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