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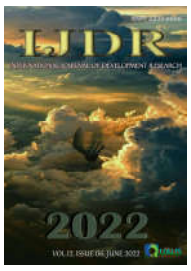

RESEARCH ARTICLE OPEN ACCESS

OLEORESIN FROM *COPAIFERA MARTII* HAYNE: *IN VITRO* ANTIBACTERIAL ACTIVITY AGAINST PLANKTONIC AND BIOFILM CELLS OF VANCOMYCIN-RESISTANT *ENTEROCOCCUS FAECIUM*

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ABSTRACT

Enterococcus faecium is one of the major causative agents of healthcare-associated infections worldwide. This bacterium exhibits a remarkable capacity of acquiring new determinants of resistance, including to the last resort antimicrobials. The present study reports the *in vitro* antibacterial effect of *Copaifera martii* Hayne oleoresin against vancomycin-resistant *E. faecium* strains and *E. faecium* ATCC6965, a vancomycin-susceptible strain. Oleoresin inhibited the growth of planktonic cells, displaying a minimal inhibitory concentration (MIC) of 0.060 mg/mL for most strains. Time-kill kinetics revealed that this oleoresin exhibited a bactericidal activity against planktonic cells. MIC of kaurenoic acid, a diterpene present in *C. martii* oleoresin, ranged from 0.025 to 0.050 mg/mL, indicating that this substance seems to be one of the active components of *C. martii* oleoresin related to the antibacterial activity against *E. faecium*. A significant reduction in metabolic activity and biomass of established biofilms of *E. faecium* strains was observed after treatment with MIC and 2xMIC of oleoresin. The oleoresin did not display *in vitro* toxicity to mammalian cells at concentrations that were effective in killing planktonic and biofilm cells of *E. faecium*. These results indicate the potential of *C. martii* oleoresin for the development of new control strategies for *E. faecium* infections.

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INTRODUCTION

Enterococcus species are a ubiquitous group of Gram-positive bacteria. In humans, these bacteria can be found as members of the gastrointestinal microbiota and as an important causative agent of potentially fatal infections, especially in immunocompromised individuals and (García-Solache and Rice, 2019). Within this genus, *Enterococcus faecium* is a leading cause of healthcare-associated infections worldwide. This scenario is partially associated with its intrinsic resistance to many antimicrobials and its capacity of

acquiring new resistance traits (Miller *et al*., 2020). In fact, a substantial proportion of enterococcal infections are caused by vancomycin-resistant *E. faecium* (VR*Efm*), which also exhibits resistance to several other antimicrobials (Miller *et al*., 2020; Ruzon *et al*., 2010). Worryingly, isolates resistant to linezolid, tigecycline and daptomycin, the "last resort" antimicrobials for the treatment of VR*Efm* infections, have also been reported (Miller *et al*., 2020). Besides antimicrobial resistance, most enterococcal infections are associated with biofilm formation on the surface of host tissues or implanted medical devices (Ch'ng *et al*., 2019). Bacteria within biofilms are highly tolerant to most clinically used antibacterial agents and resistant to host defenses (Ch'ng *et al*., 2019). In light of this alarming scenario, in 2017 the World Health Organization ranked VR*Efm* as a high priority pathogen, for which there is an urgent need for new therapeutic options (WHO, 2017). A range of plant-derived compounds has been empirically used to treat different diseases since ancient times. Indeed, plants have been recognized as potential sources of antimicrobial agents due to their inhibitory activity both on growth and on the expression of microbial virulence factors. Thus, plant-derived compounds represent an affordable alternative to control microbial infections (Tobouti *et al*., 2017). *Copaifera* L. species (Leguminosae Juss) are typical of Amazonian rainforest and Brazilian Cerrado biomes. The oleoresin (known as copaiba oil) exuded directly from *Copaifera* spp. trunks is a major non-timber forest product sustainably exploited in Brazil, being used both in the cosmetics industry as in folk medicine. Copaiba oil has been used in Latin-American indigenous culture since the sixteenth century to treat various skin and urinary tract diseases, bronchitis, asthma, pneumonia, leucorrhoea, ulcers, leishmaniasis and syphilis (Veiga Júnior and Pinto, 2002; Tobouti *et al*., 2017), and its use is widespread until today.

Some biological properties of copaiba oil have been confirmed by pharmacological and microbiological tests, including anti-inflammatory (Teixeira *et al*., 2017), anticancer (Abrão *et al*., 2015; da Moraes *et al*., 2016), wound-healing (Guimarães *et al*., 2016), antinociceptive (Gomes *et al*., 2007; Dalenogare *et al*., 2019) and antimicrobial (Abrão *et al*., 2015; da Moraes *et al*., 2016; Guimarães *et al*., 2016; Santos *et al*., 2008a; Santos *et al*., 2008b; Otaguiri *et al*., 2017; Pfeifer Barbosa *et al*., 2019; Morguette *et al*., 2019; Andrade *et al*., 2020). The antibacterial effect of oleoresin from *C. cearensis*, *C. lucens*, *C. paupera* and *C. reticulate* (Santos *et al*., 2008a; Pfeifer Barbosa *et al*., 2019), *C. langsdorfii* (Abrão *et al*., 2015; Santos *et al*., 2008a), *C. officinalis* (Guimarães *et al*., 2016; Santos *et al*., 2008a; Morguette *et al*., 2019), *C. oblongfolia* (da Moraes *et al*., 2016) and *C. multijuga* (Santos *et al*., 2008a; Otaguiri *et al*., 2017) has been specifically reported in Gram-positive bacteria. However, only the oleoresin from *C. reticulata* has been evaluated in planktonic cells of *E. faecium* DSM 20477, a vancomycin-susceptible strain (Pfeifer Barbosa *et al*., 2019). For the purposes of this study, the potential antibacterial effect of *C.martii* Hayne oleoresin was evaluated *in vitro* for the first time against planktonic and sessile cells of vancomycin-resistant *Enterococcus faecium*.

MATERIALS AND METHODS

***Microorganisms*:** Twelve *vanA* VR*Efm* strains isolated from blood (n = 2), rectal swab (n = 5), scrotum secretion (n = 1) and urine (n = 4) were obtained from the bacterial collection of the Laboratory of Clinical Microbiology of the Universidade Estadual de Londrina (UEL), Londrina, Paraná, Brazil. The antimicrobial susceptibility profiles of VR*Efm* strains were previously described (Ruzon *et al*., 2010) and are shown in Table 1. The vancomycin-susceptible *E. faecium* ATCC 6569 (VS*Efm*) and the *vanA*VRE*fm* UEL-170 (Tavares *et al*., 2019) strains were also included. Before the experiments, bacteria were grown in Brain Heart Infusion (BHI, Himedia, Brazil) agar at 37ºC for 24 h. To prepare a standard bacterial suspension, three colonies of each strain were transferred to BHI broth, incubated at 37ºC until a 0.5 McFarland [1.0 to 2.0 x 10^8 colony forming unit (CFU)/mL] equivalent turbidity was achieved, which was estimated using the DensiCHEK™ PLUS colorimeter (bioMérieux, Brazil). Each standard bacterial suspension was then diluted in culture medium to achieve the cell density (inoculum) used in each assay.

***Plant compounds*:** Oleoresin from *C. martii* Hayne was collected in Tapará, Pará, Brazil by direct tapping of its trunk using a manual m*et al* auger. Kaurenoic acid was purified from the dried roots of *Sphagneticola trilobata* as described previously (Miranda *et al.,* 2015). To prepare a stock solution, oleoresin (10.0 mg) and kaurenoic acid (1.0 mg) were solubilized in 100 μL dimethylsulfoxide (DMSO, Merck, Brazil) and then added to 900 μL of BHI broth. The DMSO concentration did not exceed 1.0% in allassays.

***Permission/Consent*:** The Sistema de Autorização e Informação em Biodiversidade (SISBIO) of Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) of Ministério do Meio Ambiente (MMA) of Brazil granted permission for collecting the oleoresin (Document number 30809-1/2014-SISBIO/ICMBio/MMA). Field studies did not involve endangered or protected plant species. The use of *C. martii* oleoresin and the VR*Efm* strains in this research was registered in Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN) under the number AE94ED7.

Antibacterial susceptibility testing in planktonic cells

Minimal inhibitory concentration: The minimal inhibitory concentrations (MIC) of copaiba oil and kaurenoic acid for planktonic *E. faecium* cells were determined by broth microdilution assay according to the Clinical and Laboratory Standards Institute recommendations (CLSI, 2015), except the medium. Bacteria were added to wells of 96-wells U-bottom microtiter plates (Techno Plastic Products, Switzerland) containing two-fold serial dilutions of copaiba oil (0.015 to 1.0 mg/mL) or kaurenoic acid (0.0015 to 0.1 mg/mL) in BHI broth. Wells containing medium *plus* bacteria or medium *plus* 1% DMSO *plus* bacteria, and wells without bacterial cells in each plate served as growth and sterility controls, respectively. MIC of copaiba oil was determined at total inhibition of visual growth after 24 h incubation compared to untreated cells.

***Time–kill kinetics*:** The time-kill assay (CLSI, 1999) was used to determine the nature of the inhibitory effect of copaiba oil against *E. faecium* strains. Planktonic cells (5 x 10^5 CFU/mL) were added in BHI broth containing copaiba oil at MIC values and incubated statically at 37ºC. At specific time points (0, 4, 8, 12 and 24 h), 20 μL were removed from each well and serially diluted (ratio 1:10) in 0.15 M phosphate-buffered saline pH 7.2 (PBS). An aliquot of 10 μL of each dilution was inoculated on BHI agar (Morguette *et al*., 2019) and the CFU counts were determined after incubation at 37ºC for 24 h. Data were averaged and plotted as $\log_{10}$ CFU/mL *versus* time (h). The viability of copaiba oil-treated (at MIC values for 6 and 24 h) and -untreated cells was evaluated using the LIVE/DEAD®*Bac*Light™ staining kit (Molecular Probes, Invitrogen) according to the manufacturers' recommendations. Bacteria were incubated with SYTO® 9 and propidium iodide and analyzed by fluorescence microscopy (Olympus BX53, Brazil) using the excitation/emission of 480/635 nm and 490/635 nm, respectively.

Antibacterial susceptibility testing in sessile cells

***Biofilm formation*:** The ability of *E. faecium* strains to form biofilm on polystyrene surface was analyzed according to Ruzon *et al*. (2010) with minor modifications. Briefly, a standard bacterial suspension was prepared as described above, except the BHI broth which was supplemented with 1% glucose (BHI+glu). A 20 μL-aliquot of standard bacterial suspension was placed in each well of a clear flat-bottomed 96-well microtiter plates (Greiner CELLSTAR® 96 well plates, Sigma-Aldrich, Brazil) containing 180 μL of BHI+glu, and the plates were incubated statically at 37ºC for 24 h. Negative control wells containing only BHI+glu were included. After incubation, the medium was aspirated off and the biofilm was washed once with PBS. The adherent biofilm layer was fixed with methanol (15 min),

dried at room temperature and stained with 2% (w/v) crystal violet (15 min). The stained biofilm was washed three times with PBS and biofilm-bound dye was removed by the addition of 95% methanol (150 μL). A volume of 100 μL of bleaching solution was transferred to another plate and the optical density (OD) was measured at 570 nm (OD_{570nm}) with a microtiter plate reader (Synergy™ HT, BioTek, USA). Experiments were carried out in quintuplicate on three different occasions. The mean OD values were analyzed and interpreted according to the criteria proposed by Stepanovic *et al*. (2007).

***Antibiofilm susceptibility testing*:** The effect of copaiba oil was evaluated against the established biofilm (after 24 h of incubation). After biofilm formation, the medium was aspirated off and sessile cells were gently washed with sterile PBS before adding fresh BHI+glu (200 μL) containing different concentrations of the copaiba oil, and the plates were incubated for further 24 h. For all assays, copaiba oil-free wells and biofilm-free wells were included as controls. The metabolic activity of treated sessile cells was compared to untreated cells using the 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT)-reduction assay. A 200-μL aliquot of XTT-menadione [0.5 mg/mL XTT, 1 mM menadione (Merck, Brazil)] was added to each well and the plates were incubated in the dark at 37°C for 90 min. The supernatants were transferred to a new microtiter plate and the OD_{490} was measured with a microtiter plate reader (Morguette *et al*., 2019). Experiments were carried out in quintuplicate on two different occasions.

***Confocal laser scanning microscopy*:** Bacterial biofilms were formed on CELLview™ cell culture dish with glass bottom (Greiner Bio One, Brazil) as described above. The biofilms were gently washed once with sterile 0.85% NaCl solution (saline) and were treated with copaiba oil at MIC and 2xMIC for 24 h at 37°C. The biofilms were washed and incubated in 1 mL of PBS containing SYTO® 9 (10 μM) and propidium iodide (10 μM) from the Live/DEAD® *Bac*Light™ kit at 4°C for 15 min in the dark. After washing and drying, the copaiba oil-treated and -untreated biofilms were observed in confocal laser scanning microscope (CLSM, Leica Microsystems, Germany) with the excitation and emission wavelengths, respectively: 483 and 500 nm for SYTO® 9 and 490 and 635 nm for propidium iodide, both with long-pass filter.

***Scanning electron microscopy*:** Morphological alterations induced by copaiba oil on biofilm of *E. faecium* were analyzed by scanning electron microscopy (SEM). Strips of polystyrene (surface area 0.5 cm^2) were aseptically cut and placed in wells of 24-well tissue culture plates (Techno Plastic Products, Switzerland). An inoculum (3.0 x 10^6 cells) was prepared in 1 mL of BHI+glu and used to form biofilms. The strips were then immersed in these cell suspensions and incubated statically at 37 ºC for 24 h. Afterwards, non-adherent bacteria were removed by gently washing three times with PBS before adding fresh 1.0 mL BHI+glu containing copaiba oil at MIC and 2xMIC values, and the plates were incubated for further 24 h. Copaiba oil-treated and -untreated biofilms were fixed with 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 4 h, and post-fixed in 1% OsO_4 for 2 h. The cells were dehydrated with a series of ethanol washes (30, 50, 70, 90 and 100%), critical-point dried in CO_2 (BALCTED CPD 030 Critical Point Dryer), coated with gold (BALTEC SDC 050 Sputter Coater) and observed under a FEI Quanta 200 scanning electron microscope.

***Cytotoxicity to mammalian cells*:** The toxicity of copaiba oil was evaluated on LLC-MK2 cells (*Macaca mulata* kidney epithelial cells, ATCC® CCL-7™). Epithelial cells were grown in Roswell Park Memorial Institute 1640 medium (RPMI, Invitrogen-Gibco, USA) supplemented with 10% (v/v) heat-inactivated f*et al* bovine serum, 2 mM *L*-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B. The cells were grown in a flat-bottomed 96 well microtiter plate at a density of 2.5 x10^4 cells/well for 24 h in 5% CO_2 at 37°C. At confluence, non-adherent cells were removed by washing with sterile PBS. The medium (200 μL), containing different concentrations of copaiba oil (0.002 – 1.0 mg/mL), was added and the plates were incubated for 24 h. The metabolic activity of the cells was determined by the dimethylthiazol diphenyl tetrazolium bromide (MTT, Merck, Brazil) reduction method according to the manufacturer's recommendations. The concentration of the compounds which inhibited the metabolic activity of the cells number by 50% was determined by regression analysis and corresponds to the 50% cytotoxic concentration ($CC_{50/24h}$).

***Statistical analysis*:** Data were analyzed using GRAPHPAD Prism software version 6.0 (GRAPHPAD Software, San Diego, CA). Kruskal-Wallis and Dunn's multiple comparison *post-hoc* tests were used to analyze MIC values and time-kill curves. One-way ANOVA and Tukey's multiple comparison *post-hoc* tests were used to analyze biofilm data. Two-way ANOVA and Bonferroni *post-hoc* tests were used to analyze the differences between the biofilm treatments. *p* values less than 0.05 were considered significant in all cases.

RESULTS AND DISCUSSION

Copaifera martii Hayne oleoresin exhibits bactericidal activity against planktonic cells of Enterococcus faecium: In the present study, the antibacterial effect of *C. martii* oleoresin toward *E. faecium*, including VRE*fm,* was reported for the first time. This oleoresin inhibited the planktonic growth of all *E. faecium* strains. Except for VR*Efm*161, whose MIC value was 0.03 mg/mL, for the other strains of VR*Efm* and VSE*fm* the MIC values were 0.06 mg/mL (Table 1). In a previous study, Pfeifer Barbosa *et al*. (2019) described the inhibitory effect of *C. reticulata* oleoresin on growth of planktonic cells of *E. faecium* DSM 20477, displaying a MIC_{50} (MIC capable to inhibit 50% of the cells) value of 0.0042 mg/mL. Most of the biological effects of oleoresin from *Copaifera* species have been attributed to their sesquiterpene and diterpene contents (Abrão *et al*., 2015; Tobouti *et al*., 2017; Dalenogare *et al*., 2019; Pfeifer Barbosa *et al*., 2019; Morguette *et al*., 2019). The terpene content of different copaiba oils has already been described in the literature, and by using high-resolution chromatographic analysis our research group was able to determine that *C. martii* oleoresin collected in Tapará is mainly composed of the sesquiterpenes beta-bisabolene (10.7%) and alfa-zingiberene (7.2%) and the diterpenes kaurenoic (7.9%) and kovalenic (29.0%) acids (Santos *et al*., 2008b). On the other hand, Pfeifer Barbosa *et al*.(2019) identified the presence of beta-bisabolene (42.3%), caryophyllene oxide (18.7%), cis-α-bergamotene (11.7%) and α-caryophyllene (10.0%) as the main sesquiterpenes; and agathic acid (0.92%), (13E)-labd-8(17)-en-15,18-dioic acid (1.96%), polyalthic acid (3, 51.6%), kaurenoic acid (21.6%), kolavenic acid (8.52%) and (13E)-labda-7,13-dien-15-13 were the main diterpenes in *C. reticulata*. In addition, these authors reported that (13E)-labda-7,13-dien-15-oic acid, kaurenoic acid and kolavenic acid were the most active phytochemicals against *E. faecium*.

We also evaluated the effect of kaurenoic acid on the growth of VR*Efm* and VS*Efm*, and the MIC values ranged from 0.025 to 0.050 mg/mL (Table 1), which were comparable to the values obtained for *C. marttii* oleoresin, indicating that the antibacterial activity may be attributed to this compound. However, we cannot rule out the possibility that the antibacterial effect observed against *E. faecium* is due to the combined effect of several phytochemicals present in the *C. marttii* oleoresin. To identify the nature of the antibacterial effect of copaiba oil on *E. faecium*, planktonic cells of VS*Efm* (Figure 1-A1) and VR*Efm* UEL-170 (Figure 1-B1) were grown in presence of MIC values and CFU counts were determined at time-intervals during 24 h. Overall, a gradual decrease in CFU counts was observed over the time and 100% of both strains were killed after 24 h of copaiba oil treatment ($p<0.01$), indicating a time-dependent bactericidal effect. The bactericidal effect of *C. Martii* oleoresin also collected in Tapará, Pará, Brazil has been described for other Gram-positive bacteria, such as *Staphylococcus aureus* (including methicillin-resistant *S. aureus*), *Staphylococcus epidermidis*, *Bacillus subtilis* and *Enterococcus faecalis*.

Table 1. Characteristics of *Enterococcus faecium*: clinical source, antimicrobial susceptibility profile and biofilm formation capacity

Bacteria	Source[a]	Resistance[b]	Oleoresin[c]	Kaurenoic[c]	Biofilm[d]
ATCC6569	F		0.06	0.05	+
UEL-170	U	amp,cip,eri,str,tet,tei,van	0.06	0.025	+++
5	S	amp,cip,eri,tet,tei,van	0.06	0.025	++
15	RS	amp,cip,eri,tet,tei,van	0.06	0.05	+++
17	U	amp,cip,eri,tei,van	0.06	0.05	+
35	U	amp,cip,eri,tet,tei,van	0.06	0.05	++
37	B	amp,cip,eri,tet,tei,van	0.06	0.05	+
47	U	amp,cip,eri,tet,tei,van	0.06	0.05	+
158	RS	amp,cip,eri,gen,rif,tei,van	0.06	0.05	+
161	RS	amp,cip,eri,gen,rif,tei,van	0.03	0.025	++
162	RS	amp,cip,eri,tet,tei,van	0.06	0.025	++
167	B	amp,cip,eri,tet,tei,van	0.06	0.05	+
172	U	amp,cip,eri,tet,tei,van	0.06	0.025	+++
176	RS	amp,cip,eri,tet,tei,van	0.06	0.05	++

[a]Source of bacterial strain – B: blood; F: feces; RS: rectal swab; S: scrotum secretion; U: urine.[b]The antimicrobial susceptibility profile was previously determined by the automated broth microdilution panel of the MicroScan Walk Away 96 Instrument (Ruzon *et al.,* 2010). amp: ampicillin; cip: ciprofloxacin; eri: erythromycin; gen: gentamicin; rif: rifampicin; str: streptomycin; tei: teicoplanin; tet: tetracycline; van: vancomycin. [c]Minimal inhibitory concentrations of *C. marttii* oleoresin and kaurenoic acid were determined by broth microdilution according to the CLSI (2015) recommendations, with minor modifications.The values are expressed as mg/mL.[d]24 h-biofilm was formed on polystyrene surface and the biomass was estimated by the crystal violet assay. A semi-quantitative classification was determined according to Stepanovic et al. (2007) +, weak biofilm-producer; ++, moderate biofilm-producer; +++, strong biofilm-producer.

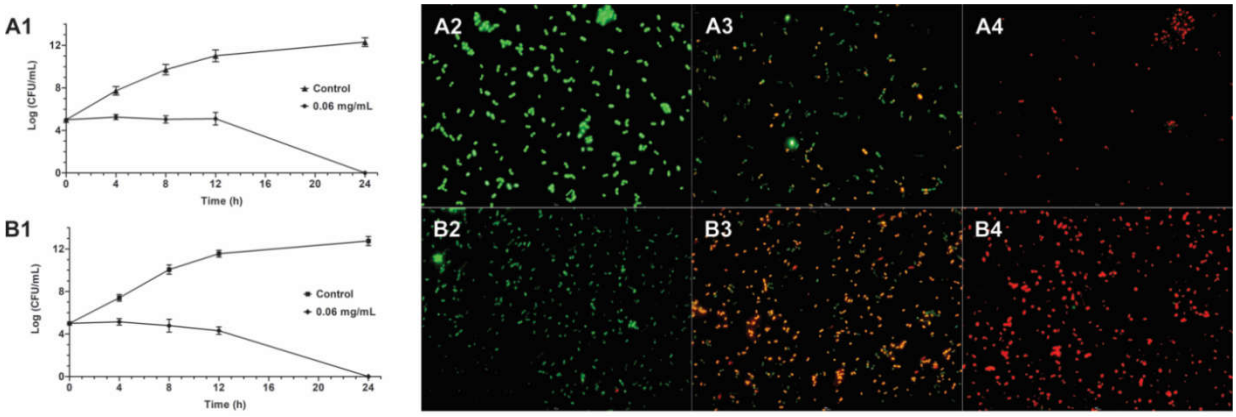

Figure 1. Bactericidal activity of *Copaifera marttii* oleoresin against *Enterococcus faecium* susceptible (*E. faecium* ATCC 6569, VS*Efm*) and resistant to vancomycin (VR*Efm* UEL-170). Growth kinetics of VS*Efm* (A1) and VR*Efm* (B1) in presence of the oleoresin at MIC (0.06 mg/mL) at 37 °C. The CFU counts were determined at specified time points until 24 h. Metabolic activity of the cells were determined with LIVE/DEAD® BacLight™ staining kit. Untreated cells of VS*Efm* (A2) and VR*Efm* (B2) were green-fluorescent, indicating the presence of metabolically active cells with intact membrane integrity. After 6 and 24 h-incubation in presence of MIC, VS*Efm* (A3 and A4, respectively) and VR*Efm* (B3 and B4, respectively) were red-fluorescent, reflecting dead bacteria with damaged membranes. Magnification: 1,000x

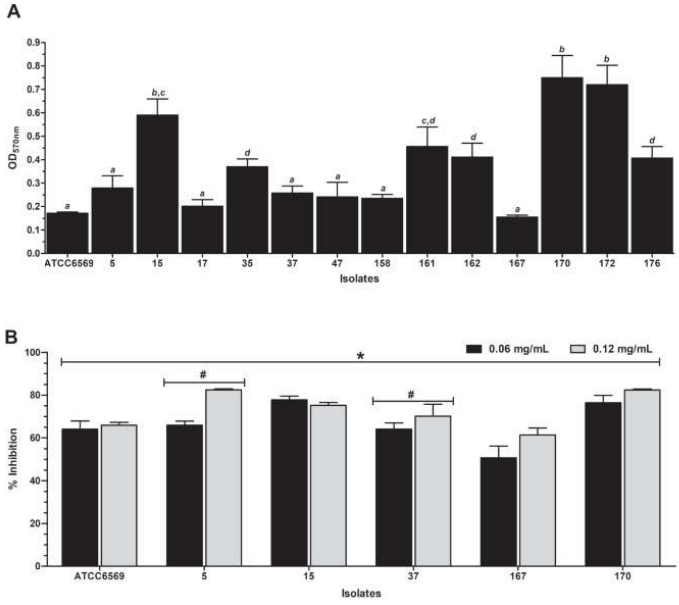

Figure 2. Antibiofilm activity of *Copaifera marttii* oleoresin on biofilms of *Enterococcus faecium*. (A) Total biofilm biomass on polystyrene surface after 24 h of incubation at 37 ºC and quantified using a crystal violet-based assay. Bars not sharing a letter differ significantly (p<0.05). (B) Inhibitory activity of the oleoresin, as assessed by XTT-reduction assay, on established biofilm of VS*Efm* (*E. faecium* ATCC 6569) and VR*Efm* (5, 15, 37, 167, 170 strains). Values are mean ± standard deviation of two experiments. Asterisk (*) indicates a significant reduction of metabolically active sessile cells treated with *C. marttii* oleoresin compared to untreated cells (p<0.05). Hashtag (#) indicates a significant difference between MIC and 2xMIC treatments (p<0.01)

The MIC values ranged from 0.016 to 0.063 mg/mL, being *B. subtilis* the most susceptible (Santos *et al*., 2008a). A previous study reported that the bactericidal effect of *C. martii* oleoresin on planktonic cells of *S. aureus* was due to the disruption of its cell membrane and cell wall, resulting in leakage of intracellular components (Santos *et al*., 2008a). Although the mechanism by which the oleoresin from *C. martii* induced *E. faecium* death was not investigated in this study, its effect on bacterial cell membrane integrity was also observed. Thus, copaiba oil-untreated and -treated planktonic cells were visualized after incubation with a combination of two fluorescent nucleic acid dyes, SYTO® 9 (green-fluorescent dye that labels live and dead bacteria) and propidium iodide (red-fluorescent dye that selectively labels bacteria with permeable/damaged membranes). At 6 h of incubation in presence of MIC value of copaiba oil most planktonic VS*Efm* (Figure 1-A3) and VR*Efm* (Figure 1-B3) showed red fluorescence, reflecting dead bacteria with damaged membranes. After 24 h of incubation, no viable cells were detected, corroborating the bactericidal effect of oleoresin from *C. martii* on VS*Efm* (Figure 1-A4) and VR*Efm* UEL-170 (Figure 1-B4) strains. On the other hand, the majority of untreated cells was green-fluorescent, indicating the presence of metabolically active cells after 24 h-incubation (Figure 1-A2, VS*Efm* and Figure 1-B2, VR*Efm*).

Copaifera martii Hayne oleoresin reduces the biomass and cell viability of established biofilms of Enterococcus faecium: Along with the antimicrobial resistance of planktonic cells, biofilms are among the main challenges of infectious diseases therapy. Bacteria within these communities (sessile cells) can resist antimicrobial concentrations higher than those required to kill planktonic free-floating counterpart cells, contributing to both the persistence of infection and high mortality rates (Ch'ng *et al*., 2019). In this study, all *E. faecium* strains were capable of forming biofilms on polystyrene surface.The biomass of 24-h biofilms was measured after crystal violet staining, and the OD_{570nm} ± standard deviation ranged from 0.16 ± 0.01 to 0.75 ± 0.10. Significant differences (p<0.05) in biofilm biomass were observed among the different strains (Figure 2A), and three categories were detected according to the Stepanovic *et al*. (2007) criteria (Table 1). Five VR*Efm* (5, 15, 37, 167, 170) and the VS*Efm* strains were selected to evaluate the effect of *C. martii* oleoresin on biofilms. The VR*Efm* were selected according to the categories established for biofilm formation capacity. The oleoresin treatment resulted in a considerable (p<0.05) reduction of metabolically active cells within the established biofilms of all strains. At 0.06 mg/mL and 0.12 mg/mL, which corresponded respectively to MIC and 2xMIC values, for planktonic cells of all strains, a mean percentage reduction ranged from 35.6 to 83.0% and 80.3 to 99.5%, respectively (Figure 2B.). To further confirm the effect of copaiba oil on *E. faecium* biofilms, the cell viability within the 24 h-biofilms of VS*Efm* and VR*Efm* UEL-170 strains was analyzed by CLSM after staining with the SYTO® 9 and propidium iodide. Copaiba oil-untreated biofilms of both strains consisted of a dense aggregate of intensely greenish cells, indicating the presence of metabolically active cells (Figure 3 A1-A3 and D1-D3). Conversely, following the treatment with copaiba oil at 0.06 mg/mL (Figure 3 B1-B3 and E1-E3) and 0.12 mg/mL (Figure 3 C1-C3 and F1-F3), biofilms showed reduced biomass and metabolic activity, as judged by an increase in reddish cells. SEM images also revealed a robust biofilm of untreated VS *Efm* (Figure 4A) and VR*E fm* (Figure 4D) strains with cells exhibiting typical spherical morphology after 24 h-incubation.

In contrast, a remarkable decrease in the number of cells within the biofilms treated with copaiba oil at MIC (Figure 4B and E) and 2xMIC (Figure 4C and F) was readily visualized. Moreover, withered cells were observed in both biofilms in presence of copaiba oil. A limited number of studies have sought to analyze the effect of copaiba oil on bacterial biofilms. The diterpene copalic acid from *C. langsdorfii* reduced 50% of biofilm biomass in concentrations of 0.063 mg/mL for *S. aureus*, *Staphylococcus capitis*, *Streptococcus pneumoniae*; 0.25 mg/mL for *Staphylococcus haemolyticus*; 1.0 mg/mL for *E. faecalis*; and 2.0 mg/mL for *S. epidermidis* (Abrão *et al*., 2015).

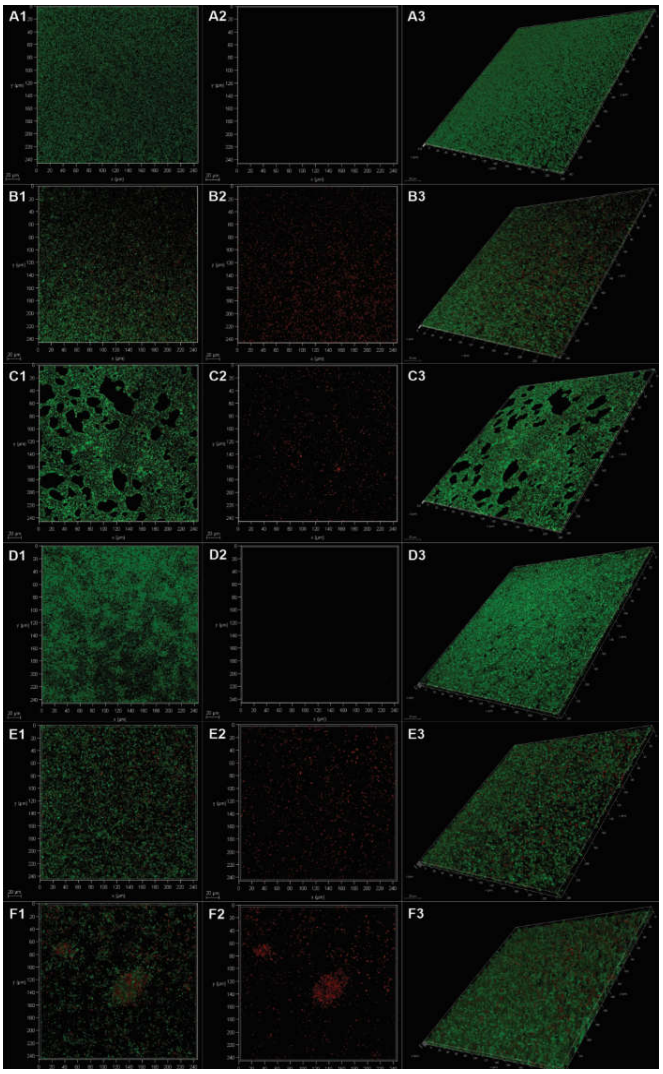

Figure 3. Confocal laser scanning microscopy images of 24 h-biofilm of *Enterococcus faecium* ATCC 6569 (VS*Efm*, A1-C3) and vancomycin resistant *E. faecium* UEL-170 (VR*Efm*, D1-F3). Biofilms were formed on CELLview™ cell culture dish with glass bottom during 24 h at 37 ºC before the treatments with MIC and 2MIC values. Cells were stained with SYTO 9 (green-fluorescent) and propidium iodide (red-fluorescent). Untreated biofilms (A1-A3 and D1-D3); treated with MIC (B1-B3 and E1-E3) and with 2xMIC (C1-C3 and F1-F3). Panoramic view of biofilms labeled with SYTO 9 (A1, B1, C1, D1, E1, F1). Panoramic view of biofilms labeled with propidium iodide (A2, B2, C2, D2, E2, F2). Three-dimensional biofilms reconstitution (A3, B3, C3, D3, E3, F3)

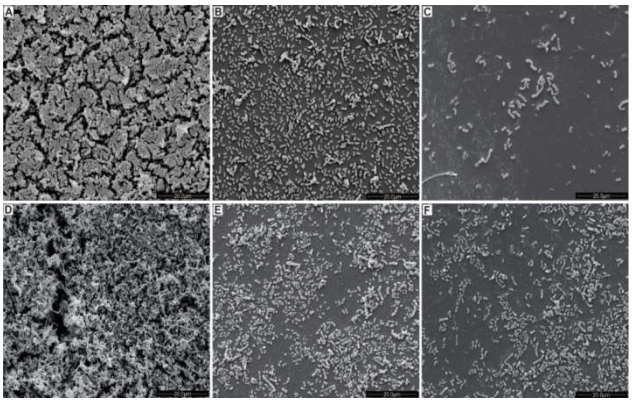

Figure 4. Scanning electronic microscopy images of *Enterococcus faecium* ATCC 6569 (VS*Efm*, A-C) and vancomycin resistant *E. faecium* UEL-170 (VR*Efm*, D-F) biofilms formed on polystyrene surface for 24 h at 37ºC. Untreated biofilms (A and D), treated with MIC (B and E) and with 2MIC (C and F). Bars: 20 µm.

The *C. oblongifolia* oleoresin reduced the biomass of mature bacterial biofilms associated with oral diseases, with sessile MIC_{50} values of 0.4 mg/mL for *Lactobacillus casei* and *Peptostreptococcus micros*; 0.2 mg/mL for *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans*; and 0.1 mg/mL for *Streptococcus mitis* and *Porphyromomas gingivalis* (da Moraes *et al*., 2016). The treatment of mature biofilms of *Streptococcus agalactiae* with *C. multijuga* oleoresin at 0.5 and 1.0 mg/mL concentrations resulted in a mean percentage reduction of metabolic activity of sessile cells ranging from 19.40% ± 5.02 to 54.19% ± 11.37, and 23.66% ± 7.30 to 60.60% ± 0.05, respectively (Otaguiri *et al*., 2017). Finally, *C. officinalis* oleoresin also reduced the metabolic activity of mature biofilms of *Streptococcus agalactiae*, exhibiting sessile MIC_{80} ranging from 0.06 to 0.5 mg/mL (Morguette *et al*., 2019).

Copaifera martii Hayne oleoresin presents selective bactericidal activity toward Enterococcus faecium: To assess the effect of *C. martii* oleoresin on mammalian cells viability, LLC-MK2 cells were incubated in presence of increasing concentrations of copaiba oil. This plant compound did not display toxicity to these mammalian cells at concentrations that were effective in killing planktonic and biofilm cells of *E. faecium* strains, indicating that its membrane-damaging activity might not target the mammalian cell membrane at bactericidal concentrations (data not shown). At concentrations of 0.12 mg/mL, only a slight reduction (8.3%) in mammalian cell viability was observed. However, at 0.5 mg/mL no viable cells were observed. After 24 h of incubation with copaiba oil, the estimated CC_{50} was 0.35 mg/mL. Based on CC_{50} and MIC, SI value of 5.8 was detected, indicating that *C. martii* oleoresin was more toxic toward *E. faecium*. Previous studies have reported the safety and biocompatibility to mammalian cells and tissues of oleoresin from various *Copaifera* species *in natura* or incorporated into pharmaceutical formulations and applied by different routes. For instance, the oral administration of *C. multijuga* and *C. reticulata* (Gomes *et al*., 2007) and *C. officinalis* (Dalenogare *et al*., 2019) oleoresins in mice did not provoke acute toxicity, nor any lesion in gastric mucosa after five and seven days of treatment, respectively. Moreover, the oral administration of *C. martii* (dos Santos *et al*., 2011) and *C. officinalis* (Dalenogare *et al*., 2019) oleoresins did not cause genotoxicity or mutagenicity in mice. These findings were further supported by Damasceno *et al*. (2019) who described that oleoresin, leaf extract and kaurenoic acid from different *Copaifera* species did not exhibit mutagenic activity by Ames test. In rats, acute toxicity was also not observed after oral administration of *C. reticulata* oleoresin (Teixeira *et al*., 2017) and by intraperitoneal route, this oleoresin induced neuroprotection after an acute injury of the motor cortex (Guimarães-Santos *et al*., 2012). Lastly, the addition of copaiba oil in formulation, such as: a) vaginal cream (*C. duckei*) did not cause maternal or f*et al* toxicity in pregnant rats (Lima *et al*., 2011); b) ointment (*C. langsdorfii*) improved the tissue repair in a dorsal cutaneous rat flap model (Estevão *et al*., 2013); c) eye drop (*C. multijuga*) induced corneal epithelization without damage to the ocular surface in rats with alkali-induced acute superficial corneal ulcers. Moreover, oleoresin treatment did not cause cytotoxicity and genotoxicity in rats (Gonçalves Dias *et al*., 2017); d) vaginal hydrogel (*C. officinalis*) did not cause damage in cervicovaginal mucosa of mice (Morguette *et al*., 2019).

CONCLUSION

The results of the present study revealed the bactericidal effect of *C. martii* Hayne oleoresin on planktonic and biofilm cells of VSE*fm* and VRE*fm* strains, indicating its potential for the development of new strategies for the treatment of infections caused by these bacteria.

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REFERENCES

Abrão, F., de Araújo Costa, L.D., Alves, J.M., Senedese, J.M., de Castro, P.T., Ambrósio, R.S., Veneziani, R.C.S., Bastos, J.K., Tavares, D.C., and Martins, C.H.G. 2015. *Copaifera langsdorffii* oleoresin and its isolated compounds: antibacterial effect and antiproliferative activity in cancer cell lines. *BMC Complement Altern Med,* 15, pp.443.

Andrade, G., Orlando, H.C.S., Scorzoni, L., Pedroso, R.S., Abrão, F., Carvalho, M.T.M., Veneziani, R.C.S., Ambrósio, S.R., Bastos, J.K., Mendes-Giannini, M.J.S., Martins, C.H.G., and Pires, R.H. 2020. Brazilian *Copaifera* species: Antifungal Activity against Clinically Relevant *Candida* Species, Cellular Target, and *in vivo* Toxicity. *J Fungi Basel,* 6, pp.153.

Ch'ng, J-H. Chong, K.K.L. Lam, L.N., Wong, J.J., and Kline, K.A. 2019. Biofilm-associated infection by enterococci. *Nat Rev Microbiol,* 17, pp.82-94.

Clinical and Laboratory Standards Institute. Methods for determining bactericidal activity of antimicrobial agents; Approved Guideline. CLSI document M26-A. Wayne, PA: CLSI, 1999.

Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard - Tenth Edition. CLSI document M07-A10. Wayne, PA: CLSI, 2015.

da Moraes, T.S., Leandro, L.F., de O. Silva, L., Santiago, M.B., Souza, A.B., Furtado, R.A., Tavares, D.C., Veneziani, R.C.S., Ambrósio, S.R., Bastos J.K., and Martins, C.H.G. 2016. *In vitro* Evaluation of *Copaifera oblongifolia* Oleoresin Against Bacteria Causing Oral Infections and Assessment of Its Cytotoxic Potential. *Curr Pharm Biotechnol,* 17, pp.894-904.

Dalenogare, D.P., Ferro, P.R., de Prá, S.D.T., Rigo, F.K., de David Antoniazzi, C.T., de Almeida, A.S., Damiani, A.P., Strapazzon, G., de Oliveira Sardinha, T.T., Galvani, N.C., Boligon, A.A., de Andrade, V.M., da Siva Brum, E., Oliveira S.M., and Trevisan, G. 2019. Antinociceptive activity of *Copaifera officinalis* Jacq. L oil and kaurenoic acid in mice. *Inflammo pharmacology*, 27, pp. 829-844.

Damasceno, J.L., Arnet, Y.F., Fortunato, G.C., Girotto, L. Marena, G.D., Rocha, B.P., Resende, F.A., Ambrósio, S.R., Veneziani, R.C.S., Bastos J.K., and Martins, C.H.G. 2019. Investigation of Safety Profile of Four *Copaifera* species and of Kaurenoic Acid by *Salmonella*/Microsome *Test. Evid Based Complement Alternat Med,* 2019, pp.7631531.

dos Santos, A.O. Costa, M.A. Ueda-Nakamura, T. Dias-Filho, B.P. da Veiga-Júnior, V.F. de Souza Lima M.M. and Nakamura, C.V. 2011. *Leishmania amazonensis*: effects of oral treatment with copaiba oil in mice. *Exp Parasitol,* 129, 145-151.

Estevão, L.R., Medeiros, J.P., Baratella-Evêncio, L., Simões, R.S., Mendonça, F.de S., and Evêncio-Neto, J. 2013. Effects of the topical administration of copaiba oil ointment *Copaifera langsdorffii* in skin flaps viability of rats. *Acta Cir Bras*, 28, pp.863-869.

García-Solache, M. and Rice, L.B. 2019. The *Enterococcus*: a Model of Adaptability to its Environment. *Clin Microbiol Rev*, 32, pp.e00058-18.

Gomes, N.M., Rezende, C.M., Fontes, S.P., Matheus M.E., and Fernandes, P.D. 2007. Antinociceptive activity of Amazonian Copaiba oils. *J Ethnopharmacol*, 109, pp. 486-492.

Gonçalves Dias, G.G., Jorge, A.T., de Freitas Pereira, L., Furtado, R.A., Ambrósio, S.R., Bastos, J.K., Ramos, S.B., Chahud, F., Dias, L.G.G.G., Honsho, C.S., and Tavares, D.C. 2017. Use of *Copaifera multijuga* for acute corneal repair after chemical injury: A histopathological and toxicogenetic study. *Biomed Pharmacother,* 96, pp.1193-1198.

Guimarães, A.L., Cunha, E.A., Matias, F.O., Garcia, P.G., Danopoulos, P., Swikidisa, R., Pinheiro V.A., and Nogueira, R.J.L. 2016. Antimicrobial Activity of Copaiba *Copaifera officinalis* and Pracaxi *Pentaclethra macroba* Oils against *Staphylococcus aureus*: Importance in Compounding for Wound Care. *Int J Pharm Compd*, 20, pp. 58-62

Guimarães-Santos, A., Santos, D.S., Santos, I.R., Lima, R.R., Pereira, A., de Moura, L.S., Carvalho Jr, R.N., Lameira O., and Leal, W.G. 2012. Copaiba oil-resin treatment is neuroprotective and reduces neutrophil recruitment and microglia activation after motor cortex excitotoxic injury. *Evi Based Complement Alternat Med,* 2012, pp.918174.

Lima, C.S., de Medeiros, B.J.L., Favacho, H.A.S., dos Santos, K.C., de Oliveira, B.R., Taglialegna, J.C., da Costa, E.V.M., de Campos, K.J., andCarvalho, J.C.T. 2011. Pre-clinical validation of a vaginal cream containing copaiba oil reproductive toxicology study. Phytomedicine, 18, pp.1013-1023.

Miller, W.R., Murray, B.E., Rice, L.B., and Arias, C.A. 2020. Resistance in Vancomycin-Resistant Enterococci. Infect Dis Clin North Am, 34, pp.751-771.

Miranda, M.M., Panis, C., da Silva, S.S., Macri, J.A., Kawakami, N.Y., Hayashida, T.H., Madeira, T.B., Acquaro Jr, V.R., Nixdorf, S.L., Pizzatti, L., Ambrósio, S.R., Cecchini, R., Arakawa, N.S., Verri Jr, W.A., Costa, I.C., and Pavanelli, W.R. 2015. Kaurenoic Acid Possesses Leishmanicidal Activity by Triggering a $NLRP_{12}$/IL-1β/cNOS/NO Pathway. Mediators Inflamm, 2015, pp.392918.

Morguette, A.E.B., Bigotto, B.G., Varella, R.L., Andriani, G.M., Spoladori, L.F.A., Pereira, P.M.L., de Andrade, F.G., Lancheros, C.A.C., Nakamura, C.V., Syogo Arakawa, N., Bruschi, M.L., Tomaz, J., Lonni, A.A.S.G., Kerbauy, G., Tavares, E.R., Yamauchi, L.M., and Yamada-Ogatta, S.F. 2019. Hydrogel Containing Oleoresin From *Copaifera officinalis* Presents Antibacterial Activity Against *Streptococcus agalactiae*. Front Microbiol, 10, pp.2806.

Otaguiri, E.S., Morguette, A.E.B., Biasi-Garbin, R.P., Morey, A.T., Lancheros, C.A.C., Kian, D., de Oliveira, A.G., Kerbauy, G., Perugini, M.R.E., Duran, N., Nakamura, C.V., da Veiga, V.F., Nakazato, G., Pinge-Filho, P., Yamauchi, L.M., and Yamada-Ogatta, S.F. 2017. Antibacterial Combination of Oleoresin from *Copaifera multijuga* Hayne and Biogenic Silver Nanoparticles Towards *Streptococcus agalactiae*. *Curr Pharm Biotechnol,* 18, pp.177-190.

Pfeifer Barbosa, A.L., Wenzel-Storjohann, A., Barbosa, J.D., Zidorn, C., Peifer, C., Tasdemir, D., and Çiçek, S.S. 2019. Antimicrobial and cytotoxic effects of the *Copaifera reticulata* oleoresin and its main diterpene acids. J Ethnopharmacol, 233, pp.94-100

Ruzon, F.I., de Paula, S.B., Kanoshiki, R.L., Pereira-Santos, J. Kerbauy, G., Kobayashi, R.K.T., Yamauchi, L.M. Perugini, M.R.E., and Yamada-Ogatta, S.F. 2010 Virulence determinants in vancomycin-resistant *Enterococcus faecium vanA* isolated from different sources at University Hospital of Londrina, Paraná, *Brazil. J Microbiol,* 48, pp. 814-821.

Santos, A.O., Ueda-Nakamura, T., Dias-Filho, B.P., Veiga Júnior, V.F., Pinto A.C., and Nakamura, C.V. 2008a. Antimicrobial activity of Brazilian copaiba oils obtained from different species of the *Copaifera* genus. *Mem Inst Oswaldo Cruz,* 103, pp.277-278.

Santos, A.O., Ueda-Nakamura, T., Dias-Filho, B.P., Veiga Júnior, V.F., Pinto A.C., and Nakamura, C.V. 2008b. Effect of Brazilian copaiba oils on *Leishmania amazonensis*. *J Ethnopharmacol,* 120, pp.204-208.

Stepanovic, S., Vukovic, D., Hola, V., Di Bonaventura, G., Djukic, S., Cirkovic I., and Ruzicka, F. 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS,* 115, pp.891-899.

Tavares, E.R., da Silva, L.F., Morey, A.T., de Oliveira, A.G., da Rocha, S.P.D., Ribeiro, R.A., Hungria, M., Thihara, I.R.T., Perugini, M.R.E., Yamauchi, L.M., and Yamada-Ogatta, S.F. 2019. Draft Genome Sequence of Vancomycin-Resistant *Enterococcus faecium* UEL170 Sequence Type 412 Isolated from a Patient with Urinary Tract Infection in a Tertiary Hospital in Southern Brazil. *Microbiol Resour Announc*, 8, pp.e01365-18.

Teixeira, F.B., de Brito Silva, R., Lameira, A.O,.Webber, L.P., D'Almeida Couto R.S., Martins, M.D., and Lima, R.R. 2017. Copaiba oil-resin *Copaifera reticulate* Ducke modulates the inflammation in a model of injury to rats' tongues. *BMC Complement Alternat Med*, 17, pp.313.

Tobouti, P.L., de Andrade Martins, T.C., Pereira T.J., and Mussi, M.C.M. 2017. Antimicrobial activity of copaiba oil: A review and a call for further research. *Biomed Pharmacother*, 94, pp.93-99.

Veiga Júnior, V.F. and Pinto, A.C. 2002. O gênero *Copaifera* L. Quimica Nova, 25, pp.273-286.

World Health Organization. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Geneva: WHO; 2017. https://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/

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