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Research Paper

Antimicrobial activity of *Aspilia latissima* (Asteraceae)

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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 stigmasterol, 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

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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 (Bohlmann *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 57°1'11" 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:H_2O (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:H_2O (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-$CHCl_3$	3.32
Leaves – ethyl acetate fraction	L-EtAc	4.72
Leaves – ethanol fraction	L-EtOH	61.2
Leaves – ethanol:water fraction	L-EtOH:H_2O	26.04
Roots – hexane fraction	R-HX	0.22
Roots – chloroform fraction	R-$CHCl_3$	0.43
Roots – ethyl acetate fraction	R-EtAc	4.52
Roots – ethanol fraction	R-EtOH	63.87
Roots – ethanol:water fraction	R-EtOH:H_2O	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 BHI (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 $CHCl_3$: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-$CHCl_3$ and R-$CHCl_3$ were submitted to TLC on glass plates (20 cm x 20 cm) prepared with silica $60G_{F254}$, with 150 mg divided among three plates for each fraction were applied. The TLC plates were eluted using $CHCl_3$: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-$CHCl_3$ and six bands for R-$CHCl_3$. Bioautography showed inhibition zones in two of the bands for L-$CHCl_3$ and three of the bands for R-$CHCl_3$. 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 ^{13}C spectra were obtained using a Bruker DPX -300 (300/75 MHz) spectrometer and $CDCl_3$ 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.

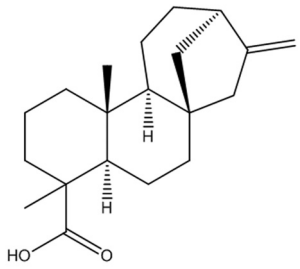
Results

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*.

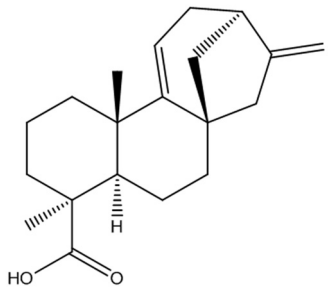
The two test concentrations of the root extract inhibited the growth of *C. tropicalis* and *C. krusei*. For those micro-organisms, the leaf extract showed activity only at the higher concentration, so it was considered dose-dependent. Only the root material showed activity against *E. coli*.

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:H_2O as being the most effective against *C. parapsilosis,* with its halo of inhibition statistically similar to the positive control.

We observed in Table 3 that the inhibition halos of the active fractions against *S. aureus* showed a broad spectrum



kaurenoic acid



kaura-9(11),16-dien-18-oic acid

Figure 1 - Diterpenes identified in *Aspilia latissima*.

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	-	-	-	-	-	-	-	-
FLU	32 ± 3^{b}	22 ± 3^{b}	27 ± 3^{b}	16 ± 2^{b}	NA	NA	NA	29 ± 1^{b}
CFT	NA	NA	NA	NA	23 ± 3^{b}	NA	NA	NA
OXA	NA	NA	NA	NA	NA	$25. \pm 0^{b}$	NA	NA
VAN	NA	NA	NA	NA	NA	NA	22 ± 3^{b}	NA
Leaves 250 μg	$8 \pm 2^{a.b}$	$8 \pm 1^{a.b}$	-	-	$8 \pm 1^{a.b}$	$7 \pm 1^{a.b}$	$7 \pm 1^{a.b}$	-
Leaves 1,000 μg	$11 \pm 1^{a.b}$	$8 \pm 1^{a.b}$	$10 \pm 2^{a.b}$	$8 \pm 1^{a.b}$	$8 \pm 0^{a.b}$	$7 \pm 0^{a.b}$	$8 \pm 1^{a.b}$	-
Roots 250 μg	$9 \pm 1^{a.b}$	$9 \pm 1^{a.b}$	$10 \pm 1^{a.b}$	$10 \pm 0^{a.b}$	$8 \pm 1^{a.b}$	$8 \pm 1^{a.b}$	$9 \pm 0^{a.b}$	$7 \pm 1^{a.b}$
Roots 1,000 μg	$10 \pm 2^{a.b}$	$8 \pm 0^{.b}$	$11 \pm 1^{a.b}$	$11 \pm 1^{a.b}$	$10 \pm 1^{a.b}$	$11 \pm 1^{a.b}$	$11 \pm 0^{a.b}$	$9 \pm 1^{a.b}$

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: fluconazole (25 μg), CFT: ceftazidime (30 μg), OXA: oxacillin (1 μg) and VAN: vancomycin (30 μg).

Table 3 - Antimicrobial activity of *A. latissima* fractions ($CHCl_3$, EtAc, EtOH and EtOH:H2O) 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	-	-	-	-	-	-	-	-
FLU	32 ± 3^{b}	25 ± 0^{b}	$28 \pm 3^{b.c}$	16 ± 2^{b}	NA	NA	NA	NA
CFT	NA	NA	NA	NA	24 ± 1^{b}	NA	NA	29 ± 1^{b}
OXA	NA	NA	NA	NA	NA	25 ± 0^{b}	NA	NA
VAN	NA	NA	NA	NA	NA	NA	21 ± 1^{b}	NA
L-HX	$9 \pm 1^{a.b}$	$10 \pm 0^{a.b}$	13 ± 1^{b}	$12 \pm 0^{a.b}$	$10 \pm 0^{a.b}$	$12 \pm 1^{a.b}$	$11 \pm 1^{a.b}$	$9 \pm 0^{a.b}$
L-$CHCl_3$	$8 \pm 1^{a.b}$	$10 \pm 0^{a.b}$	$11 \pm 0^{a.b}$	$11 \pm 2^{a.b}$	$9 \pm 1^{a.b}$	$15 \pm 0^{a.b}$	$10 \pm 1^{a.b}$	$9 \pm 1^{a.b}$
L-EtAc	-	-	$10 \pm 0^{a.b}$	$10 \pm 0^{a.b}$	-	-	$10 \pm 1^{a.b}$	-
L-EtOH	$11 \pm 1^{.b}$	$9 \pm 1^{a.b}$	$10 \pm 1^{a.b}$	$10 \pm 0^{a.b}$	-	-	$11 \pm 0^{a.b}$	$8 \pm 1^{a.b}$
L-EtOH:H_2O	$10 \pm 0^{a.b}$	-	$10 \pm 1^{a.b}$	$8 \pm 0^{a.b}$	-	-	$9 \pm 1^{a.b}$	-
R-$CHCl_3$	-	-	$8 \pm 1^{a.b}$	$10 \pm 0^{a.b}$	$10 \pm 1^{a.b}$	$20 \pm 1^{a.b}$	$10 \pm 1^{a.b}$	$9 \pm 1^{a.b}$
R-EtAc	$10 \pm 0^{a.b}$	-	$10 \pm 0^{a.b}$	-	$9 \pm 1^{a.b}$	$15 \pm 1^{a.b}$	7 ± 1^{a}	$10 \pm 1^{a.b}$
R-EtOH	$9 \pm 1^{a.b}$	$9 \pm 1^{a.b}$	-	$10 \pm 2^{a.b}$	$10 \pm 0^{a.b}$	-	$11 \pm 1^{a.b}$	-
R-EtOH:H_2O	$10 \pm 1^{a.b}$	11 ± 1^{b}	$10 \pm 1^{a.b}$	$10 \pm 2^{a.b}$	$10 \pm 1^{a.b}$	-	$11 \pm 0^{a.b}$	-

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: fluconazole (25 μg), CFT: ceftazidime (30 μg), OXA: oxacillin (1 μg) and VAN: vancomycin (30 μg).

of inhibition. We noted that the activities of L-$CHCl_3$ and R-$CHCl_3$, 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-$CHCl_3$, L-EtAc and R-EtOH:H_2O) and bacteria (L-HX, L-$CHCl_3$, R- $CHCl_3$ and R-EtAc) were chosen for determining MICs. The only fractions that showed a significant MIC were L-$CHCl_3$ and R-$CHCl_3$, with an MIC of 500 μg/mL when tested against *S. aureus*.

Bioautography was used to characterize the compounds within L-$CHCl_3$ and R-$CHCl_3$ 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-$CHCl_3$ and R-$CHCl_3$ activity against *S. aureus* observed by bioautography test.

L-$CHCl_3$	Activity	Compounds	R-$CHCl_3$	Activity	Compounds
L-A	+	n-Hexadecanoic acid	R-A	-	NI
L-B	+	Stigmasterol kaura-9(11), 16-dien-18-oic kaurenoic acid	R-B	-	NI
L-C	-	NI	R-C	+	kaurenoic acid and acid kaura-9(11), 16-dien-18-oic
L-D	-	NI	R-D	+	Stigmasterol kaurenoic acid acid kaura-9(11), 16-dien-18-oic
L-E	-	NI	R-E	+	kaurenoic acid kaura-9(11), 16-dien-18-oic acid
			R-F	-	NI

+ Positive; - Negative; NI = Not Identified.

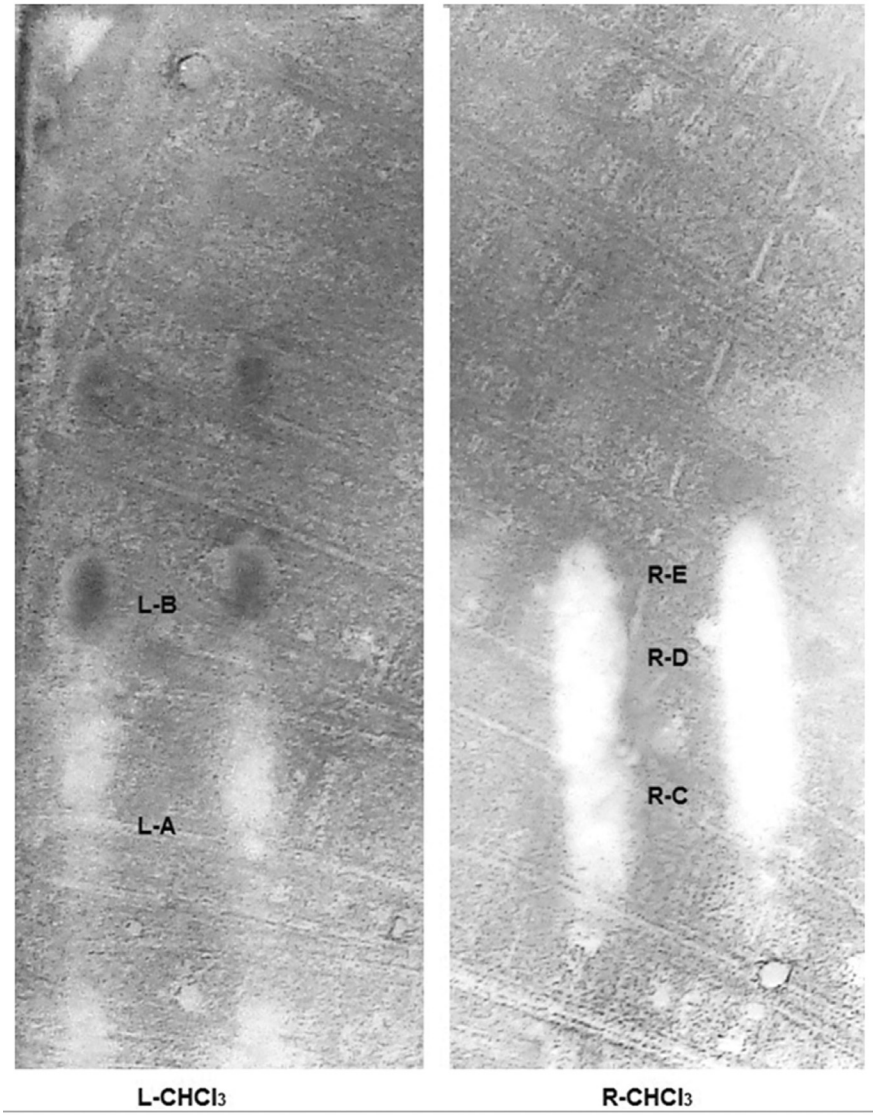


Figure 2 - Bioautography plates of L-CHCl3 and R-CHCl3 against *S. aureus*, stained with INT (2 mg/mL). **L-A**= *n*-hexadecanoic acid, **L-B**= kaura-9(11), 16-dien-18-oic, **R-C=** mixture of kaurenoic acid and kaura-9(11), 16-dien-18-oic acid, **R-D**= stigmasterol, **R-E**= mixture of kaurenoic acid and kaura-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 [$M^{\cdot+}$].

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 steroid stigmasterol, having a molecular formula of $C_{29}H_{48}O$ and an *m/z* of 412 [$M^{\cdot+}$]. Other compounds identified in L-B were the acid kaura-9(11), 16-dien-18-oic ($C_{20}H_{28}O_2$) and kaurenoic acid ($C_{20}H_{30}O_2$), with m/z ratios of 302 [$M^{\cdot+}$] and 300 [$M^{\cdot+}$], 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 kaurenoic 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, and kaura-9(11),16-dien-18-oic acid from R-E accounted for 16.97% of area. The major compound within R-D, representing 60% of the area of the chromatogram, was stigmasterol, with an MS *m/z* ratio of 412 [$M^{\cdot+}$].

The kaurenoic acid and kaura-9(11), 16-dien-18-oic acid were characterized by ^{1}H-NMR and ^{13}C-NMR, and the results were compared with literature data (Batista *et al.*, 2005). The results of that analysis are presented in Tables 5 and 6.

In the ^{13}C-NMR spectrum of R-E, we observed five signals of kaura-9(11), 16-dien-18-oic acid at δ = 105.47, δ = 114.87, δ = 155.89, δ = 158.56 and δ = 184.20.

For stigmasterol from R-D, we also observed five signals in the ^{13}C-NMR spectrum that were characteristic of its structure: δ = 140.74, δ = 138.3, δ = 129.27, δ = 121.71 and δ = 71.82. In kaurenoic acid from R-D, we observed in the ^{13}C-NMR spectrum three signals characteristic of its structure: δ = 182.55, δ = 155.9 and δ = 102.97.

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_2O 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)

Better results of the disk diffusion test were obtained for L-$CHCl_3$ and R-$CHCl_3$ against *S. aureus*, with an inhibition similar to that caused by the positive control. This bacterium is considered an important food pathogen, and it is the primary bacterium in human infections, some of which can be fatal (Kelsey *et al.*, 2006).

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 µg/mL should be discarded.

Bioautography was performed to identify antimicrobial compounds and simplify the isolation process (Rahalison *et al.*, 1991). The presence of *n*-hexadecanoic acid in L-A contributed to its antibacterial activity against *S. aureus* and, in previous work, plants containing this fatty acid had exhibited strong activity against *S. aureus* and other Gram-positive bacteria (Keawsa-ard *et al.*, 2012; Pasdaran *et al.*, 2012; Yagi *et al.*, 2012).

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

H	kaurenoic acid				kaura-9(11), 16-dien-18-oic acid			
	R-C[a]	R-D[a]	R-E[a]	(Batista *et al.*, 2005)[a]	R-C[a]	R-D[a]	R-E[a]	(Batista *et al.*, 2005)[b]
11	-	-	-	-	5.22 (1H)	5.22 (1H)	5.22 (1H)	5.24(1H)
13	2.61 (1H, sl)	2.61 (1H, sl)	2.61 (1H, sl)	2.64 (1H, m)	2.75 (1H, sl)	2.75 (1H, sl)	2.75 (1H, sl)	2.77(1H, s)
17a	4.72 (1H, s)	4.72 (1H, s)	4.72 (1H, s)	4.73(1H, s)	4.78 (1H, s)	4.78 (1H, s)	4.78 (1H, s)	4.79(1H, s)
17b	4.78 (1H, s)	4.78 (1H, s)	4.78 (1H, s)	4.79(1H, s)	4.89 (1H, s)	4.89 (1H, s)	4.89 (1H, s)	4.91(1H, s)
18	1.22 (3H, s)	1.21 (3H, s)	1.22 (3H, s)	1.24(3H, s)	1.23 (3H)	1.23 (3H)	1.23 (3H)	1.24(3H)
20	0.95 (3H, s)	0.92 (3H, s)	0.95 (3H, s)	0.95(3H, s)	1.02 (3H)	1.01 (3H)	1.02 (3H)	1.02(3H)

[a]300 MHz, $CDCl_3$; [b]50 MHz, $CDCl_3$.

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-D[a]	R-E[a]	(Batista *et al.*, 2005)[b]	R-D[a]	R-E[a]	(Batista *et al.*, 2005)[b]
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

[a]75 MHz, $CDCl_3$; [b]50 MHz, $CDCl_3$.

In L-$CHCl_3$, 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 (IC_{50} 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.

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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References

Ambrosio SR, Furtado NAJC, Martins CHG *et al.* (2008) Antimicrobial activity of kaurane diterpenes against oral pathogens. Z. Naturforsch. C: J Biosci 63:326-330.

Batista R, Braga FC, Oliveira AB (2005) Quantitative determination by HPLC of ent-kaurenoic and grandiflorenic acids in aerial parts of *Wedelia paludosa* D.C. Rev Bras Farmacogn 15:119-125.

Batista R, Garcia PA, Castro MA *et al.* (2013) Synthesis, cytotoxicity and antiplasmodial activity of novel ent-kaurane derivatives. Eur J Med Chem 62:168-176.

Berdy J (2005) Bioactive microbial metabolites - A personal view. J Antibiot 58:1-26.

Bohlmann F, Ziesche J, King RM *et al.* (1981) Naturally-occurring terpene derivatives .300. eudesmanolides and diterpenes from wedelia-trilobata and an ent-kaurenic acid-derivative from *Aspilia parvifolia*. Phytochemistry 20:751-756.

CLSI (2008) M27-A3: Reference method for broth dilution antifungal susceptibily testing of yeasts. Approved Standard, third edition, 28:25.

CLSI (2009) Publication M44-A2: Method for antifungal disk diffusion susceptibility testing of yeasts. Approved guideline, 29:22.

Guilhon GMSP, da Silva ES, Santos LD *et al.* (2012) Volatile and non-volatile compounds and antimicrobial activity of *Mansoa difficilis* (Cham.) Bureau & K. Schum. (Bignoniaceae). Quim Nova 35:2249-2253.

Keawsa-ard S, Liawruangrath B, Liawruangrath S *et al.* (2012) Chemical constituents and antioxidant and biological activities of the essential oil from leaves of *Solanum spirale*. Nat Prod Commun 7:955-958.

Kelsey JA, Bayles KW, Shafii B *et al.* (2006) Fatty acids and monoacylglycerols inhibit growth of *Staphylococcus aureus*. Lipids 41:951-961.

Kingston DGI (2011) Modern natural products drug discovery and its relevance to biodiversity conservation. J Nat Prod 74:496-511.

Lewinsohn TM (2006) Avaliação do estado do conhecimento da biodiversidade brasileira. Ministerio do Meio Ambiente 2:145-192.

Mishra BB, Tiwari VK (2011) Natural products: An evolving role in future drug discovery. Eur J Med Chem 46:4769-4807.

Mizokami SS, Arakawa NS, Ambrosio SR *et al.* (2012) Kaurenoic acid from *Sphagneticola trilobata* Inhibits Inflammatory Pain: effect on cytokine production and activation of the NO-cyclic GMP-protein kinase G-ATP-sensitive potassium channel signaling pathway. J Nat Prod 75:896-904.

NCCLS (2003) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard. Sixth Edition. NCCLS document M7-A6.

Newman DJ, Cragg GM (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 75:311-335.

Okoye TC, Akah PA, Okoli CO *et al.* (2012) Antimicrobial effects of a lipophilic fraction and kaurenoic acid isolated from the root bark extracts of *Annona senegalensis*. Evid-Based Compl Alt.

Ortholand JY, Ganesan A (2004) Natural products and combinatorial chemistry: back to the future. Curr Opin Chem Biol 8:271-280.

Page JE, Balza F, Nishida T *et al.* (1992) Biologically active diterpenes from *Aspilia mossambicensis*, a chimpanzee medicinal plant. Phytochemistry 31:3437-3439.

Paiva LA, Gurgel LA, Silva RM *et al.* (2002) Anti-inflammatory effect of kaurenoic acid, a diterpene from *Copaifera langsdorffi* on acetic acid-induced colitis in rats. Vascul Pharmacol 39:303-307.

Pasdaran A, Delazar A, Nazemiyeh H *et al.* (2012) Chemical composition, and antibacterial (against *Staphylococcus aureus*) and free-radical-scavenging activities of the essential oil of *Scrophularia amplexicaulis* Benth. Rec Nat Prod 6:350-355.

Peixoto AL, Morin MP (2003) Coleções botânicas: documentação da biodiversidade brasileira. Ciência e Cultura 55:21-24.

Pereira S, Taleb-Contini S, Coppede J *et al.* (2012) An ent-kaurane-type diterpene in *Croton antisyphiliticus* Mart. Molecules 17:8851-8858.

Rahalison L, Hamburger M, Hostettmann K *et al.* (1991) A bioautographic agar overlay method for the detection of antifungal compounds from higher-plants. Phytochem Analysis 2:199-203.

Rana NF, Sauvageot N, Laplace JM *et al.* (2013) Redox balance via lactate dehydrogenase is important for multiple stress resistance and virulence in *Enterococcus faecalis*. Infect Immun 81:2662-2668.

Rice LB (2009) The clinical consequences of antimicrobial resistance. Curr Opin Microbiol 12:476-481.

Rios JL, Recio MC (2005) Medicinal plants and antimicrobial activity. J Ethnopharmacol 100:80-84.

Roemer T, Xu DM, Singh SB *et al.* (2011) Confronting the challenges of natural product-based antifungal discovery. Chem Biol 18:148-164.

Ruiz LdS, Khouri S, Hahn RC *et al.* (2013) Candidemia by species of the *Candida parapsilosis* complex in children's hospital: prevalence, biofilm production and antifungal susceptibility. Mycopathologia 175:231-239.

Santos AO, Izumi E, Ueda-Nakamura T *et al.* (2013) Antileishmanial activity of diterpene acids in copaiba oil. Memorias do Instituto Oswaldo Cruz 108:59-64.

Scorzoni L, Pilar dLM, Mesa-Arango AC *et al.* (2013) Antifungal efficacy during *Candida krusei* infection in non-conventional models correlates with the yeast *in vitro* susceptibility profile. Plos One 8:e60047.

Valgas C, de Souza SM, Smania EFA *et al.* (2007) Screening methods to determine antibacterial activity of natural products. Braz J Microbiol 38:369-380.

Yagi S, Chretien F, Duval RE *et al.* (2012) Antibacterial activity, cytotoxicity and chemical constituents of *Hydnora johannis* roots. South African Journal of Botany 78:228-234.

Yongabi KA, Mbacham WF, Nubia KK *et al.* (2009) Yeast strains isolated from HIV-seropositive patients in Cameroon and their sensitivity to extracts of eight medicinal plants. Afr J Microbiol Res 3:133-136.

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