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ORIGINAL ARTICLE

Potent and selective inhibitors of class A β-lactamase: 7-prenyloxy coumarins

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Class A and D β-lactamases are the main causes of resistance against β-lactam antibiotics, especially the penam group, in *Staphylococcus aureus*. On the basis of the potentiator property of ethanolic extracts of *Ferula szowitsiana* root on penicillin, MIC values observed for resistant *S. aureus*, the main naturally occurring compounds in these extracts, auraptene, umbelliprenin and galbanic acid, were evaluated for β-lactamase inhibitory activity. Amongst them auraptene showed the most potent inhibitory activity ($IC50 = 21 \pm 1.5\,\mu\text{M}$) toward class A β-lactamase, whereas no inhibition was observed for class D β-lactamase. To obtain the structure activity relationship of the mentioned compounds and rationalize the enzyme inhibitory results, docking analysis was performed for both groups of β-lactamases. Docking analysis showed that the compounds have 100–500-fold lower bonding affinity toward the class D β-lactamase than toward the class A enzyme.

INTRODUCTION

Staphylococcus aureus is now the major cause of nosocomial pneumonia and surgical infections, and is the most common source of nosocomial bloodstream infections behind coagulase-negative staphylococci.[1] Infections and outbreaks in nursing homes[2,3] and among outpatient populations[4,5] are common throughout the world. Since the introduction of antibiotics against bacterial infections (from 1940), microorganisms have shown a substantial ability to protect themselves by creating and acquiring antibiotic resistance. By 1942, penicillin resistance was observed in *S. aureus* after 3 months of clinical trials.[6] By 1953, as prescription of penicillin became more widespread, more than 60% of *S. aureus* isolates were resistant to penicillin, with development of resistance to other classes of antibiotics such as tetracyclines and macrolides beginning to emerge.[6] By 1960, despite taking careful surveillance, antibiotic-resistant *S. aureus* had become the most common cause of hospital-acquired infection.[6,7] Penicillin-resistant *S. aureus* has been mainly a nosocomial problem until now.

By 1952, introduction of β-lactam antibiotics for the outgrowth of *S. aureus* strains, possessing or having acquired β-lactamases expression ability, led to a resistance rate of 75%.[8] The catalytic function of β-lactamases is the primary cause of resistance to β-lactam antibiotics. These enzymes hydrolyze the β-lactam ring in this series of antibiotics, a process that deactivates the drugs. The outgrowth of β-lactamase producing *S. aureus* led to the commercial development of β-lactamase-resistant derivatives of penicillin, such as methicillin, possessing a dimethoxybenzoyl side chain that prevents hydrolysis of the β-lactam ring. But methicillin-resistant *S. aureus* was detected shortly after the introduction of these derivatives.

Over 470 β-lactamases have been recognized to date and are categorized into four distinct functional classes (classes A, B, C and D).[9–11] Whereas classes A, C and D have evolved dependence on an active-site serine as their key mechanistic feature, class B enzymes are zinc dependent and hence different. The catalytic process resulting in turnover of the former group members involves acylation at the active-site serine by the β-lactam antibiotic, followed by deacylation of the acyl-enzyme species. It is noteworthy that these enzymes do not share any sequence homologies, structural similarities or mechanistic features with serine or zinc-dependent proteases.

Class A β-lactamases generally prefer penicillins as substrates, whereas class C enzymes prefer cephalosporins as the substrate. Class B enzymes can hydrolyze a broad range of substrates including carbapenems, which are resistant to hydrolysis by most of the other classes of enzymes. On the other hand, class D β-lactamases efficiently hydrolyze oxacillin-type β-lactams. Classes A and C β-lactamases are the most common enzymes, among them class A is mostly expressed in *S. aureus*.[12–14]

Another approach to overcoming resistance mediated by β-lactamase production has been to combine penicillin with a β-lactamase inhibitor. Clavulanic acid is one of the favorite inhibitors

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used for therapeutic purposes in combination with amoxicillin. Unfortunately, the emergence of clavulanic acid-resistant variants of class A β-lactamase significantly compromise the efficacy of this combination. A single amino-acid mutation at catalytic position Ser[130] (Ser→Gly) results in resistance to clavulanate in class A β-lactamase.

Research for discovery of new β-lactamase inhibitors to produce the combined antibiotic is now under investigation. In this work, we discovered a new series of natural prenylated coumarins, isolated from *Ferula szowitsiana*, as class A β-lactamase inhibitors and showed their potentiating activity on penicillin against some clinical isolates of penicillin-resistant *S. aureus*.

MATERIALS AND METHODS
Molecular modeling, docking and SAR STUDY

Structure optimization. Three dimensional structures of the natural coumarins were simulated in Hyper Chem7.5 using the MM+ method (RMS gradient = 0.1 kcal mol^{-1}) (HyperChem Release 7, Hypercube Inc., http://www.hyper.com/). In the second optimization, output files were minimized under Semiempirical AM1 methods (Convergence limit = 0.01; Iteration limit = 50; RMS gradient = 0.1 kcal mol^{-1}; Polak-Ribiere optimizer algorithm).[15]

Crystal structures of class A and D β-lactamases (EC 3.5.2.6) were retrieved from RCSB Protein Data Bank (PDB entry: 1BLC and 1K6S, respectively).

Molecular docking. The geometry-minimized structures were docked into the active site of 1BLC and 1K6S with AutoDock Tools version 4.2 (revision 30)[16] using the Lamarckian genetic algorithm.[17] The efficiency of this method to produce bonding models similar to the experimentally results has been reported.[18,19]

The docking regions of 1BLC and 1K6S were defined with the considering Cartesian chart 4.08, −8.312, −12.31 and 5.53, 13.81, 64.95 as the center of a grid with 52, 44, 63 and 50, 50, 50 points in X, Y and Z axes, respectively. The docking parameter files were generated using Genetic Algorithm and Local Search Parameters while the number of generations (Genetic Algorithm and Local Search Parameters runs) and the maximum number of energy evaluations was set to 200 and 2,500,000, respectively (number of individuals in the population = 300). The 200 docked complexes were clustered with a root-mean-square deviation tolerance of 2.0 Å. Docking results were submitted to Accelrys DS Visualizer 2.0.1 (http://www.accelrys.com/products/discovery-studio) for further evaluations.

Preparation of β-lactamase

Desired bacteria from the exponential growth phase were harvested by centrifugation for 10 min at 5000 g, washed once and treated with lysozyme in 0.1 M phosphate buffer (pH 7.0) to destroy the cell wall. The lysed cells were sonicated at 20 kHz for 10 min. After the cellular debris was removed by centrifugation, the supernatant was used to test for β-lactamase activity.[20] One unit of β-lactamase activity was defined as 1 μmol of nitrocefin hydrolyzed.

β-lactamase inhibitory assessment

β-lactamase inhibitory activity was determined according to a previously published study.[21,22] A 1-mM nitrocefin solution is prepared by dissolving 5.16 mg powder in 0.5 ml dimethyl sulfoxide and then diluting with 9.5 ml of 0.1 M phosphate buffer (pH 7.0). β-lactamase activity is indicated by measuring the absorbance at 486 nm for 15 min after addition of 50 μl prepared β-lactamase (β-lactamase activity = 0.035 U ml^{-1}) to each tube containing inhibitor and nitrocefin (50 μM) in 1 ml 0.1 M phosphate buffer (incubated at 37 °C). The IC$_{50}$ values were determined from the % activity of the enzymes at 0.4, 2, 10, 50 and 250 μM concentrations (three replicates for each concentration) of synthetic inhibitors using a sigmoidal dose-response curve in Graphpad Prism 3.0.

Table 1 β-lactamase inhibitory assessment data of the *O*-prenylated coumarins and docking analysis data of the consensus conformers for both class A (1BLC) and D (1K6S) β-lactamase

| Compound | IC$_{50}$ (μM) | 1BLC | | 1K6S | |
		Ki (μM)	Number of conformers in cluster	Ki (μM)	Number of conformers in cluster
7IPC	119 ± 6.1	0.689	27	147.3	33
Umbelliprenin	54 ± 2.9	0.173	37	91.7	37
Auraptene	21 ± 1.5	0.042	34	14.1	32
Galbanic acid	47 ± 3.1	0.087	39	10.7	41
Clavulanic acid	24.1 ± 2.1	—	—	—	—

The IC$_{50}$ values are given as ± s.e. (three replicates for each concentration). The P-values of the IC$_{50}$ data, computed by one-way ANOVA Tukey multiple comparison test are $P < 0.001$, except umbelliprenin vs galbanic acid and auraptene vs clavulanic acid ($P > 0.05$).
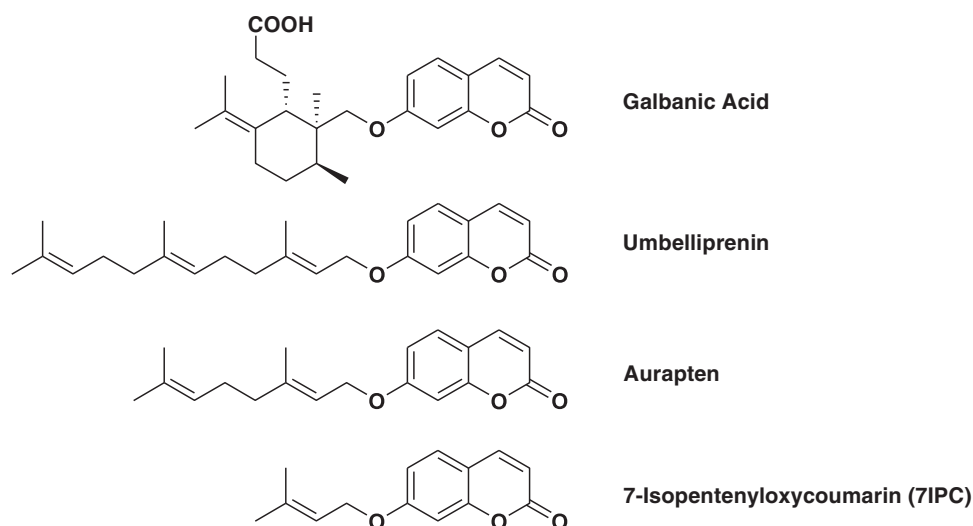

Figure 1 Chemical structure of the main natural coumarins isolated from *F. szowitsiana* root.

Determination of MICs

The MICs were determined by the tube dilution method, introduced by the National Committee for Clinical Laboratory Standards (NCCLS).[23] A serial dilution of tested compounds (final concentrations of 400 to 4 μM), were added to the test bacteria in Mueller–Hinton broth and were incubated at 37 °C for 24 h (5×10^5 CFU ml^{-1}). After sufficient incubation (24 h), the tubes are examined for turbidity indicating growth of the microorganism. For further confidence, the samples were cultured onto Petri dishes containing Muller–Hinton agar (24 h at 37 °C). The lowest drug concentration of the agent that prevented growth of the test organism was designated the MIC.

β-lactamase inhibitors

Auraptene, Umbelliprenin and 7IPC were synthesized by reaction of 7-hydroxycoumarin with the appropriate prenyl bromide according to the procedure reported in the previous work.[24] Galbanic acid was isolated from the air-dried roots of *F. szowitsiana* according to the previous work.[25]

RESULTS AND DISCUSSION

In this work we found the ethanolic extract of *F. szowitsiana* root to have potentiating activity on penicillin bactericidal potency. This was observed using the disc diffusion assay and was confined to β-lactamase producers, mainly Gram positives. Then the β-lactamase inhibitory activity of the concentrated extract (final concentrations = 5.0, 2.0 and 1.0 mg ml^{-1}) was evaluated against 30 lysed clinical isolates of penicillin-resistant *S. aureus* using penicillin-iodine-starch and nitrocefin tests.[26] During treating time, 26 samples were not able to hydrolyze penicillin G in comparison to the control group. Lack of penicillinase activity in these samples indicated the β-lactamase inhibitory activity of the extract components. The major natural compounds of the extract were three prenylated coumarins: auraptene, umbelliprenin and galbanic acid[25] (Figure 1). Afterward, the penicillinase inhibitory activity of the isolated
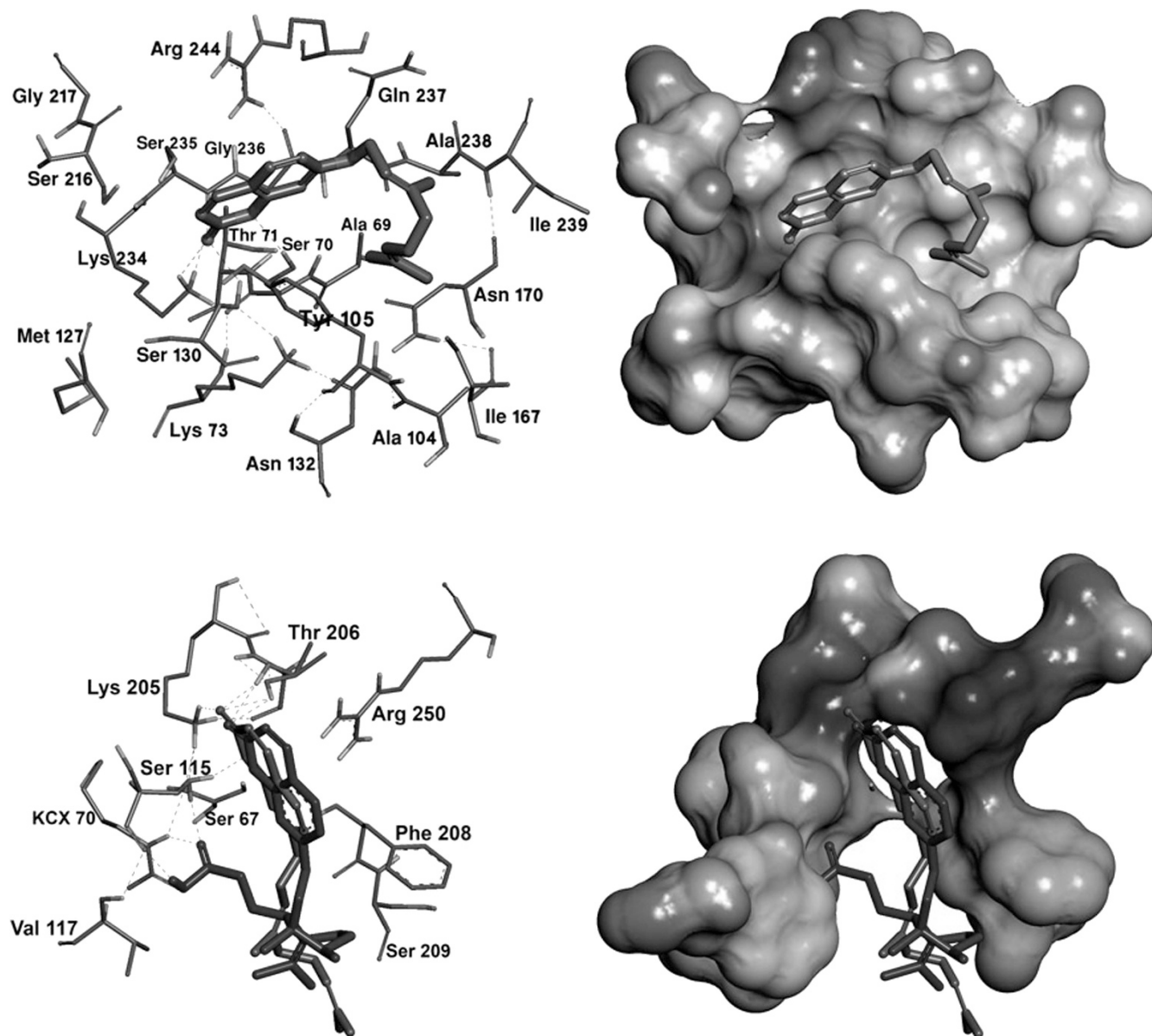

Figure 2 (above) Stick (left) and solvent surface (right) view of the active site pocket residues of 1BLC interacting with auraptene. (below) Stick (left) and solvent surface (right) view of the active site pocket residues of 1K6S interacting with auraptene (green stick) and galbanic acid (blue stick). In this figure hydrogen bonds are shown as dashed line. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

coumarins (100 μM) was evaluated on the mentioned S. aureus samples by using penicillin–iodine–starch and nitrocefin tests. The compounds inhibited the penicillinase activity in each of these 26 samples.

The β-lactamase type of the sample bacteria was determined by using the method that had been introduced by Ambler.[27] Amongst the 30 samples, 26 cases which had shown penicillinase activity belonged to class A (meticillin resistant) and the remaining belonged to class D. These results imply that the natural coumarin analogs are the class A β-lactamase inhibitors.

Amongst the 26 samples, one with highest nitrocefin hydrolysis rate was selected for enzyme inhibitory study. β-lactamase inhibitory potency of the three isolated coumarins was measured in comparison with 7-isopentenyloxycoumarin (7IPC) by using the nitrocefin method (Table 1).[21,22]

Amongst the compounds, auraptene showed the best inhibitory activity ($IC_{50} = 21 \pm 1.5$ μM) and the lowest inhibition was observed for 7IPC ($IC_{50} = 119 \pm 6.1$ μM). The IC_{50} of clavulanic acid, a control inhibitor run under the same assay conditions, was 24.1 ± 2.1 μM. The IC_{50} values showed that the coumarin moiety of the test compounds is the pharmacophore for enzyme inhibition and that the prenyl length adversely affects the potency. The results also showed that the
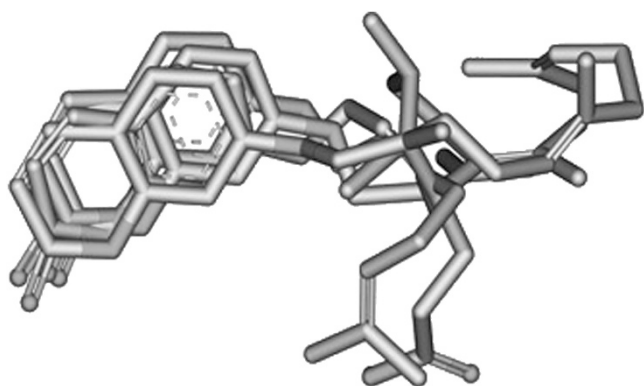
structural changes on the farnesyl moiety such as cyclization and carboxylation seen in galbanic acid did not have any effect on its β-lactamase inhibitory activity (umbelliprenin vs galbanic acid: $P > 0.05$; Table 1). For further study of the structure activity relationships, all of the test compounds were docked into the class A β-lactamase (pdb entry: 1BLC). For each compound, docking analyses showed that most of the docked models (number of conformers in cluster) with the lowest estimated inhibitory constant (Ki) (Table 1) belonged to a cluster in which the coumarin ring was covered by the conserved residues Ser70, Ser130, Lys234, Ser235 and Gly236 (Figure 2). In the desired cluster the residues Ser70, Ser130 and Lys234 have hydrogen bonds with the lacton portion of the coumarin ring and the prenyl moieties form a hydrophobic interaction with Tyr105, Ile167 and Ile239 (Figure 2). Amongst all of the conformers in the mentioned cluster, the ones with the least Ki was named as a 'consensus structure' and used for further analyses (Figure 3).

In the mentioned cluster, the side chain of Tyr105 rotated to make a hydrogen bond with the Tyr129–Ser130 amide bond (Figure 4). Hydrophobic nature of the prenyl moieties of the docked molecules led to formation of this rotation. The new orientation stabilizes the cavity of the bonding pocket (Figure 4).

The bonding affinity of the coumarins toward class D β-lactamase (pdb entry: 1K6S) was also studied. The docking results showed higher Ki (100–500 folds) for the similar orientations, which had been seen in the selected cluster of 1BLC docking outputs (Table 1).

In this orientation the lacton portion of the coumarin ring had hydrogen bonds with conserved residues Ser115, Lys205 and Thr206 (Figure 2). The steric hindrance prevents hydrogen bond formation with Ser67 unlike that seen for Ser70 in 1BLC. This finding can explain the inactivity of the coumarins against class D β-lactamases in S. aureus.

It was interesting that, unlike that seen in prenyl length, the carboxylic moiety had no effect on bonding affinity of galbanic acid toward both enzymes in the desired clusters.

The penicillin potentiating activity of the four compounds (50 μM) was tested against all of the penicillinase-positive S. aureus (26 cases) by using the serial dilution method according to the NCCLS. The MIC of penicillin G alone averaged 244.2 ± 12.3, whereas in individual combination with 7IPC, umbelliprenin, auraptene and galbanic acid the average MICs were significantly different ($P < 0.001$) (Table 2).

Figure 3 Stick view of the consensus structure of auraptene, umelliprenin, galbanic and 7IPC in 1BLC active site pocket. A full color version of this figure is available at The Journal of Antibiotics journal online.
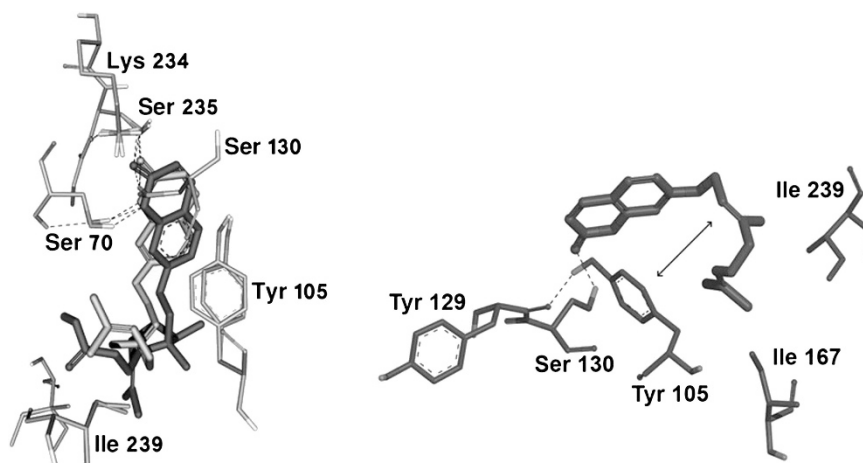

Figure 4 (left) Stick view of consensus structure of auraptene (green stick) and galbanic acid (blue stick) interacting with flexible residue in the active site pocket of 1BLC. (right) hydrophobic interaction of auraptene prenyl moiety with side chain of Ile 167, Ile 239 and phenyl moiety of Tyr 105. In this figure hydrogen bonds are shown as dashed line. A full color version of this figure is available at The Journal of Antibiotics journal online.

Compound	Average MIC (μM)	P-value
Penicillin-7IPC	78.2 ± 7.3	$P < 0.001$
Penicillin-umbelliprenin	21.3 ± 4.3	$P > 0.05$
Penicillin-auraptene	14.2 ± 4.5	$P > 0.05$
Penicillin-galbanate	18.2 ± 5.6	$P > 0.05$
Penicillin-clavulate	10.2 ± 4.6	—
Penicillin	244.2 ± 12.3	$P < 0.001$

The MICs of all _O_-prenylated coumarins alone are > 300 μM. The P-values were calculated by one-way ANOVA Dunnett's multiple comparison test (comparison between Penicillin-clavulate and other samples).

The MIC of penicillin–clavulate was significantly close to penicillin–auraptene, penicillin–galbanate and penicillin–umbelliprenin results ($P > 0.05$) (Table 2). It was interesting that the _O_-prenylated coumarins, themselves, had no antibacterial activity even at high concentrations (> 300 μM).

The MIC results make the last three natural coumarins candidates for _in vivo_ evaluation against infections caused by resistant _S. aureus_. These compounds could decrease the MIC of penicillin G by 11–16-fold. Among the β-lactam antibiotics, penicillin has been a safe and cheap agent for the treatment of _S. aureus_ infections; hence, applying this antibiotic with a suitable β-lactamase inhibitor could still make it an effective therapeutic agent for curing these infections.

ACKNOWLEDGEMENTS

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