SUPPLEMENTARY MATERIAL

First report on the chemical composition of leaf essential oil of *Myrciaria pilosa* Sobral & Couto and its antimicrobial and antivirulence activities against *Staphylococcus aureus*

Wêndeo Kennedy Costa[a*], Nandara Ohana da Costa Gomes[a], Bruno Souza dos Santos[a], Clóvis Macêdo Bezerra Filho[a], Alisson Macário de Oliveira[a], Graziela Cláudia da Silva[a], Bruno Oliveira de Veras[b], Fernanda Granja da Silva Oliveira[a], Júlio César Ribeiro de Oliveira Farias de Aguiar[c], Daniela Maria do Amaral Ferraz Navarro[c], Maria Tereza dos Santos Correia[a], Márcia Vanusa da Silva[a]

[a] *Universidade Federal De Pernambuco – Departamento de Bioquímica, Recife PE, 50670-901, Brazil.*
[b] *Universidade Federal De Pernambuco – Departamento de Medicina Tropical, Recife PE, 50670-901, Brazil.*
[c] *Universidade Federal De Pernambuco – Departamento de Química Fundamental, Recife PE, 50670-901, Brazil.*

Abstract

Myrciaria pilosa is a tree species of the Brazilian Caatinga biome. This paper is the first report on the chemical composition and the antimicrobial and antivirulence activities of essential oil extracted from its leaves. The oil was extracted by hydrodistillation. Chemical composition determined by GC-MS and CG-FID, revealed 63 compounds; the sesquiterpenes guaiol (13.17%) and (E)-β-caryophyllene (11.26%) dominated. Antimicrobial activity against strains of *Staphylococcus aureus* was evaluated by the broth microdilution method. It showed minimum inhibitory concentrations (MIC) of 5 μg/mL against evaluated strains and minimum bactericidal concentrations (MBC) ranging from 10–20 μg/mL. Evaluation of antivirulence activity showed reductions of 92.0% and 47.2%, respectively, in haemolytic action and production of staphyloxanthin. These findings show that the essential oil of *M. pilosa* has potential as an antimicrobial drug to control infection by multi-resistant strains of *S. aureus*.

Keywords: *Myrciaria pilosa*, Myrtaceae, caatinga, sesquiterpenes, antimicrobial activity, *S. aureus*

1. Experimental

1.1. Herbal material and essential oil extraction

Myrciaria pilosa leaves were collected in March 2016, in the Vale do Catimbau National Park, in the municipality of Buíque (8 ° 30 '57 "S 37 ° 20' 59" W), Pernambuco, Brazil, a region of the Caatinga biome. Access was registered (A08E18B) in the National Management System for Genetic Heritage and Associated Traditional Knowledge (SisGen). The botanical identification was carried out by Dr Alexandre Gomes da Silva and a specimen (78,996) was deposited at the Herbário Dárdano de Andrade Lima of the Agronomic Institute of Pernambuco (IPA), Brazil.

The leaves (500 g) were washed, dried at room temperature and then processed in industrial shredder. Then, the plant material was hydrodistilled (Clevenger) for 3 h. The essential oil layer was separated, dried using anhydrous sodium sulfate and stored in airtight amber glass bottle at -5 ° C until needed for the test. The oil yield was reported as the quotient between the mass of oil collected and the fresh weight of the extracted plant material.

1.2 Chemical characterization of leaf essential oil of Myrciaria pilosa

The essential oil was analyzed on Agilent Technologies Gas Chromatograph (Palo Alto, CA, USA) 5975C series, with quadrupole detection system equipped with apolar column DB-5 9Agilent J&W, 60 m x 0,25 mm x 0,25 µm. Aliquots 1 µL of in split 1:50 of the essential oil with concentrations varying from 2,000 to 3,000 ppm were injected in the gas chromatography coupled to CG/MS mass spectrometry. Subsequently, 1 µL split (1:50) of the mixture of hydrocarbon standards: C9-C30 was injected. The essential oil mixture and the mixture of hydrocarbon standards, 1 µL (0.2 µL of alkanes and 0.8 µL of the oil) were then injected under the spitless mode. The GC temperature was maintained at 60°C for 3 min, then increasing from 2.5 °C.min-1 to 240°C and held for 10 min at this temperature. The helium flow was maintained at a constant pressure of 100 kPa. The MS interface was set at 200°C and injector at 250 °C and the mass spectra recorded at 70 eV (in EI mode) with a scan rate of 0.5 scan-s of m/z 20-350. From the analysis of the retention times of the compounds present in the essential oil sample, the hydrocarbon standards and the combination of the essential oil with the pattern mixture, the retention index (RI) for each component of the oil were calculated. Compounds were identified by comparing their mass

spectra (MassFinder 4, NIST08 and Wiley Registry ™ 9th Edition, integrated into the software Agilent MSD Productivity ChemStation (Agilent Technologies, Palo Alto, EUA) and RI to those of authentic standards available in Adams (2007). The oil samples were quantified in gas chromatography with flame ionization detector (GC-FID), under the same GC-MS conditions, in triplicate for calculation of standard deviation of peak area percentage of each compound in the chromatogram. This was used to determine the proportion of the compounds in the oil.

1.3 Bacterial strains

The microorganisms tested were; standard strain *S. aureus* (ATCC 6538) and two clinical isolates of *S. aureus* (UFPEDA 679 and UFPEDA 683), obtained from the collection of microorganisms of the Department of Antibiotics of the Federal University of Pernambuco. The susceptibility of these isolates was previously assessed using the disk diffusion technique and interpreted according to the Clinical and Laboratory Standards Institute (2016) as described in Table S3. The MAR index was calculated using the formula MAR = x / y, where "x" was the number of antibiotics to which the isolate demonstrated resistance; And "y" was the total number of antibiotics tested (Table S3).

1.4 Antimicrobial activity

The antimicrobial activity of the essential oil of *M. pilosa* against the standard strain (ATCC 6538) and clinical isolates (UFPEDA 679 and UFPEDA 683) of *S. aureus* was evaluated by determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). MIC was assessed by the broth microdilution assay, using a 96-well culture plate with Mueller-Hilton broth as growth medium. The microorganisms (5×10^5 CFU / mL) and different concentrations (1–1,000 µg/mL) of the essential oil of *M. pilosa* or Chloramphenicol were added to each cell prior to incubation for 24 h at 37 °C. Wells containing microorganism in the absence of the essential oil of *M. pilosa* or antibiotic corresponded to the growth control. Resazurin solution (0.01%) was used as an indicator of bacterial growth. The lowest concentration at which the solution showed no colour change (from purple to pink) was taken as the MIC. To assess the MBC, an aliquot (10 µL) of the well suspension was transferred to Mueller-Hinton agar plates and incubated for 24 h at 37 °C. The MBC was determined as the lowest concentration of essential oil at which bacterial growth did not occur.

1.5 Antihaemolytic evaluation

Haemolysin is a cytotoxin produced by *S. aureus*, capable of lysing red blood cells, allowing bacterial growth and dissemination, as well as evading the immune response (Lo et al. 2011). The *S. aureus* clinical isolate UFPEDA 683 was used to investigate the effects of essential oil of *M. pilosa* on the haemolysis inhibition in human blood because it shows a high index of multiple antibiotic resistance and a high capacity to produce haemolysis. The haemolysis test was performed as described by Almaaytah et al. (2014). In summary, the log phase cultures were re-inoculated [1:100 (v/v)] in trypticase soy broth (TSB) medium with subinhibitory concentrations of the essential oil of *M. pilosa* (2.5, 1.25 and 0.625 μg/mL, corresponding respectively to MIC/2, MIC/4 and MIC/8). After 16 h, 500 μL of these cultures were added to 1 mL of human erythrocyte solution (3%) and incubated at 37 ± 1 °C for 1 h with shaking (250 rpm). The supernatant was collected by centrifugation (3200 rpm for 10 min) and the optical density was measured at 540 nm. The tests were performed in triplicate.

1.6 Staphyloxanthin (STX) inhibition assessment

S. aureus also secretes staphyloxanthin, another virulence factor (Holt et al. 2011). *S. aureus* UFPEDA 683 cultures were inoculated in brain heart infusion (BHI) medium and then incubated for 24 h at 37 °C. Subsequently, the inoculum was diluted (1:100) in BHI and the samples (1 mL) of this suspension were incubated with subinhibitory concentrations of the essential oil of *M. pilosa* (2.5, 1.25 and 0.625 μg/mL, corresponding respectively to MIC/2, MIC/4 and MIC/8) and incubated for 24 h at 37 ºC. The bacterial cells were then harvested by centrifugation (10 000 rpm for 10 min), washed twice with saline and re-centrifuged. The pellet was then photographed to compare the production of staphyloxanthin with control. For the extraction of carotenoid pigments, the pellet was resuspended in 0.2 mL of methanol before heating at 55 °C for 30 min. Extracted pigment was separated from cell debris by centrifugation (16 600 rpm for 10 min). The pigment extraction procedure was three times to maximise the extraction of staphyloxanthin. The optical densities of the collected extractions were measured at 465 nm (Bezerra Filho et al. 2020).

3.7 Statistical analysis

The data obtained were checked for their normal distribution and analyzed using (one-way ANOVA) comparing the values for each concentration of the essential oil, point by point, with different letters mean statistical difference, followed by Tuckey's post hoc test.

Reference

Adams RP. 2007. Identification of essential oil components by gas chromatography/mass spectrometry 4th Edition. Carol Stream, IL: Allured Publishing Corporation.

Almaaytah A, Tarazi S, Alsheyab F, Al-Balas Q, Mukattash T. 2014. Antimicrobial and antibiofilm activity of mauriporin, a multifunctional scorpion venom peptide. Int. J. Pept. Res. Ther. 20(4), 397-408.

Bezerra Filho CM, Silva LCN, Silva MV, Løbner-Olesen A, Struve C, Krogfelt KA, Correia MTS, Oliva MLV. 2020. Antimicrobial and Antivirulence Action of *Eugenia brejoensis* Essential Oil *in vitro* and *in vivo* Invertebrate Models. Front. Microbiol. 11: 424-435.

CLSI. 2016. Performance standards for antimicrobial susceptibility testing. Clinical Lab Standards Institute, 2016.

Table S1: Chemical composition of the *Myrciaria pilosa* essential oil.

Components	RI[a]	RI[b]	%	Components	RI[a]	RI[b]	%
α-Thujene	924	924	0.28	Aromadendrene	1439	1439	0.55
α-Pinene	930	932	1.76	Guaia-6.9-diene	1444	1442	0.60
Camphene	949	946	0.01	α-Humulene	1454	1452	1.66
Sabinene	970	969	0.83	Alloaromadendrene	1462	1458	1.26
β-Pinene	973	974	2.40	γ-Muurolene	1477	1478	0.45
β-Myrcene	988	990	0.29	Germacrene D	1482	1480	2.28
α-Phellandrene	1002	1002	0.09	β-Selinene	1487	1489	0.84
α-Terpinene	1014	1014	0.29	δ-Selinene	1492	1492	0.01
p-Cymene	1020	1022	1.43	Bicyclogermacrene	1497	1500	3.97
Limonene	1024	1026	1.69	δ-Amorphene	1508	1511	0.33
1,8-cineole	1026	1028	1.46	γ-Cadinene	1515	1514	0.26
γ-Terpinene	1054	1057	0.47	δ-Cadinene	1524	1522	1.89
α-Terpinolene	1086	1087	0.13	α-Calacorene	1544	1544	0.43
Linalool	1095	1099	0.08	Elemol	1550	1548	4.40
Endo-Fenchol	1111	1114	0.01	Germacrene B	1558	1559	0.46
p-Menth-2-en-1-ol	1118	1118	0.07	Palustrol	1569	1567	0.24
Trans-Pinocarveol	1137	1135	0.17	Spathulenol	1579	1577	3.87
Pinocarvone	1161	1160	0.01	Caryophyllene oxide	1585	1583	3.81
Terpinen-4-ol	1176	1174	4.06	Viridiflorol	1593	1592	0.01
α-Terpineol	1189	1186	0.38	Guaiol	1600	1600	13.70
Myrtenol	1195	1194	0.17	5-epi-7-epi-α-Eudesmol	1607	1607	0.01
Trans-Piperitol	1207	1207	0.01	10-epi-γ-Eudesmol	1621	1622	0.01
δ-Elemene	1337	1335	0.80	γ-Eudesmol	1634	1630	6.56
α-Cubebene	1350	1348	0.01	Caryophylla-4(12),8(13)-dien-5β-ol	1638	1639	0.01
α-Ylangene	1372	1373	0.05	β-Eudesmol	1653	1649	9.20
α-Copaene	1376	1374	0.71	α-Eudesmol	1656	1652	4.90
β-Bourbonene	1385	1387	0.65	Bulnesol	1669	1670	3.06
β-Elemene	1392	1390	0.91	Cadalene	1677	1675	0.15
(Z)-Caryophyllene	1407	1408	0.01	Eudesma-4(15),7-dien-1β -ol	1688	1687	0.06
α-Gurjunene	1410	1409	0.05	Eudesm-7(11)-en-4α-ol	1697	1700	0.09
(E)-β-caryophyllene	1421	1417	11.26	Kaurene	2039	2042	0.13
γ-Elemene	1434	1434	0.24	**Total**			**95,98**

RI[a] = Retention rate determined; RI[b] = Retention index obtained from from Adams (2007); % = area of compost

relative to *M. tenella* Essential Oil.

Table S2. Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) values of *Myrciaria pilosa* essencial oil and Chloramphenicol (µg/mL).

Staphylococcus aureus	***Myrciaria pilosa* essencial oil (µg/mL)**			**Chloramphenicol (µg/mL)**		
	MIC	**MBC**	**MBC/MIC**	**MIC**	**MBC**	**MBC/MIC**
ATCC 6538	5	10	2	10	20	2
UFPEDA 679	5	20	4	40	80	2
UFPEDA 683	5	20	4	60	120	2

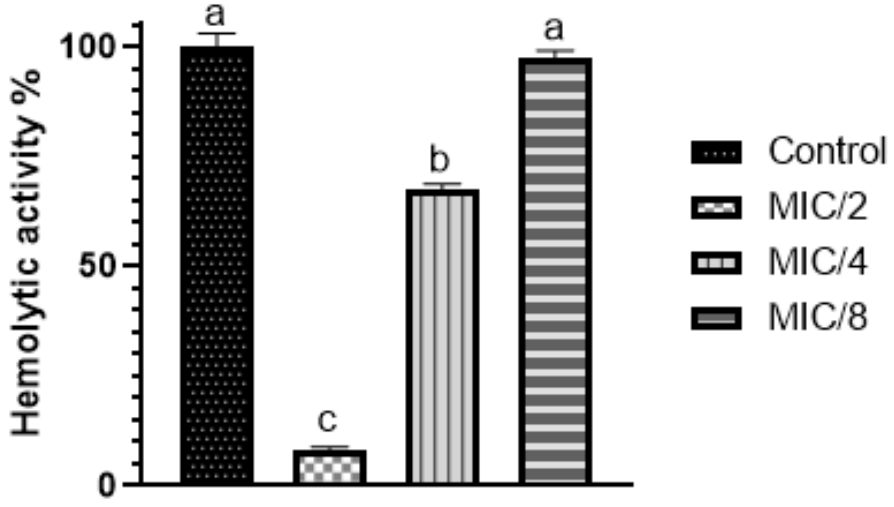
MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

Table S3. Antimicrobial susceptibility profile of *S. aureus* ATCC 6538, *S. aureus* UFPEDA 679 and *S. aureus* UFPEDA 683.

Staphylococcus aureus	**Antibiotics**										**MAR**
	PEN	**VAN**	**OXA**	**GEN**	**CFL**	**CFO**	**CTX**	**CPM**	**CIP**	**CLI**	
ATCC 6538	S	S	S	S	S	S	S	S	S	S	0
UFPEDA 679	R	S	S	S	S	S	S	S	S	S	0.1
UFPEDA 683	R	S	S	S	R	R	I	S	R	R	0.55

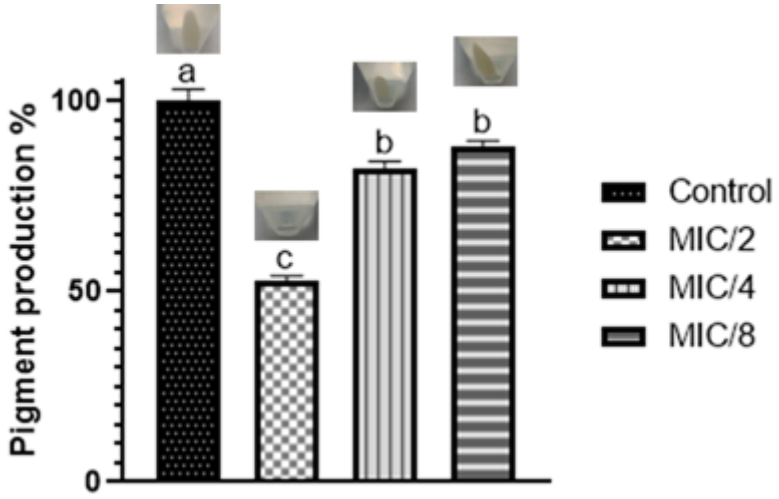
S: sensitive; R: resistant; PEN: penicillin; VAN: vancomycin; OXA: oxacillin; GEN: gentamicin; CFL: cephalothin; CFO: cefoxitin; CTX: cefotaxime; CPM: cefepime; CIP: ciprofloxacin; CLI: clindamycin. MAR: Index of resistance to multiple antibiotics.

Figure S1. Inhibition of hemolysis by the essential oil of *Myrciaria pilosa*.



Results expressed as mean ± standard error of three independent tests. Different letters indicate significant difference ($p<0.05$) between the groups, one-way ANOVA followed by Tuckey's Test.

Figure S2. Inhibition of staphyloxanthin by the essential oil of *Myrciaria pilosa*.



Results expressed as mean ± standard error of three independent tests. Different letters indicate significant difference ($p<0.05$) between the groups, one-way ANOVA followed by Tuckey's Test.