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Antibacterial activity of chalcones, hydrazones and oxadiazoles against methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

The increase in antibiotic resistance due to multiple factors has encouraged the search for new compounds which are active against multidrug-resistant pathogens. In this context, chalcones, dihydrochalcones, hydrazones and oxadiazoles were tested against *Staphylococcus aureus* ATCC 25923 and methicillin-resistant *S. aureus* (MRSA) isolates, which were obtained from clinical laboratories and were characterized as MRSA using traditional and molecular methods. Among 65 tested compounds, two chalcones, one dihydrochalcone and two hydrazones were active against MRSA. Based on the minimal inhibitory concentration and cytotoxicity, hydrazones provided a better selectivity index than chalcones. Active hydrazones are promising antibiotic-like substances and they should be the subject of further microbiological studies.

Since the introduction of antibiotics in clinical medicine, there are over 60 years, this became the main strategy for controlling bacterial infections.[1] However, already in 1942 was reported penicillin resistant *Staphylococcus aureus*,[2] and shortly after the introduction of methicillin in 1961, has been reported strains of *S. aureus* resistant to this drug.[3] Currently the most strains of *S. aureus* that cause infection are resistant to penicillin[4] and methicillin- or oxacillin-resistant *S. aureus* (MRSA or ORSA) bacterial strains are recognized as among the most important pathogens causing nosocomial infections worldwide.[5] The concern regarding of the increased incidence of MRSA is not only related to the fact that there are strains resistant to these chemotherapy, but, showing resistance simultaneously to all beta-lactam antibiotics (penicillins, cephalosporins and carbapenems), and often, for other classes of antimicrobials used in therapy, such as aminoglycosides, chloramphenicol, clindamycin, fluoroquinolones and macrolides; this fact limit the treatment options for patients, representing a threat to public health.[6] Thus, the discovery of new molecules with antimicrobial activity is important, especially to control hospital infections by such multidrug-resistant strains, which are responsible for high morbidity and mortality.

In this context, the chemistry of chalcones and hydrazones is of particular interest since these compounds present a variety of biological activities. Chalcones are essential intermediate compounds in flavonoid biosynthesis, presenting a benzal-acetophenone fundamental core.[7] Several reports have documented the biological properties of natural or synthesized chalcones which include anti-inflamatory, antitumoral, antifungal and antibacterial.[8] Licochalcone A, a natural hydroxylated compound, has been shown to possess activity against Gram-positive strains of bacteria.[9]

Hydrazones are compounds structurally related to chalcones, the propanone group being replaced by a hydrazide group. Biological

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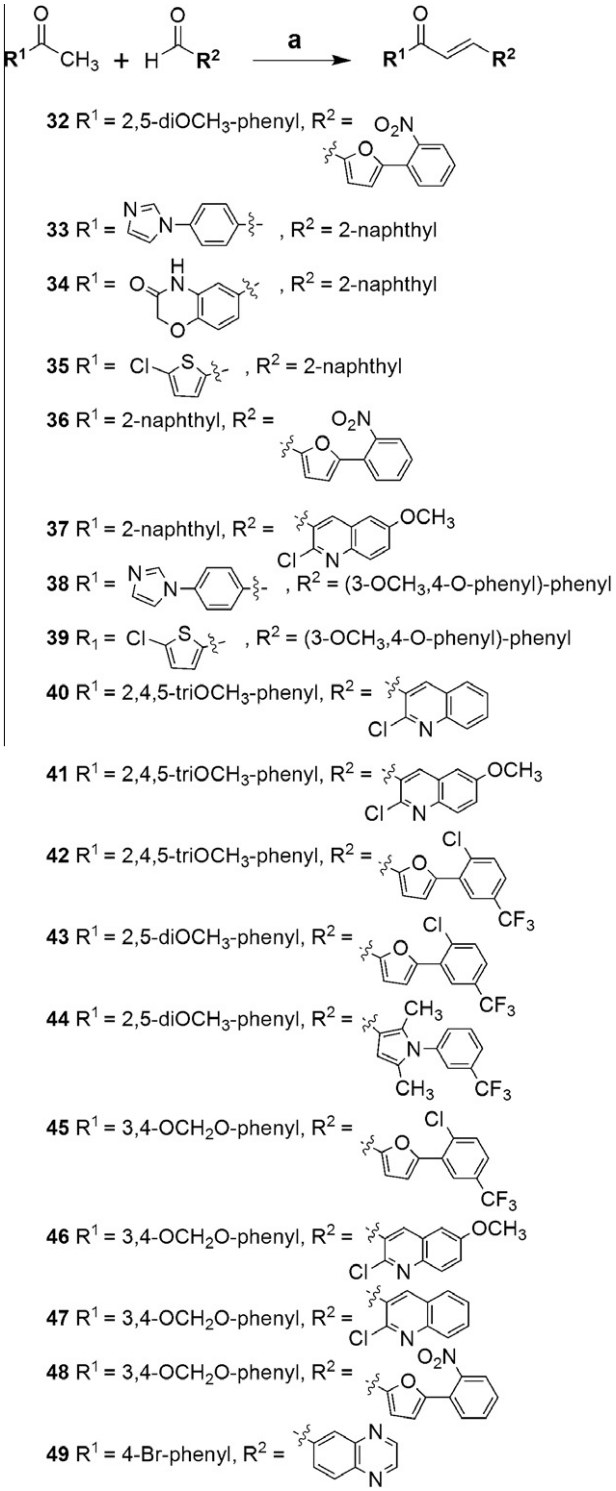
activities which have been associated with hydrazones include antimicrobial, anti-inflammatory, antitumoral, analgesic, antimalarial, anti-platelet and inhibition of cruzain from Trypanosoma cruzi.[10] Oxadiazoles are heterocyclic compounds that can be easily obtained by the cyclization of hydrazones with acetic anhydride.[11]

The increase in antibiotic resistance due to multiple factors has encouraged the search for new compounds which are active against multidrug-resistant pathogens.[12] Thus, the purpose of this study was to evaluate the antimicrobial activity of 49 chalcones, seven oxadiazoles and nine hydrazones against fourteen strains of methicillin-resistant *S. aureus* (MRSA), obtained in clinical laboratories in the city of Florianópolis (Southern Brazil).

Thirty-one natural or synthetic chalcones (**1–15**, **17–27**, **29** and **30**) and dihydrochalcones (**16**, **28** and **31**) have been previously published by our group (Table 1).[13] In this study 18 new chalcones (**32–49**) were synthesized by aldolic condensation between acetophenones and aldehydes, under basic conditions (Scheme 1),[14] with good yields (55–99%). Compounds **33–35** are derived from 2-naphthaldehyde; compounds **36** and **37** are derived from 2-naphthylacetophenone; compounds **38** and **39** are derived from benzylated vanillin; compounds **40–42** are derived from 2,4,5-trimethoxyacetophenone; compounds **32**, **43** and **44** are derived from 2,5-dimethoxyacetophenone; compounds **45–48** are derived from 3,4-methylenedioxyacetophenone and compound **49** are derived from quinoxaline-6-carbaldehyde. The reagents used were commercially available (Sigma–Aldrich®), except benzylated vanillin (3-methoxy-4-(phenylmethoxy)-benzaldehyde) (prepared as previously described with yield of 88%),[15] 2,4,5-trimethoxyacetophenone (synthesized as previously described with yield of 81%)[16] and quinoxaline-6-carbaldehyde (synthesized as previously described with yield of 80%).[17]

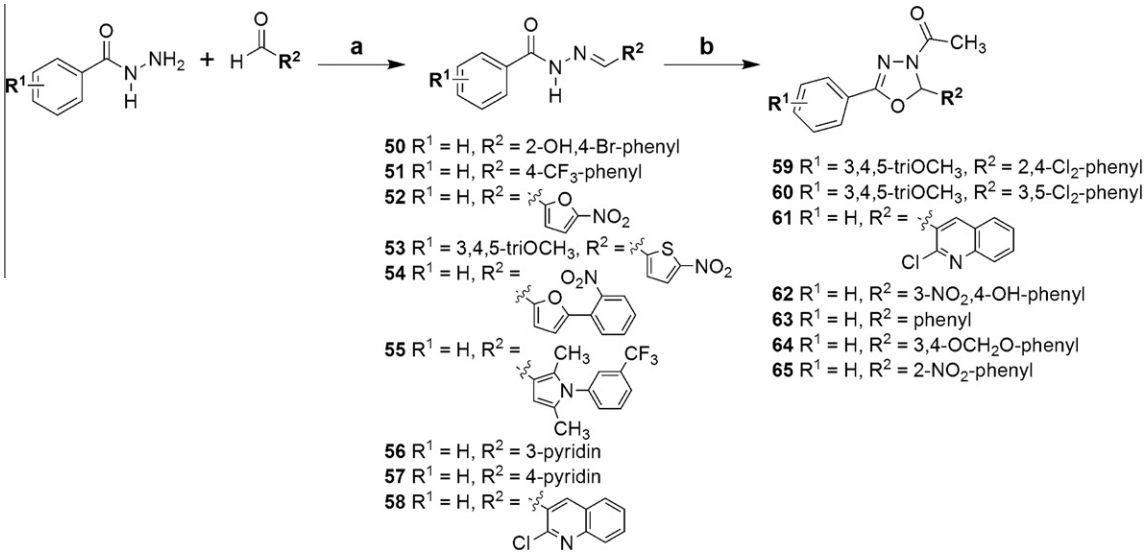
Table 1
Natural or synthetic chalcones and dihydrochalcones[13]

	Compounds
1	2′-Hydroxy-4,4′,5′-trimethoxychalcone
2	3′,4′-Dimethoxychalcone
3	2-Hydroxy-3′,4′-dimethoxychalcone
4	2′,4-Dihydroxy-4′-prenyloxy-chalcone (4-hydroxycordoin)
5	2′-Hydroxy-3′-prenyl-4′-methoxy-chalcone (derricin)
6	2′,2-Dihydroxy-3′-prenyl-4′-methoxy-chalcone (2-hydroxyderricin)
7	2′,3-Dihydroxy-3′-prenyl-4′-methoxy-chalcone (3-hydroxyderricin)
8	2′,4-Dihydroxy-3′-prenyl-4′-methoxy-chalcone (4-hydroxyderricin)
9	2′-Hydroxy-3′-prenyl-4′,4-dimethoxy-chalcone (4-methoxyderricin)
10	2′,4,4′-Trihydroxychalcone
11	2′-Hydroxy-3′,4′-(2,2,-dimethyl-3,4-dihydropyranyl)-chalcone (lonchocarpin)
12	2′,4-Dihydroxy-4′,5′-(2,2,-dimethyl-3,4-dihydropyranyl)-chalcone (4-hydroxy-isolonchocarpin)
13	2′-Hydroxy-4′,5′-(2,2,-dimethyl-3,4-dihydropyranyl)-chalcone (isolonchocarpin)
14	2′,4′,4-Trihydroxy-3′-prenyl-chalcone (4-hydroxy-isocordoin)
15	4-Hydroxy-4′-methoxychalcone
16	2′,4′,5′-Trimethoxy-3,4-methylenedioxy-dihydrochalcone
17	2,2′,4,4′,5,5′-Hexamethoxychalcone
18	2′,4-Dihydroxy-3′,4′-(2,2,-dimethyl-3,4-dihydropyranyl)-chalcone (4-hydroxy-lonchocarpin)
19	3,4-Methylenedioxy-2′,3′,4′,6′-tretramethoxychalcone
20	4,4′-Dimethoxychalcone
21	2′,4,4′-Trihydroxy-3′-geranyl-3-prenyl-chalcone
22	2′,4,4′-Trihydroxy-3′-geranyl-chalcone
23	2′-Acetoxy-3′,4,4′,6′-tetramethoxychalcone
24	2,3′,4,4′,5-Pentamethoxychalcone
25	2,2′,4′,5′-Tetramethoxychalcone
26	2,3,3′,4,4′,6-Hexamethoxychalcone
27	2′-Hydroxy,4′-prenyloxy-chalcone (cordoin)
28	2-Hydroxy-dihydrochalcone
29	2′,4′-Dihydroxy-3′-prenyl-chalcone (isocordoin)
30	2,2′,4′-Trihydroxy-chalcone
31	2′,4,4′,6′-Tetrahydroxy-dihydrochalcone



Scheme 1. Synthesis of chalcones (**32–49**). Reagents and condition: (a) KOH 50%, methanol, rt, 24 h.

Nine hydrazones (**50–58**) and seven oxadiazoles (**59–65**) were also prepared (Scheme 2), with yields varying between 25% and 84%. The hydrazones were prepared by condensation of hydrazide and the appropriate aldehyde, under reflux.[18] The 1,3,4-oxadiazoles were prepared by cyclization of the previously obtained hydrazones with acetic anhydride under reflux.[18] Reagents used were commercially available (Sigma–Aldrich®), except 3,4,5-trimethoxybenzohydrazide, prepared as previously described, with yield of 80%.[19]



Scheme 2. Synthesis of hydrazones (**50**–**58**) and oxadiazoles (**59**–**65**). Reagents and conditions: (a) Methanol, reflux, 2 h; (b) acetic anhydride, reflux, 3 h.

Table 2
Anti-staphylococcal activity of chalcones, dihydrochalcones and hydrazones

Microorganisms	Compounds (MIC μg/mL)									
	14	**18**	**21**	**22**	**28**	**30**	**31**	**52**	**53**	Vancomicin
MSSA[a]	31.25	7.80	31.20	31.20	500.00	31.25	250.00	62.50	15.62	0.20
MRSA1[b]	62.50	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	125.00	125.00	0.80
MRSA2	62.50	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	62.50	125.00	0.80
MRSA3	62.50	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	62.50	125.00	0.80
MRSA4	15.62	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	15.62	15.62	0.80
MRSA5	62.50	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	62.50	125.00	0.40
MRSA6	62.50	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	62.50	125.00	0.40
MRSA7	125.00	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	125.00	125.00	0.80
MRSA8	125.00	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	125.00	125.00	0.80
MRSA9	62.50	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	125.00	125.00	0.80
MRSA10	125.00	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	15.62	125.00	0.80
MRSA11	62.50	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	125.00	125.00	0.80
MRSA12	62.50	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	62.5	125.00	0.80
MRSA13	125.00	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	62.5	125.00	0.80
MRSA14	62.50	⩾1000	⩾1000	⩾1000	⩾1000	62.50	250.00	62.5	125.00	0.80

[a] Methicillin-sensitive *S. aureus* (ATCC 25923).
[b] MRSA = Methicillin-resistant *S. aureus*.

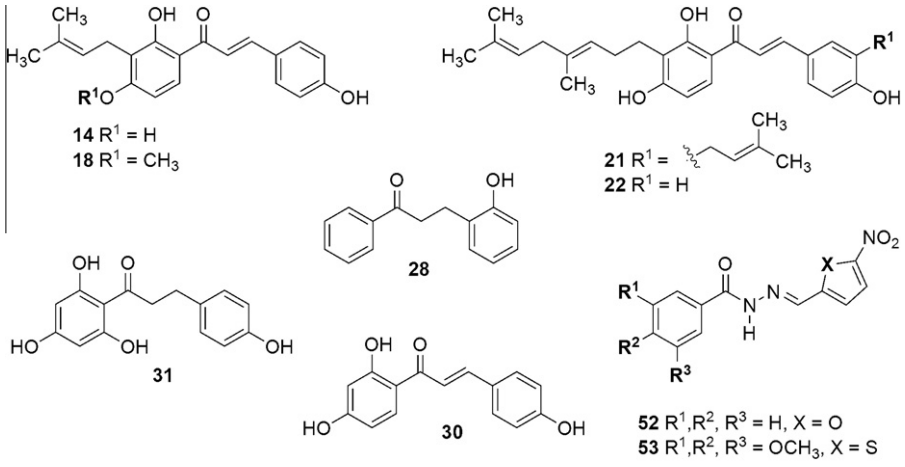


Figure 1. Chemical structures of bioactive compounds.

Among these synthesized compounds, nine (**51**, **52**, **54**, **56**–**58**, **60**, **61** and **63**) have been previously published[11,20] and 25 are novel (**32–50**, **53**, **55**, **59**, **62**, **64** and **65**). All compounds were characterized by ^{1}H NMR, ^{13}C NMR and IR. Detailed spectral characterization (^{1}H NMR, ^{13}C NMR, IR and elementary analysis) for novel compounds (**32–50**, **53**, **55**, **59**, **62**, **64** and **65**) is presented as Supplementary data. 1H NMR spectra of chalcones revealed that all structures are *E* configured ($J_{H\alpha-H\beta}$ = ~16 Hz).

Thus, 46 natural or synthetic chalcones, three natural dihydrochalcones, seven synthetic oxadiazoles and nine synthetic hydrazones (Table 1 and Schemes 1 and 2) were screened against *S. aureus* ATCC 25923 strain (methicillin-sensitive *S. aureus*—MSSA) (results not shown). Five chalcones (**14**, **18**, **21**, **22** and **30**), two dihydrochalcones (**28** and **31**) and two hydrazones (**52** and **53**) showed significant activity (Table 2 and Fig. 1). Subsequently, these seven bioactive compounds were tested against 14 clinical isolates of methicillin-resistant *S. aureus* (MRSA) (Table 2),[13,21] obtained from two clinical microbiology laboratories (University Hospital of Federal University of Santa Catarina and Santa Luzia Laboratory), both located in the city of Florianópolis, Southern Brazil.

The clinical isolates, referred to as MRSA1–MRSA14, were selected on the basis of their resistance to oxacillin in a disk diffusion assay. The bacteria were maintained on brain heart infusion (BHI) at −80 °C until use. The inoculum was an overnight culture of each bacterial species in Müeller-Hinton broth diluted with the same medium to give final concentrations of approximately 10^{8} or 10^{7} CFU/mL (diffusion and microdilution assays, respectively).[13]

Isolate identification was primarily carried out in the respective laboratories, and later by Gram staining, the coagulase test and detection of the nuc gene in the authors' laboratories. The diffusion assay was also repeated using cefoxitin in addition to oxacillin.[22] Genotypic characterization of the MRSA was carried out by detection of the mec gene[23] and by the randomly amplified polymorphic DNA (RAPD) reaction.[24] The isolates were confirmed as *S. aureus* by detection of the nuc gene in their genomes, and the results obtained for oxacillin qualify all isolates as being MRSA, according to the values contained in document M100-S20.[25] The majority of the isolates showed inhibition zones of ⩽21 mm for cefoxitin and were considered as resistant. Isolates 4 and 5 showed inhibition zones of 22 and 33 mm, respectively, and were considered as sensitive.

Cefoxitin was included in this study because recent evidence has revealed that this antibiotic is an important marker to predict the presence of the mecA gene in clinical isolates. The results obtained in the cefoxitin disc diffusion test were in agreement with those obtained by PCR, which showed the presence of the gene mecA (or PBP2a-positive) in the genome of the majority of the isolates, with the exception of the isolates MRSA 4 and MRSA 5. These results are consistent with the hypothesis that cefoxitin is a better marker than oxacillin in terms of discriminating the presence of the mecA gene in MRSA strains.[26] The isolates were also tested against vancomycin because this antibiotic is considered a gold treatment for infections caused by this group of organisms;[27] all isolates were sensitive to this antibiotic. The isolates were inhibited at concentrations ranging from 0.4 to 0.8 μg/mL.

With the results of the diffusion test and PCR it was possible to conclude that the resistance to oxacillin indicates that the isolates can be classified as MRSA, and the resistance to cefoxitin and the results of the PCR confirmed that of the 14 MRSA isolates, 12 had the mecA gene. The results obtained by random amplified polymorphic DNA revealed that different patterns of amplification products were generated by different primers, allowing the genotyping of the MRSA isolates. Some primers (AP-1, AP-1026 and Eric 1) were not useful in terms of distinguishing differences between the isolates. Based on the differences in the patterns, dendograms were constructed using the primers AP-7 and Eric 2 (Fig. 2A and B).[28]

Primer AP-7 allowed us to observe an evolutionary correlation between the isolates, except for isolates MRSA 1 and MRSA 2, which were not amplified by this protocol. However, the primer most suitable for the differentiation of the isolates was Eric 2 (Fig. 2). Samples MRSA 7 and MRSA 8 showed distinct origins when compared to the others. Indeed, these isolates were collected from outpatients. These isolates showed the highest percentage of similarity (100%) between them, and they may have originated from the same clone, since the biological specimens were collected in the same place (laboratory). The isolate MRSA 2 was also collected outside of the hospital, but its genetic profile correlated with that of hospital origin and was very similar to the profile of the isolate used as the reference (isolate 14).

Regarding the microbiological assays, the two hydrazones that were active against MSSA (**52** and **53**) were also active against the MRSA isolates. However, of the other compounds which were active against MSSA, only two chalcones (**14** and **30**) and one dihydrochalcone (**31**) were active against the MRSA isolates (Table 2).

The antimicrobial activity of hydrazones against MSSA, but not against MRSA, has been described elsewhere.[10a] Also, 4-hydroxybenzoic acid[(5-nitro-2-furyl)methylene]-hydrazide (nifuroxazide) and analogs have been assayed against *S. aureus* ATCC 25923[29] and 4-fluorobenzoic acid[(5-nitro-2-furyl)methylene]-hydrazide against *S. aureus* ATCC 29213.[30] These compounds are similar to the active hydrazones of this study (**52** and **53**), differing only in terms of the substituent at the *para* position of the phenyl ring and the heteroatom of the heterocyclic ring. Another similar compound is 4-acetylbenzoic acid [(5-nitro-thiophene-2-yl)methylene]hydrazide, which is reportedly active against a multidrug-resistant 3SP/R33 *S. aureus* strain.[31]

As mentioned above, only one dihydrochalcone and two chalcones inhibited the growth of the MRSA isolates: **14 (2′,4′,4-trihy-**

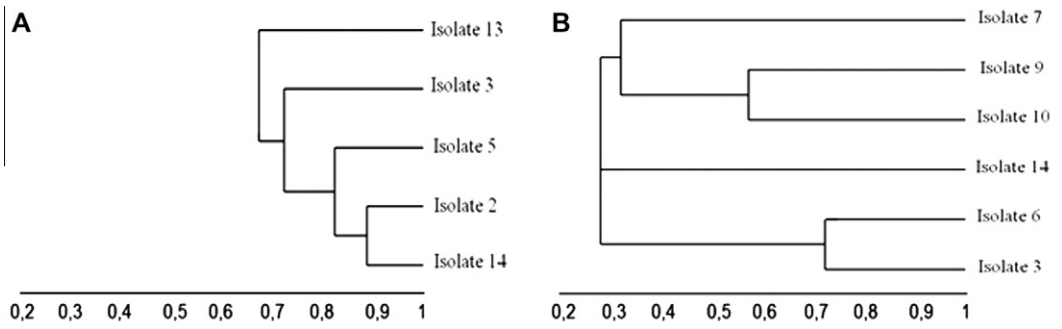


Figure 2. (A) Dendogram obtained from banding patterns of RAPD analysis with AP-7 primer. (B) Dendogram obtained from banding patterns of RAPD analysis with Eric-2 primer.

droxy-3′-prenyl-chalcone), 30 (2,2′,4′-trihydroxy-chalcone) and **31 (2′,4,4′,6′-tetrahydroxy-dihydrochalcone).** These three anti-MRSA compounds have in common a hydroxyl group at positions 2′,4′ and 4. In addition to the hydroxyl substituents, the bioactive chalcone **14** also has a prenyl substituent at position 3′. The antimicrobial activity of natural compounds structurally related to this chalcone has been previously described; for erypostyrene minimal inhibitory concentration (MIC) values of 50 μg/mL and 6.25 μg/mL in relation to *Candida albicans* and MRSA, respectively, and for angolensin an MIC of 50 μg/mL in relation to MRSA have been reported.[32] Chalcones **21** and **22**, despite having hydroxyl groups in the same positions of the active compounds **14**, 30 and **31**, have a geranyl substituent at position 3′, which interfere with their action against MRSA strains probably due to their considerable size. Of the three chalcones which were active against the MRSA isolates, dihydrochalcone **31** was the least active, possibly due to a loss of rigidity of the molecule, resulting in a difficulty in maintaining the conformation required for interaction with the biological target.

The presence of the hydroxyl group at position 2′ is important due to its intramolecular interaction with the carbonyl group, and the hydroxyl group at position 4′ activates the region that includes the neighboring hydroxyl group at 2′ and the α,β-unsaturated carbonyl group.[33] Nielsen and col. assayed licochalcone A analogues against *S. aureus* and concluded that the presence of hydroxyl group at 4′ position is important to antibacterial activity.[34] This may be the reason for the low activity of dihydrochalcone **28** against the MSSA strain and absence of activity against the MRSA isolates, since it lacks the hydroxyl groups in these positions, as well as the double bond.

Chalcone **18** showed very good antimicrobial activity against the MSSA strain, but surprisingly was inactive against the MRSA isolates; the only difference between **18** and **14** is the presence of a methoxyl group instead of hydroxyl group at position 4′. However, erypostirene, active compound against MRSA in a previous work,[32] also has a methoxy group at position 4′.

All compounds that showed a bacteriostatic effect on the MRSA isolates were also evaluated in terms of their lytic activity. The MIC values were the same as those obtained for the minimum bactericidal concentration (MBC).[13,35] The cytotoxicity of the five active compounds against the MRSA isolates (**14**, **30**, **31**, **52** and **53**), at concentrations equal to or less than 500 μg/mL, was evaluated against VERO cells using the MTT method.[36] The results are shown in Table 3 and from these values it was possible to calculate the selectivity index (SI), to assess whether the compounds are toxic at the minimal inhibitory concentration. A high IS value indicates low toxicity of the compound. Thus, based on the IS indices obtained, it is possible to suggest that the hydrazones studied are clinically safer than chalcones and dihydrochalcone. This result is in agreement with the findings of Dimmock et al.,[37] who reported that the presence of hydroxyl groups in the structure of the chalcones increases their cytotoxic effect; dihydrochalcone **31** contains four hydroxyl groups, and is the most toxic compound among those tested in this study. This indicates that hydrazones may be more promising than chalcones in terms of their future potential as antibacterial drugs.

Table 3
Cytotoxicity of chalcones and hydrazones toward VERO cells

Compounds	VERO cells CC_{50}[a] (μg/mL ± SD)	IC_{50}[b] (μg/mL)	IS[c]
14	726.82 ± 14.63	31.25	23.25
30	463.65 ± 53.00	31.25	14.80
31	735.47 ± 68.83	125.00	5.90
52	488.71 ± 67.13	7.81	62.60
53	856.71 ± 15.13	7.81	109.70

[a] Average of three independent assays ± standard deviation; statistical test was conducted to assess the significance of the results, with $p = 0.05$, all results were significant.
[b] IC_{50} = concentration that showed 50% cellular cytotoxic effect.
[c] $IS = CC_{50}/IC_{50}$.

In summary, the genotypic characterization of the MRSA isolates revealed that the mecA gene was found in 12 of the 14 isolates, and the randomly amplified polymorphic DNA (RAPD) reaction allowed the discrimination of nosocomial bacteria from community isolates. The tested compounds can be distributed into three categories related to the spectrum of activity: compounds inactive against *S. aureus*, compounds active against sensitive *S. aureus* and compounds active against methicillin-resistant isolates. Chalcones **14** and **30**, dihydrochalcone **31** and hydrazones **52** and **53** were the compounds which were most active against MRSA isolates, and hydrazones showed the best selectivity indices. From the 65 tested compounds, **52** and **53** are potentially promising as antibiotic-like substances, and microbiological studies to better evaluate them should be continued.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.11.059.

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