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Antibacterial Activity of the Plant-Derived Compounds 23-Methyl-6-*O*-desmethylauricepyrone and (*Z*,*Z*)-5-(Trideca-4,7-dienyl)resorcinol and Their Synergy with Antibiotics against Methicillin-Susceptible and -Resistant *Staphylococcus aureus*

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ABSTRACT: The present study investigated the antibacterial activity of two plant-derived compounds, 23-methyl-6-*O*-desmethylauricepyrone (**1**) and (*Z*,*Z*)-5-(trideca-4,7-dienyl)resorcinol (**2**), and their synergistic effects with erythromycin and gentamicin against methicillin-susceptible (MSSA) and gentamicin- and methicillin-resistant *Staphylococcus aureus* (MRSA). Studies of the individual antibacterial activity of each plant-derived compound and synergy experiments were carried out, by the microdilution test in agar and by the checkerboard method, respectively. Compound **1** showed minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 2 and 8 μg/mL, respectively, against both strains of *S. aureus*, while compound **2** exhibited anti-MSSA and anti-MRSA activity with MICs and MBCs of 4 and 8 and 2 and 8 μg/mL, respectively. Time-kill curves showed that, while compound **1** produced complete killing of both strains at 24 h from the beginning of the experiment, **2** produced the same effect in the first hour. Combinations of **1** with erythromycin or gentamicin showed a notable synergism against MSSA, which enabled the antibiotic concentration to decrease by up to 300 or 260 times, respectively. When the aminoglycoside was placed together with compound **2,** only an additive effect was observed. The assayed compounds did not produce erythrocyte hemolysis or genotoxicity and they did not affect macrophage viability at the effective or higher concentrations. These results suggest that both compounds could be considered as promising antibacterial agents while compound **1** could be used in combinatory therapies with erythromycin and gentamicin.

KEYWORDS: 23-methyl-6-*O*-desmethylauricepyrone, (*Z*,*Z*)-5-(trideca-4,7-dienyl)resorcinol, MRSA, MSSA, synergy

INTRODUCTION

Since the emergence of antibiotics, it has been possible to eradicate a wide range of infectious diseases that threaten human health. However, microorganisms, such as bacteria, have developed a wide variety of resistance mechanisms for evading their inhibitory effects.

As science has advanced toward the development of new therapeutic agents, the number of bacterial strains resistant to the last line of antibiotic defense has increased.[1] With the emergence of multidrug-resistant bacteria, the antibiotic of choice has had to be replaced or its dose increased leading to a parallel increase in its side effects.[2,3]

This global increase in bacterial resistance involves, among other bacteria, different *Staphylococcus aureus* strains. This bacterium causes a wide range of infectious diseases in humans and animals, from relatively mild skin infections to life-threatening conditions.[4,5] These bacterium has been frequently reported as a contaminant of a great variety of foods and beverages.[6] Methicillin-resistant *S. aureus* (MRSA) has become a major nosocomial pathogen in the past two decades,[7] and, together with the escalating rates of foodborne infections in ambulatory patients, this bacterium has come to be considered a major public health problem.[4] Despite the range of antibacterial agents currently available, the development of new therapeutic alternatives to deal with this pathogen is therefore of great interest to researchers and pharmaceutical industries,[8] and plant-derived products remain a rich source of new antibiotics.[9] In fact, plants synthesize a broad range of secondary metabolites that are toxic to microbial pathogens[10,11] and thus can be exploited as chemotherapeutics.[12] On some occasions, plant antibacterial metabolites show moderate or low potency. However, plants fight infections successfully due to the presence of synergism between their components,[1,13] either because these possess antibacterial properties themselves or because they act by increasing the solubility and/or resorption rate of the antimicrobial principles of the plant.[13] Therefore, it would be no surprise to find that many of these molecules can enhance the activity of conventional antibiotics.[14,15]

We have previously reported the isolation of two antibacterial compounds from two Argentine native plants, *Achyrocline satureioides* and *Lithrea molleoides*, which were identified as 23-methyl-6-*O*-desmethylauricepyrone [16] and (*Z*,*Z*)-5-(trideca-4,7-dienyl)resorcinol,[17] respectively.

Achyrocline satureioides (Lam.) DC. (Compositae), commonly known as "Marcela", is an aromatic annual herb native to South América.[18] Its infusion has been used for gastrointestinal inflammation and spasms, respiratory problems, gallbladder and liver disorders, viral infections and as antiatherosclerotic and sedative.[19] Many research studies have provided experimental evidence that its extracts may act as smooth muscle relaxant,[18]

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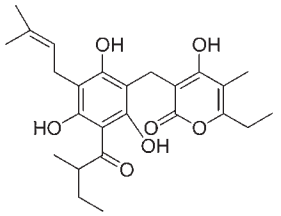

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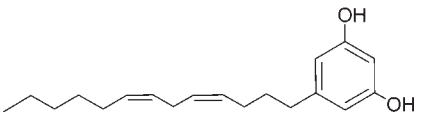

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Figure 1. Chemical structure of 23-methyl-6-*O*-desmethylauricepyrone (**1**) and (*Z*,*Z*)-5-(trideca-4,7-dienyl)resorcinol (**2**).

antioxidant and free radical scavenger,[20] cytoprotectant,[21] hepatoprotectant[22] and immunomodulator.[19]

Lithrea molleoides (Vell.) Engl. (Anacardiaceae), commonly named "molle de beber", is a native tree that is easily accessible and widely distributed in the central region of Argentina. Decoctions of this tree are used in the treatment of cough, bronchitis, and stomach disorders and as hemostatic, diuretic or tonic.[23,24] Biological properties of its extracts, such as inhibitory effects on tumor cell growth,[25] antiulcerogenic activity[23] and inhibitory effects against microorganisms including respiratory syncytial virus, herpes simplex virus type 1,[26] dermatophytes[27] and Gram-positive and -negative bacteria,[24] have been reported.

In this study, we report the inhibitory effect of both these isolated compounds against methicillin-susceptible and gentamicin- and methicillin-resistant *S. aureus,* and the synergistic behavior found between these compounds and gentamicin or erythromycin.

MATERIALS AND METHODS

Antibiotics and Isolated Compounds. Gentamicin sulfate (potency: 550−590 μg/mg) and erythromycin (potency: 863 μg/mg) were purchased from Laboratorio Fabra S.A. (Buenos Aires, Argentina) and Unifarma Laboratories (Buenos Aires, Argentina), respectively.

The antibacterial compounds 23-methyl-6-*O*-desmethylauricepyrone (**1**) (97% purity by HPLC, Figure 1) and (*Z*,*Z*)-5-(trideca-4,7-dienyl)-resorcinol (**2**) (92% purity by HPLC, Figure 1) were isolated from the ethanol extracts of *Achyrocline satureioides*[16] and *Lithrea molleoides*,[17] respectively.

Bacterial Strains. Methicillin-susceptible *S. aureus* (ATCC 6538) and gentamicin- and methicillin- resistant *S. aureus* (ATCC 33592) were purchased from Medicatec SRL (Buenos Aires, Argentina). Both strains were maintained in plate count agar (PCA) medium (Oxoid Ltd., Basingstoke, Hampshire, U.K.). Bacterial suspensions were prepared in sterile saline from each overnight organism grown. Turbidity was spectrophotometrically adjusted to the 0.5 McFarland standard. Dilutions with sterile saline were then carried out to give an adjusted concentration of 1.5×10^7 CFU/mL.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC). The MICs of the isolated compounds and of the commercial antibiotics were determined in multiwell plates by the agar microdilution test according to Joray et al.[16] Briefly, PCA culture medium was added to the appropriate amount of each pure compound or antibiotic, previously dissolved in ethanol or water as appropriate, to reach the final desired concentrations. The final concentration of ethanol was 2% (no adverse effects were observed at this concentration). After solidification, 2 μL of each bacterial suspension was placed on the agar surface. At least two replicates were used for each treatment. Plates containing only the culture medium, with or without the addition of the dissolution solvent, were used as controls for each bacterium studied. After incubation, the MIC, determined as the lowest concentration that produces complete growth inhibition of the bacteria, was visually determined. Nitro blue tetrazolium (Sigma-Aldrich Corporation, St Louis, MO) solution in saline phosphate buffer (PBS) was applied on each spot for confirmation.

To study the bactericidal effect, those portions of agar showing negative growth in the MIC experiments were transferred to brain−heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, Hampshire, U.K.). Tubes were incubated aerobically at 37 °C for five days. At the end of this period, the MBC values, defined as the lowest concentration with absence of turbidity, were recorded.

Time-Kill Analysis. These assays were conducted according to the technique described in Joray et al.[16] Briefly, BHI containing ethanol solutions (final concentration of ethanol: 1−2%) of the tested products in an amount sufficient to reach final concentrations ranging from MIC to 4 × MIC was inoculated with MSSA or MRSA suspensions to finally reach 5×10^5 CFU/mL. Controls containing only ethanol were simultaneously run. The suspensions were exhaustively mixed, and samples of each were removed, 100-fold diluted and plated onto PCA medium. Remaining suspensions were then incubated at 37 °C with shaking, and samples of each were taken at 1, 3, 6, or 24 h. The number of viable cells was determined at these times, after plating aliquots of undiluted and 10-fold serial dilutions of each sample onto PCA medium. Plates were then incubated for 24 h at 37 °C, and CFU were counted.

Electron Microscopy. For TEM, fresh cultures of bacteria grown for 24 h in the absence or presence of 8 μg/mL of compounds **1** or **2** were centrifuged at 9280 g for 10 min. The resulting sediment was resuspended, fixed with formaldehyde (4% v/v) and stained with 2% uranyl acetate. Stained bacteria were viewed and photographed with a Jeol JEM 1200 EX2 (JEOL USA Inc., Peabody, MA) electron microscope operated at 80 kV, and were analyzed with the digital imaging software, Digital Micrograph 4.2.

Checkerboard Assay. The interactions between the plant-derived compounds and the antibiotics were evaluated by the checkerboard method.[14,15] Bacterial growth inhibition resulting from the interactions was determined by the microdilution agar test. The concentrations of each tested agent used in the combinations corresponded to serial 2-fold dilutions from their MIC values. The fractional inhibitory concentration (FIC) was calculated using the MIC from the checkerboard assay and the MIC of each compound alone, obtained in parallel in the same assay, according to the following formula: FIC = MIC of antimicrobial agent in combination/MIC of antimicrobial agent alone.

Then, the synergistic effect was evaluated by calculating the FIC_{index} (FICI) for each combination, by adding the individual FIC values.[15] A FICI $\leq$0.5 indicated synergy for the combination.[15,28] When it fell between 0.5 and 1, it was defined as an additive effect,[7,29] and between 1.0 and 4.0 it was classified as "no interaction".[29] Finally, a FICI >4.0 indicated antagonism between the components in the combination.[1,28,29]

Determination of the Octanol−Water Partition Coefficient. The octanol−water partition coefficient, log *P*, was calculated using ChemBio Draw 11.0. The program estimates log *P* from the uncharged molecule with specific algorithms from fragment-based methods.

Erythrocyte Hemolysis Assay. With the aim of determining the effect of the isolated compounds on erythrocyte integrity, both compounds **1** and **2** previously dissolved in ethanol (4 μL) were added to 96 μL of BHI to reach final concentrations of 1−125 μg/mL. Then

Table 1. Antibacterial Activity of 23-Methyl-6-*O*-desmethylauricepyrone (1) and (*Z*,*Z*)-5-(Trideca-4,7-dienyl)resorcinol (2)

compd	MIC/MBC (μg/mL)	
	MSSA	MRSA
23-methyl-6-*O*-desmethylauricepyrone (**1**)	2/8	2/8
(*Z*,*Z*)-5-(trideca-4, 7-dienyl)resorcinol (**2**)	4/8	2/8
erythromycin	0.3/>40	>40/>40
gentamicin	8/10	16/32

50 μL of a 3% suspension of red blood cells (rbc) in PBS was added and incubated at 37 °C for 1 h with gentle agitation. Controls with ethanol were also carried out. The maximum concentration at which hemolysis was not observed was determined as the highest concentration value at which visible rbc pellet was observed.

***Allium cepa* Genotoxic Study.** The chromosomal aberration assay was performed according to a modified version of Teerarak et al.[30] Briefly, commercial bulbs of *Allium cepa* L., weighing from 65 to 74 g, were placed in containers with their basal ends dipped in distilled water until the emerging roots reached a uniform length (7 days). Then the roots were exposed for 24 h to compounds **1** and **2**, which were previously dissolved in acetone and added to the distilled water in the containers (the final concentration of the solvent in the recipients was 3%), reaching concentrations of 2, 4, and 8 μg/mL. Roots from onions of the control group were treated for the same period of time with acetone, finally reaching 3% concentration in the water of the containers. Three replicates were carried out for each treatment and the corresponding controls. After this period of time, the roots were transferred to distilled water, free of both compounds and left in the containers for 24 h. In the case of compound **1**, a further experiment was carried out in which bulbs were left in clean distilled water for a further 72 h. The presence of chromosome aberrations was investigated, and the mitotic index was calculated as the ratio between the number of dividing cells and the total number of cells scored (3000 cells per treatment, 1000 cells per slide) and expressed as a percentage.

Mammalian Macrophage Cytotoxicity Assay. The cytotoxicity of the isolated compounds was evaluated by MTT assay with modifications.[31] RAW 264.7 cells were cultured in RPMI medium supplemented with 10% FBS and maintained in a 37 °C humidified incubator containing 5% CO_2. Macrophages were passaged 24 h before the assay, and cell viability was determined by trypan-blue dye exclusion. Two microliters of the tested compounds previously dissolved in DMSO was added by triplicate to the first row of a 96-well microplate containing 200 μL of RPMI medium to finally reach a concentration of 64 μg/mL. Then, 2-fold serial dilutions were performed. Controls with DMSO were simultaneously performed. Finally, 1×10^4 cells were seeded per well. After 24 h incubation, 20 μL of MTT (Sigma-Aldrich Corporation, St. Louis, MO; 5 mg/mL in PBS) was placed in each well, and the plate was incubated for an additional 4 h. Then the supernatants were removed and replaced with 200 μL of DMSO to dissolve formazan crystals. Absorbance was measured at 595 nm.

Statistical Analysis. The results were analyzed statistically by the Kruskal–Wallis test and Dunn's post test at 0.05 significance level using InfoStat software (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Córdoba, Argentina).

RESULTS

Compounds 1 and 2 Showed Antibacterial Effects against MSSA and MRSA. In previous reports the antibacterial compounds **1** and **2** were isolated from the native plants *A. satureioides*[16] and *L. molleoides*,[17] respectively. The inhibitory activity against Gram-negative and -positive bacteria was previously investigated

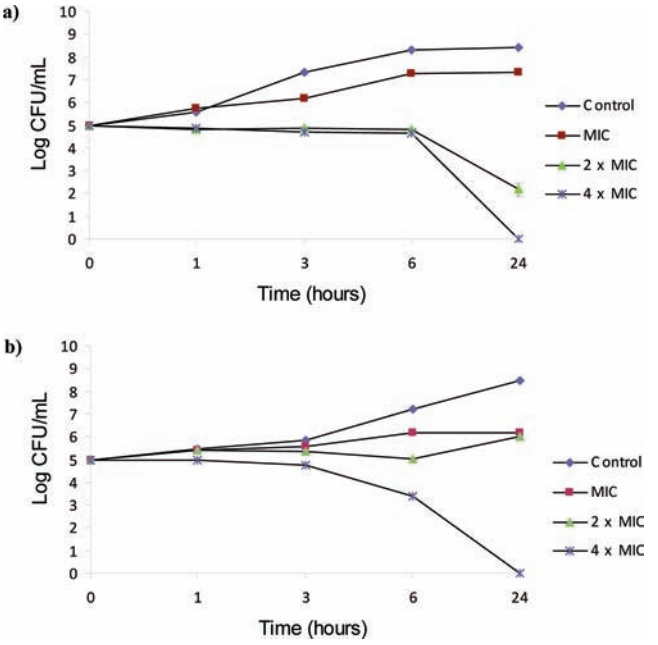

Figure 2. Time-kill kinetics of 23-methyl-6-*O*-desmethylauricepyrone (**1**) from minimum inhibitory concentration (MIC) to 4 × MIC against methicillin-susceptible *Staphylococcus aureus* (Figure 2a) and gentamicin- and methicillin-resistant *S. aureus* (Figure 2b) performed in brain–heart infusion incubated at 37 °C with agitation. Each symbol indicates the mean (±SE) for at least two replicates on separate occasions.

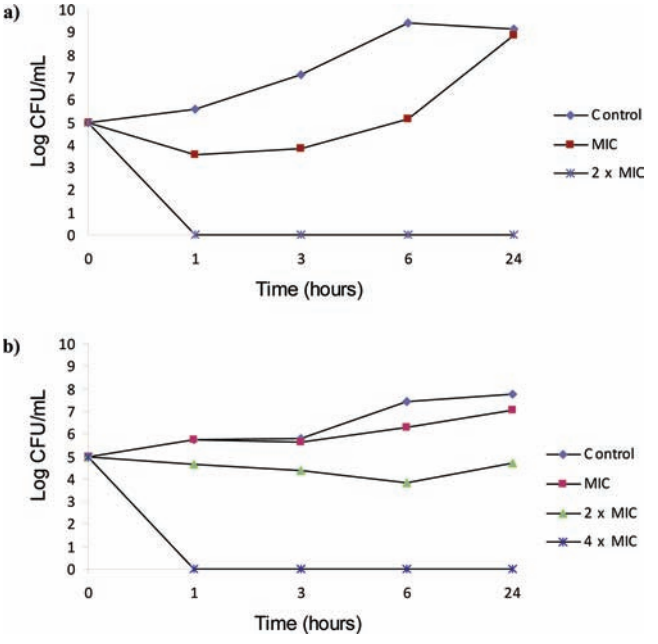

Figure 3. Time-kill kinetics of (*Z*,*Z*)-5-(trideca-4,7-dienyl)resorcinol (**2**) from minimum inhibitory concentration (MIC) to 4 × MIC against methicillin-susceptible *Staphylococcus aureus* (Figure 3a) and gentamicin- and methicillin-resistant *S. aureus* (Figure 3b) performed in brain–heart infusion incubated at 37 °C with agitation. Each symbol indicates the mean (±SE) for at least two replicates on separate occasions.

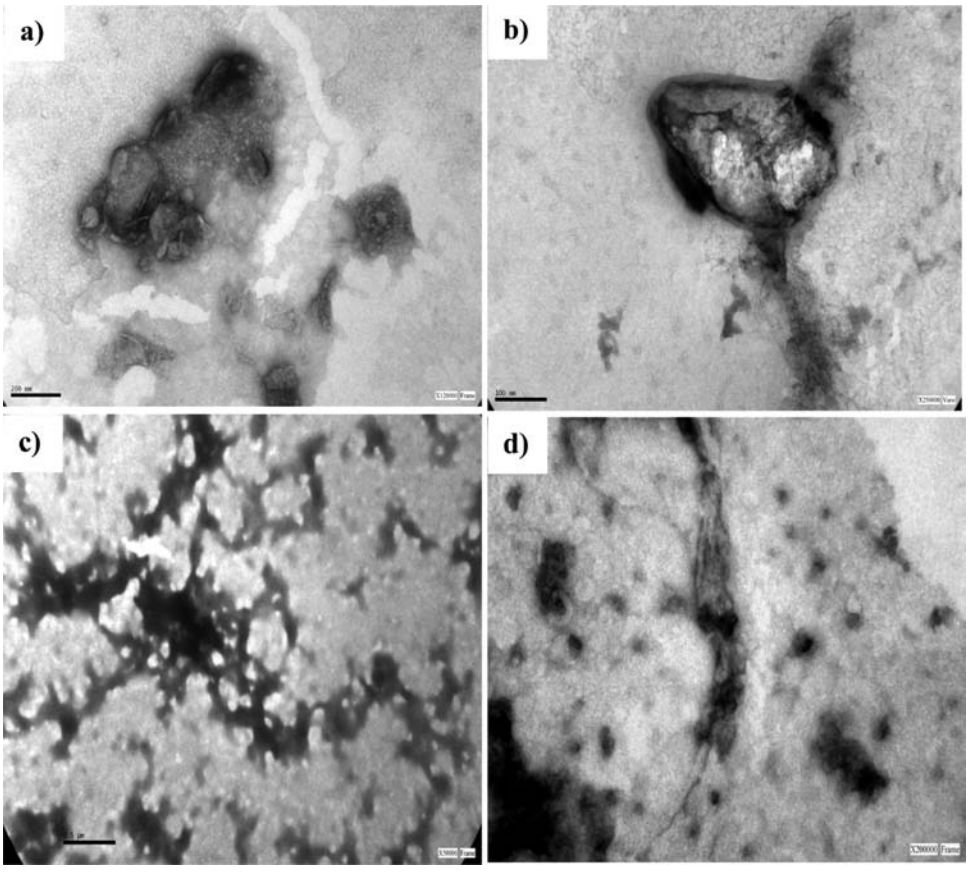

Figure 4. Effect on the cell morphology of methicillin-susceptible *Staphylococcus aureus* (a, c) and gentamicin- and methicillin-resistant *S. aureus* (b, d) treated with 8 μg/mL of 23-methyl-6-*O*-desmethylauricepyrone (**1**). Results observed at 24 h from the beginning of the experiment.

for compound **1**,[16] and the inhibition on *Proteus mirabilis* growth was determined for compound **2**.[17] This time the effectiveness of both substances against MSSA and MRSA was evaluated.

As observed in Table 1, compounds **1** and **2** showed MIC values of 2 and 4 μg/mL while the MBCs corresponded to 8 μg/mL against both MSSA and MRSA strains.

Time-Kill Study. When compound **1** was tested against susceptible *S. aureus* (Figure 2a), at 2 × MIC, a difference of 6.2 $\log_{10}$ in the number of viable cells compared to the control was observed after 24 h. At this time, a complete bactericidal effect was observed with treatments at 4 × MIC. When the phloroglucinol was assayed against the resistant strain, the treatments at MIC and 2 × MIC reached differences of ∼2.4 $\log_{10}$ with respect to the control after 24 h of exposure. When the compound was added at 4 × MIC, a difference of ∼3.8 $\log_{10}$ of viable cells with respect to control was observed at 6 h, producing a total bacterial killing at 24 h (Figure 2b).

As observed in Figure 3a, susceptible *S. aureus* treated with compound **2** at the MIC value showed a decrease of ∼1.4 and 1.2 $\log_{10}$ in the number of viable cells at the first and third hour from the beginning of the experiment, respectively. At 24 h, a recovery in the number of bacteria was detected and no significant differences were observed with respect to the control. However, when this compound was added at twice the MIC, 100% bacterial death was observed at the first hour from the beginning of the assay (Figure 3a).

The same compound was tested against the resistant strain. In treatments at 2 × MIC, the number of viable cells remained at the initial value until 24 h, in contrast to that observed in the control group ($p > 0.05$). At 4 × MIC a bactericidal effect was observed at the first hour of treatment (Figure 3b).

Electron Microscopy. In electronic micrographs, compound **1** treated susceptible and resistant *S. aureus* revealed breaks in the walls of cells (Figure 4a,b) with loss of internal cell material and evident cell debris (Figure 4c,d). Treatments with compound **2** showed altered cell membranes lacking cell contents (Figure 5a,b) with remains of lysed cells (Figure 5c,d). As seen, some cell membranes appeared completely empty of cell contents. In addition, larger MRSA cells were observed (Figure 5b) compared to those in nontreated bacteria (Figure 5f).

Checkerboard Assay. When compound **1** was combined with erythromycin for the inhibition of MSSA, an important synergistic effect ($FICI_{1+E} = 0.19$) was observed when the phloroglucinol and the antibiotic were combined at 8 and 16 times below their MIC values, respectively (Table 2). A notable synergism was still observed ($FICI_{1+E} = 0.50$) when the concentration of the macrolide was decreased to 0.001 μg/mL, equivalent to 300 times less than its MIC, and the concentration of **1** was 1 μg/mL, i.e. half of its MIC value (Table 2). When the phloroglucinol was combined with gentamicin, a surprising synergistic effect ($FICI_{1+G} = 0.25$) was observed, even when the concentration of the former was decreased 4 times below its MIC and of the antibiotic 260 times below its individual MIC (Table 3).

Compound **2**, in turn, showed only an additive effect ($FICI_{2+G} = 0.62$) when added together with gentamicin for the control of MSSA (Table 4). No effects were observed with erythromycin.

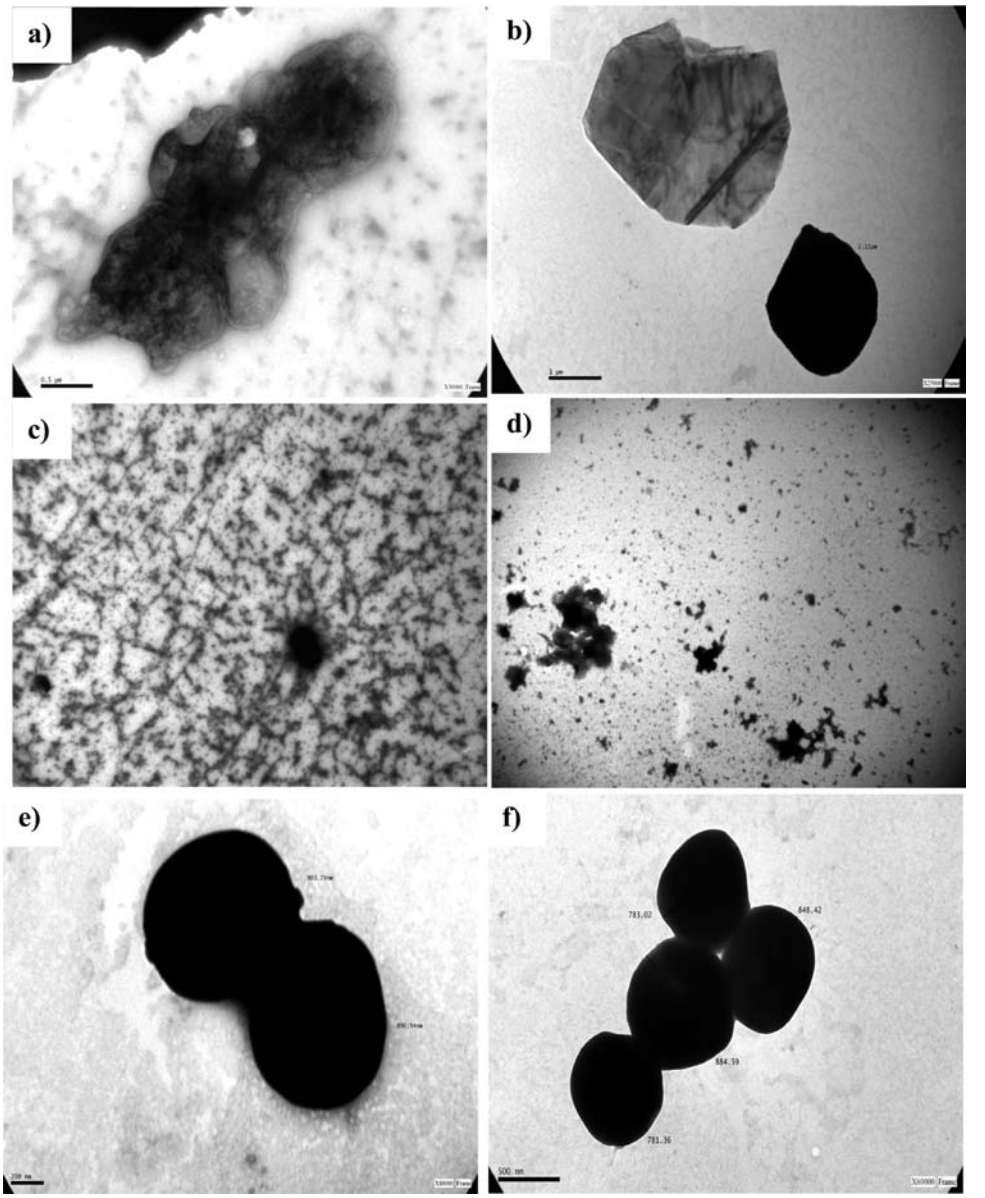

Figure 5. Effect on the cell morphology of methicillin-susceptible *Staphylococcus aureus* (a, c) and gentamicin- and methicillin-resistant *S. aureus* (b, d) treated with 8 μg/mL of (*Z*,*Z*)-5-(trideca-4,7-dienyl)resorcinol (**2**). Untreated methicillin-susceptible (e) or gentamicin- and methicillin-resistant *S. aureus* (f). Results observed at 24 h from the beginning of the experiment.

None of the compounds showed synergism with the assayed antibiotics when they were confronted with the resistant strain.

Calculated Partition Coefficient. The log *P* (ClogP) calculated for compound **1** was 5.84 while the value calculated for compound **2** was 6.69.

Hemolysis Assay. In the hemolysis assay, compound **1** did not show erythrocyte lysis at any of the assayed concentrations, while compound **2** exhibited hemolysis only as from 125 μg/mL.

Genotoxicity Analysis. In the genotoxic assay, meristematic *A. cepa* cell treatments with both plant-derived compounds showed no differences with respect to control ($p > 0.05$) either in the mitotic index or in the phase index (Table 5), except with compound **1** at 8 μg/mL, where no cell division was observed (Table 5). However, this effect was reversed when the meristematic cells were left in distilled water free of phloroglucinol for 72 h (mitotic cells = 113; mitotic index = 3.77 $\pm$ 0.15). No chromosome abnormalities were seen at any stages of the cell cycle.

Macrophage RAW264.7 Cytotoxicity. Compound **1** did not affect cell viability at the maximum concentration assayed (64 μg/mL), while compound **2** produced 76% cytotoxicity at this concentration. The latter showed a macrophage growth inhibitory concentration 50 (GIC_{50}) of 18.15 μg/mL (7.81−42.17, lower−upper limit).

DISCUSSION

Bacteria may acquire resistance to many classes of antibiotics by *de novo* mutation or via the acquisition of resistance genes from other organisms.[32] As a consequence, bacteria produce different enzymes that inactivate the antibacterial drug, overexpressed efflux pumps which extrude the therapeutic agents,[33] modify the structural conformation of the antibiotic receptors or

Table 2. Minimum Inhibitory Concentrations (MICs) in μg/mL and Fractional Inhibitory Concentration Indices (FICIs) of 23-Methyl-6-*O*-desmethylauricepyrone (1) in Combination with Erythromycin against MSSA[a]

		erythromycin (MIC = 0.3)									
compd **1** (MIC = 2)		$MICc_E$	FIC	$MICc_E$	FIC	$MICc_E$	FIC	$MICc_E$	FIC	$MICc_E$	FIC
$MICc_1$	FIC	0.150	0.500	0.075	0.250	0.019	0.063	0.005	0.017	0.001	0.003
1.000	0.500										$FICI_{1+E}$ = 0.503
0.500	0.250								$FICI_{1+E}$ = 0.267		
0.250	0.125						$FICI_{1+E}$ = 0.188				
0.125	0.062				$FICI_{1+E}$ = 0.312						
0.062	0.031		$FICI_{1+E}$ = 0.531								

[a] $MICc_1$: MIC of compound **1** in combination with erythromycin. $MICc_E$: MIC of erythromycin in combination with compound **1**. FIC: fractional inhibitory concentration. $FICI_{1+E}$: fractional inhibitory concentration index (FIC of compound **1** plus FIC of erythromycin).

Table 3. Minimum Inhibitory Concentrations (MICs) in μg/mL and Fractional Inhibitory Concentration Indices (FICIs) of 23-Methyl-6-*O*-desmethylauricepyrone (1) in Combination with Gentamicin against MSSA[a]

		gentamicin (MIC = 8)	
compd **1** (MIC = 2)		$MICc_G$	FIC
$MICc_1$	FIC	0.031	0.004
1.00	0.500	$FICI_{1+G}$ = 0.504	
0.50	0.250	$FICI_{1+G}$ = 0.254	

[a] $MICc_1$: MIC of compound **1** in combination with gentamicin. $MICc_G$: MIC of gentamicin in combination with compound **1**. FIC: fractional inhibitory concentration. $FICI_{1+G}$: fractional inhibitory concentration index (FIC of compound **1** plus FIC of gentamicin).

Table 4. Minimum Inhibitory Concentrations (MICs) in μg/mL and Fractional Inhibitory Concentration Indices (FICIs) of (*Z*,*Z*)-5-(Trideca-4,7-dienyl)resorcinol (2) in Combination with Gentamicin against MSSA[a]

		gentamicin (MIC = 8)	
compd **2** (MIC = 4)		$MICc_G$	FIC
$MICc_2$	FIC	1.000	0.125
2.000	0.500	$FICI_{2+G}$ = 0.625	

[a] $MICc_2$: MIC of compound **2** in combination with gentamicin. $MICc_G$: MIC of gentamicin in combination with compound **2**. FIC: fractional inhibitory concentration. $FICI_{2+G}$: fractional inhibitory concentration index (FIC of compound **2** plus FIC of gentamicin).

produce an alternative metabolic pathway that bypasses the action of the drug.[32,34]

With the aim of overcoming the resistance of *S. aureus* to commercially existing antibiotics, the activity of two plant-derived compounds alone or paired with erythromycin or gentamicin was evaluated against MSSA and MRSA strains.

Both compounds **1** and **2** showed a notable capability for reversibly and irreversibly inhibiting the growth of susceptible and resistant *S. aureus,* achieving, in almost all cases, better inhibition of the growth of both pathogens than the reference antibacterial agents (Table 1). These findings suggest the absence of cross-resistance toward the natural compounds, for instance through multidrug efflux pumps.[35] In relation to this, it has already been described that other phloroglucinols, such as semimyrtucommulone and myrtucommulone A isolated from *Myrtus communis,* were not a substrate of efflux transporters overexpressed in resistant *S. aureus* strains.[36] It should be noted that these phloroglucinols exhibit a highly substituted phloroglucinol core with an isobutylidene bridge to syncarpyl moieties.[36]

The effectiveness demonstrated by both natural antimicrobials is encouraging, since resistant *S. aureus* was as sensitive as the susceptible strain. Another plant-derived compound, the xanthonoid α-mangostin, which contains a phloroglucinol moiety in one of the rings, displayed similar anti-MSSA and -MRSA activity to that of the compounds tested in this work with MICs of 6.25 μg/mL.[37] Similar MIC values against MRSA were also observed with other phloroglucinols.[38]

Compound **1** at four times the MIC, on both tested strains, as well as compound **2** at two and four times the MIC on the susceptible and resistant strains, respectively, exerted bactericidal action. Although these results come from the broth dilution technique, they matched the MBC values obtained for both compounds in the agar tests, thus confirming their killing potency.

The ClogP values obtained for both natural substances (ClogP = 5.84 and 6.69 compounds **1** and **2**, respectively) indicated that they had similar liphophilicity and thus similar penetration into the bacteria membrane. However, the differences in the time at which each compound **1** and **2** produce bacterial death may suggest that they operate through different mechanisms with the bacterial membrane, a phenomenon which was also reported for natural and synthetic chalcones.[39]

Kim et al. [40] reported that, when the log *P* of compounds increases, antibacterial activity also increases, since they exhibit a higher permeability in membranes. Both phloroglucinol and resorcinol showed high liphophilicity, and thus this property may be suggested as the major contributor to antibacterial activity.[40]

Assays of antimicrobial activity performed in MRSA demonstrated that the optimum ClogP of some triketones, most of them prepared from phloroglucinols, was around 6.[38] These values agree with the ClogP values obtained for **1** and **2**. In coincidence, Kubo et al.[41] also observed potent anti-MRSA activity in anacardic acids showing log *P* around 6. On the other hand, anacardic acids showing similar log *P* values exhibited similar MBC values,[41] which was also seen for both natural compounds tested here.

Table 5. Mitotic and Phase Indices in *Allium cepa* Cells after Exposure to 23-Methyl-6-*O*- desmethylauricepyrone (1) and (*Z*,*Z*)-5-(Trideca-4,7-dienyl)resorcinol (2)[a]

				mitotic phase index (%)		
treatment	concn (μg/mL)	mitotic cells (no.)	mitotic index (%)	prophase	metaphase	anaphase−telophase
control		122*	4.1 ± 0.4*	77.9 ± 3*	9.0 ± 2.2*	13.1 ± 2.4*
compound **1**	2	117*	3.9 ± 0.2*	87.2 ± 2.7*	3.4 ± 1.6*	9.4 ± 1.3*
	4	118*	3.9 ± 0.2*	88.1 ± 10*	4.2 ± 2.8*	7.6 ± 7.5*
	8	0**				
compound **2**	2	119*	3.9 ± 0.1*	87.4 ± 2.4*	3.4 ± 1.3*	9.2 ± 1.6*
	4	121*	4.0 ± 0.4*	85.1 ± 1.6*	4.9 ± 2.4*	10.7 ± 1.2*
	8	120*	4.0 ± 0.1*	87.5 ± 2.3*	3.3 ± 1.3*	9.2 ± 1.5*

[a] Values are expressed as mean ± standard error. *Values are not significantly different ($p > 0.05$). **Value significantly different ($p < 0.05$) in comparison to control and to other treatments. Bulbs were left in distilled water free of compound for 24 h after treatments.

It has been described that hydrophobic compounds, such as terpenes from essential oils[42] or triketones,[38] may act as antibacterials by disrupting bacterial membranes.[38,42] This effect was also determined for compounds **1** and **2** in the electron microscopy studies performed in MSSA and MRSA, where it could be observed that membranes appeared strongly affected. As reported by other authors, compounds affecting membrane integrity exerted their effects within the first hours of experiments.[41] Compound **1**, which disrupts the bacterial membrane, exhibited bactericidal action at 24 h from the beginning.

The fast killing effect exerted by compound **2** in *S. aureus* differs from that previously observed in treatments against *Proteus mirabilis*, where the bactericidal action was achieved at 24 h.[17] Electron micrographs carried out with the latter bacterium showed alterations in membranes, but no lysis occurred. This may indicate that compound **2** acts with a different mechanism against Gram-positive or -negative bacteria.

The synergistic effect of compounds **1** and **2** in combination, paired with conventional antibiotics, was further examined. The results demonstrated that erythromycin could be decidedly decreased from 4 to 300 times below its MIC value when combined with different concentrations of compound **1** against MSSA. In addition, the phloroglucinol−gentamicin combination appeared synergistic, allowing a decrease in antibiotic concentration 260 times below its individual MIC. Therefore, these combinations administered in treatments against MSSA are likely to reduce the antibiotic minimum effective doses. This would minimize their undesirable side effects. Although compound **2** showed an additive effect when combined with gentamicin, it was possible to decrease the concentration of the latter 8 times, which also means a decrease in antibiotic toxicity.[2,43]

The hydrophobicity demonstrated for many compounds allows them to become partitioned into the lipid membrane, having a considerable effect on its structural and functional properties, which leads to an increase in permeability and thus enhances antibiotic intake.[42,44] However, this could not be completely demonstrated in our studies since compound **2** showed synergism against MSSA only when combined with gentamicin, and none of the tested compounds exerted this effect against MRSA. Similar results were obtained by Sakagami et al.,[37] who reported that α-mangostin showed synergism when combined with gentamicin against MSSA but not against MRSA.

The lack of potentiation in the activity of antibiotics against the resistant strain was in contrast to the strong anti-MRSA action exerted by both natural substances. This phenomenon was also reported by Smith et al.[45] in studies with epigallocatechin gallate and oxacillin. The authors reported that the significant individual activity of the natural substance and the lower combined antibacterial activity are produced by different mechanisms of action.

As demonstrated in the assay, compound **1** did not produce any alteration in erythrocyte integrity at high concentration (125 μg/mL), while compound **2** showed lysis from this concentration.

As seen, this concentration is far from those that produce killing on both MSSA and MRSA. This absence of undesirable effects was also observed in the genotoxicity assay. Our results showed no induction of chromosome aberration, or reduction in mitotic or mitotic phase indices in treated cells, except for compound **1** at 8 μg/mL, which only inhibited the number of cells undergoing division. However, this inhibition was reverted when exposure to distilled water was extended until 72 h.

It is important to remark that compound **1** did not affect the macrophage's viability at the maximum studied concentration, which was 8 times its MBC, while the concentration of compound **2** that produced a 50% inhibition was at least twice the active concentrations.

In conclusion, both compounds could be used as novel antibacterial agents due to their notable effectiveness at inhibiting *S. aureus* growth, even against the resistant strain.

The results observed in the synergism assays suggest that compound **1** was able to diminish erythromycin and gentamicin doses by acting as a codrug for combinatory anti-MSSA therapy.

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ABBREVIATIONS USED

BHI, brain−heart infusion; DMSO, dimethyl sulfoxide; FIC, fractional inhibitory concentration; FICI, FIC_{index}; GIC_{50}, growth inhibitory concentration 50; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; MSSA, methicillin-susceptible *Staphylococcus aureus*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MRSA, methicillin-resistant *Staphylococcus aureus*; PCA, plate count agar; rbc, red blood cells; TEM, transmission electron microscopy

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