SHORT REPORT

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Antibacterial and Cytotoxic Activity of Extracts and Isolated Compounds from *Myrciaria ferruginea* (Myrtaceae)

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Abstract: This study evaluated for the first time the antibacterial activity, cell viability and migration ability on 3T3 murine fibroblast cells of extracts and isolated compounds [lupeol (**1**), hexamethylcoruleoellagic acid (**2**) and a mixture of **1** and betulinaldehyde (**3**)] of *Myrciaria ferruginea*. In antibacterial assays extracts were susceptible only against *S. aureus* (MIC 500 µg/mL) and *S. epidermidis* (MIC ranging from 7.8 to 500 µg/mL) and compounds **1**-**3** have shown no significant activity. In trials for cell viability, with exception of MeOH-H_2O fraction from leaves (viable cells > 90%), both the crude extract and other fractions showed inhibition of cell growth (viable cells ≤ 80% at 15.625 and 31.25 µg/mL); while the samples from stems, with the exception of $CHCl_3$ fraction that showed strong cytotoxic effect at the lowest concentration tested (15.625 µg/mL), the other fractions were not cytotoxic. Compounds (**1-3**) inhibited cell viability in dose dependent manner (15.625 to 500 µg/mL). Mixture containing **1** and **3** showed inhibitions only in concentrations greater than 62.5 µg/mL while compound **2** decreased from the lowest concentration tested. In scratch wound assay, these compounds not increased the population of fibroblasts at concentrations less than 62.5 µg/mL.

Keywords: *Myrciaria ferruginea*; antibacterial activity; cell viability; fibroblast cells, MTT assay.

1. Plant Source

The *Myrciaria* genus comprises about 99 species distributed in several Brazilian biomes, of which 21 are native. Some of this species produce fruits that are used to make juice jams, wines and liqueurs [1] and are used in folk medicine to treat skin diseases and have antifungal and healing activities [2]. This paper describes antibacterial activity, cell viability and migration ability on 3T3 murine fibroblast cells of extracts and isolated compounds of *M. ferruginea* O. Berg. Here, we are reporting for the first time antibacterial and cytotoxic activity of extracts and isolated compounds

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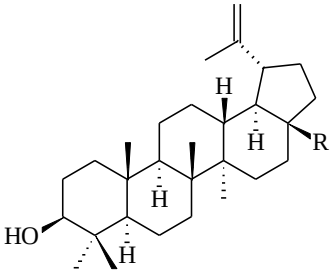
(**1-3**) (Figure 1). All these compounds are structurally known but they are being described for the first time in this species. Leaves and stems were collected in November 2011, at the Área de Proteção Ambiental da Usina Coruripe (Latitude: 9° 56' S and Longitude: 36° 05' W), Alagoas State, and collected by Mr. Nilo Pinto de Sousa and identified by Rosangela P. L. Lemos (plant taxonomist). A voucher specimen (MAC-11183) has been deposited at the Herbarium of the Instituto do Meio Ambiente do Estado de Alagoas, Brazil.

2. Previous Studies

M. ferruginea has not been previously studied and there are few studies on species of this genus. However, previous phytochemical investigations on *Myrciaria* species reported the occurrence of flavonoids, ellagic acid and anthocyanins [1], as well as mono- and sesquiterpenes as the major constituents of its essential oils [3,4]. Some of these species exhibit anti-inflammatory, antioxidant, antifungical, antiproliferative, antibacterial, antiplasmodial, and gastroprotective activities [1].

3. Present Study

The air-dried and powdered leaves (430 g) and stems (4900 g) were extracted at room temperature with acetone and ethanol (EtOH 90%), respectively. After the removal of solvents under vacuum crude extracts (acetone: 25.14 g; EtOH: 159.14 g) were suspended in MeOH-H_2O 7:3 solution and extracted successively with hexane, $CHCl_3$ and EtOAc that were stored under freezer for later use in this study. Hexane fraction from leaves (12.0 g) after successive chromatographic fractionations over silica gel column with hexane containing increasing amounts of EtOAc afforded compounds **1** (0.03 g) and **2** (0.015 g). The $CHCl_3$ fraction (9.0 g) from leaves after the same experimental procedure, gel filtration (Sephadex LH-20 with MeOH) and successive recrystallizations with MeOH afforded a mixture of **1** and **3** (0.015 g) and compound **4** (0.197 g) (Figure 1). Phytochemical investigation with stem samples will later be made.



1 R = CH_3
3 R = CHO
4 R = CO_2H

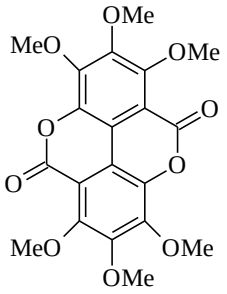


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Figure 1. Structures of isolated compounds.

3.1. Antibacterial assays

Agar diffusion method as previously described [5,6] was used for screening of extracts and its fractions. For each microorganism (*S. aureus*, *S. epidermidis*, *Enterococus faecalis*, *E. coli*, *P. aeruginosa*, and *Enterobacter aerogenes*), 1 mL of adjusted inoculum (10^6 CFU/mL) was transferred and mixed with 20 mL of Mueller Hinton Agar medium at 35°C. After that, 50 µL of each sample (5 mg/50 µL) was transferred and incubated at 35°C for 24 h. Only samples that showed promising

results [inhibition zones diameter > 9 mm] [7] against any microorganism have determined the minimum inhibitory concentrations (MIC) by broth microdilution test [7,8] and the activity determined (Table 1) according to Holetz et al. [9].

The bioactive fractions from the leaves were subjected to successive chromatographic fractionations over silica gel column. This procedure resulted in the isolation of lupeol (**1**) [10], hexamethylcoruleoellagic acid (**2**) [11,12], a mixture of **1** and betulinaldehyde (**3**) [10], and betulinic acid (**4**) [10] (Figure 1) that were identified by NMR spectral analysis and comparison with those data reported in the literature. Compounds **1-3** were tested in broth microdilution method and they show no significant antibacterial activity. In the antibacterial assays, among the six microorganisms tested, the extracts showed activity only against *S. aureus* and *S. epidermidis*, that are bacteria frequently found in intensive care units [13] and commonest cause of nosocomial infections [14], which are associated with a great deal of morbidity and mortality [15].

Table 1. Inhibition zones diameter (IZD) of extracts and fractions that provided the best results in Agar diffusion method and its minimum inhibitory concentration (MIC).

	Microorganisms			
	S. aureus		***S. epidermidis***	
Samples[a]	**IZD (mm ± DP)**[c]	**MIC** (μg/mL)	**IZD (mm ± DP)**[c]	**MIC** (μg/mL)
Leaves				
Crude acetone ext.	12.0 ± 0.0	1000	22.3 ± 0.5	125
Fr. Hexane	12.0 ± 0.0	1000	21.0 ± 0.0	125
Fr. $CHCl_3$	(-)	1000	15.7 ± 3.4	500
Fr. MeOH-H_2O	15.7 ± 1.8	1000	22.3 ± 3.3	500
Stems				
Crude EtOH ext.[b]	17.0 ± 1.6	500	14.0 ± 0.0	125
Fr. Hexane[b]	9.0 ± 0.0	1000	25.3 ± 0.5	500
Fr. $CHCl_3$	13.3 ± 1.3	1000	10.7 ± 0.5	7.8
Fr. EtOAc	14.0 ± 0.8	500	10.3 ± 0.5	7.8
Fr. MeOH-H_2O	16.0 ± 1.6	1000	12.0 ± 0.0	125

(-) Absence of inhibition zone; [a,b] solvents used to dissolve the samples ([a] 10% DMSO or [b] 10% cremophor – negative controls); [c] growth inhibition halos in mm [mean ± standard deviation (SD; result is average of triplicate experiment)]. Positive controls: ciprofloxacine (5 μg/disc; 39 mm) and ceftriaxone (30 μg/disc; 32 mm).

3.2. Cell viability assay

Murine fibroblasts cell line NIH 3T3 were grown in DEMEM supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% fetal bovine serum (FBS). The cells were cultured at 37°C in a 5% CO_2 atm., seeded into 96 well plate (1×10^5/well) and incubated for 24 h. Cells were treated with crude extract, fractions or isolated compounds (15.625 to 500 μg/mL). After treatment, plates were again incubated and the cell viability assay by MTT method [16] was performed. The formazan crystals formed were dissolved in 150 μL of DMSO and optical density was read at 540 nm in a multi-well ELISA plate reader. Four individual wells were assayed per treatment, and MTT reduction activity was determined as percentage of control cells [(absorbance of treated cells/absorbance of untreated cells) × 100].

*3.3. Scratch assay with the mixture of compounds **1** and **3***

The spreading and migration capabilities of Swiss 3T3 fibroblasts were assessed using a scratch wound assay according to Fronza et al. [17]. A linear wound was generated in the monolayer and any cellular debris was removed by washing the coverslips with phosphate buffer saline. DMEM medium with DMSO (0.25%, control group), platelet derived growth factor (2 ng/mL, as positive control). The isolated compounds (**1-3**) at 15.6, 31.25 and 62.5 μg/mL were added to a set of 3 coverslips per dose and incubated for 24 h at 37 ºC with 5% CO_2. The cells were fixed and stained overnight. Three

representative images from each coverslip of the scratched areas under each condition were photographed to estimate the relative migration cells.

As shown in Figure 2, the crude extract of leaves and its fractions in hexane and $CHCl_3$ exhibited citotoxicity in all doses evaluated. To crude extract, viable cells showed variation among 80% to 40% (at 15.625 to 500 μg/mL), while hexane and $CHCl_3$ fractions the cell viability varied among 60% to 20% (at 15.6 μg/mL to 250 μg/mL). Interestingly, the MeOH-H_2O fraction showed a slight reduction of cellular viability to 80% in concentrations of 31.2 μg/mL to 500 μg/mL. The lower concentration evaluated of MeOH-H_2O fraction did not show cytotoxic effect.

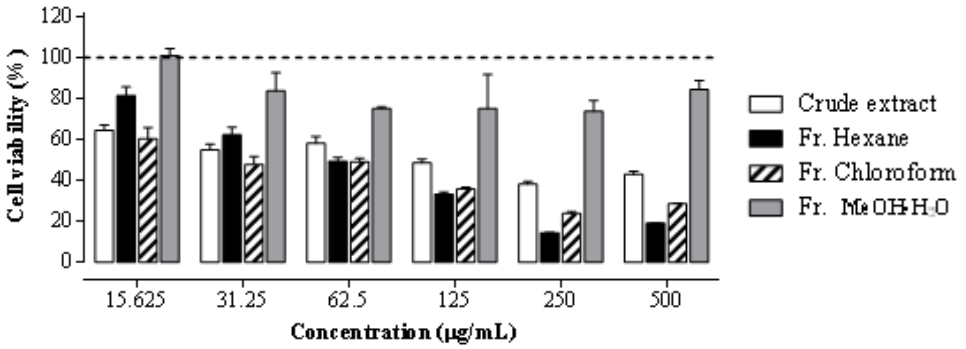

Figure 2. Effect of crude extract and fractions from leaves on viability of 3T3 cells. The bars express the mean ± SEM of three independent experiments. The dotted line represents viability of untreated cells.

Effects on cell viability of crude extract and fractions of stems are shown in Figure 3. The crude extract and its hexane fraction were able to maintain cell viability to levels above 80% at 15.6, 31.2 and 62.5 μg/mL while at 125, 250 and 500 μg/mL they reduced the cell viability to values below at 60%. The EtOAc fraction did not show cytotoxic effect at 15.6 μg/mL, but at concentrations greater than 31.2 μg/mL cell viability was reduced to below 60%. In all concentrations tested, $CHCl_3$ fraction reduced cell viability from 70% to 40%. The MeOH-H_2O fraction at 15.6, 31.2, 62.5, and 125 μg/mL did not induce cytotoxic effect, however, at 500 μg/mL cell viability decreased to 60%.

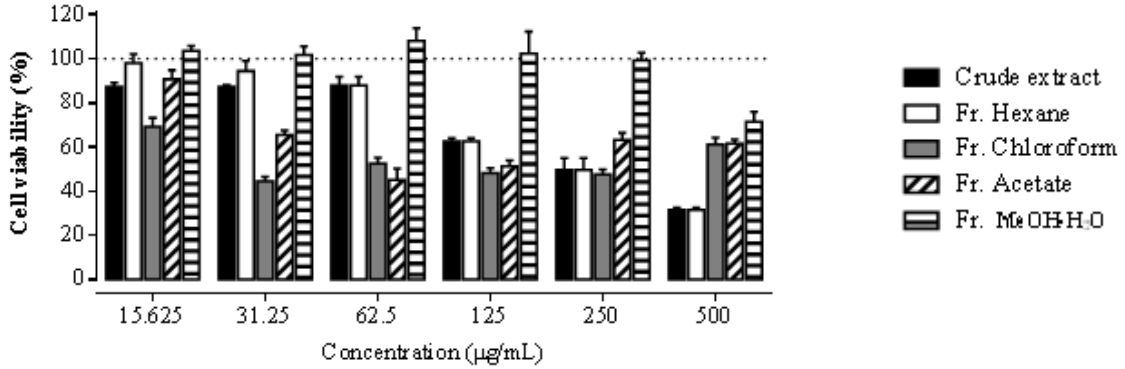

Figure 3. Effect of extract and fractions from stems on viability of 3T3 cells by MTT assay. The bars express the mean ± SEM of three independent experiments. The dotted line represents viability of untreated cells.

Isolated compounds (**1-3**) at 15.625 to 500 μg/mL inhibited cell viability in dose dependent manner. Mixture containing **1** and **3** showed inhibitions only in concentrations greater than 62.5 μg/mL while compound **2** decreased from the lowest concentration tested. No biological activity has been reported for compound **2** so far while compounds **1**, **3** and **4** are widely distributed throughout the plant kingdom and several biological activities, including anti-cancer **[**20,21], analgesic, anti-inflammatory [21,22], anti-angiogenic, immune-modulatory [22], antimicrobial, antimycobacterial, antiplasmodial [24,25], and anti-HIV [20,22,23]. Compounds **1** and **4** and many of its derivatives have

been cytotoxic to many human tumor cell lines [26], but this is the first time that these compounds have been evaluated against 3T3 murine fibroblast cells. These cells types are involved in the regulation of angiogenesis that occurs during wound healing process [27]. Although wound healing is rarely seen as a problem in healthy individuals, the increasing incidences of diseases such as diabetes and obesity worldwide, the burden of chronic wounds is expected to be in a rise [28]. However, there is increasing interest in finding natural products with wound healing efficacy. So, the results of the present study are important, taking in account the implication of the studied microorganisms in therapeutic failure. Our data indicate that extracts from *M. ferruginea* should be explored more to develop potential antibacterial drugs, although the isolated compounds so far are not the main antibacterial constituents of the leaves of the plant. Some of the extracts tested showed cytotoxic effect and the results for compound 2 suggests it is at least partly responsible for this effect, especially for the crude extract and hexane fraction from the leaves. The mixture of **1** and **3**, up to a concentration of 62.5 μg/mL not increased the population of fibroblasts in the scratched area, suggesting, therefore, that this compound has no effect on the wound healing process.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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