Brazilian Journal of Microbiology (2010) 41: 574-580
ISSN 1517-8382

EFFECT OF GALBANIC ACID, A SESQUITERPENE COUMARIN FROM *FERULA SZOWITSIANA*, AS AN INHIBITOR OF EFFLUX MECHANISM IN RESISTANT CLINICAL ISOLATES OF *STAPHYLOCOCCUS AUREUS*

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Submitted: June 03, 2009; Returned to authors for corrections: August 10, 2009; Approved: February 18, 2010.

ABSTRACT

Galbanic acid, a sesquiterpene coumarin from *Ferula szowitsiana* roots, was investigated for its potentiating effect on the antimicrobial activity of antibiotics as well as ethidium bromide, in 6 multidrug resistance (MDR) clinical isolates of *Staphylococcus aureus.* Galbanic acid had inhibitory effect on none of the isolated bacteria tested (up to 800 µg /ml). The MIC range of ciprofloxacin, tetracycline and ethidium bromide, against all tested *S. aureus* were 10-80, 10-80 and 4-16 µg/ml, respectively. These were reduced to ≤2.5-5, 2.5-5 and 0.5-2 µg/ml in the presence of galbanic acid (300 µg /ml) or verapamil (100 µg /ml). The rate of ethidium bromide (2 µg /ml) accumulation in clinical isolates was enhanced with galbanic acid (300 µg /ml). There is also a decrease in loss of ethidium bromide from bacteria in the presence of galbanic acid. Similar results were obtained when verapamil (100 µg /ml) was used as an efflux pump inhibitor. Galbanic acid, like verapamil, a typical inhibitor of efflux pump, reduced the MIC of ethidium bromide and tested antibiotics. Since efflux is the only known reported mechanism for ethidium bromide resistance, the reduction in ethidium bromide MIC and enhanced accumulation as well as decreased efflux of ethidium bromide in the presence of galbanic acid, can be attributed to this efflux inhibitory properties.

Key words: Efflux pump inhibitor, *Ferula szowitsiana*, Galbanic acid, *Staphylococcus aureus*

INTRODUCTION

Efflux of antibiotics is a clinically important resistance mechanism for bacteria, often endowing them with multiple-drug-resistance (MDR) phenotypes (13). Efflux pumps may be specific for one substrate or may transport various compounds with different structures (17). MDR efflux pumps play an important role in antimicrobial resistance, and by removal of the environmental toxins contribute to the bacterial survival (4). *Staphylococcus aureus* is a major human pathogen that produces many virulence factors which contribute to its pathogenicity (6, 20). This bacterium is a cause for considerable concern, because of its ability to acquire resistance towards the currently used antibacterial agents (19). *S. aureus* chromosomes encode a range of MDR transporters. Several of these efflux pumps have been identified and demonstrated to cause resistance to various compounds (20). Fluoroquinolone resistance of several clinical isolates of *S. aureus* is provided by the membrane protein NorA encoded in the bacterial chromosome (12, 15). Also, over-expression of

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NorB leads to decrease in the susceptibility to fluoroquinolones, tetracycline, disinfectants, and dyes (17, 20). NorB, can also facilitate bacterial survival, when over-expressed in a staphylococcal abscess. Also it may contribute to the relative resistance of abscesses to antimicrobial therapy, thus linking bacterial fitness and resistance in vivo (4). It is therefore necessary that new antibiotics, resistance-modifying agents and, more specifically, efflux pumps inhibitors (EPIs) to be identified (19). The reversal of this resistance, via inhibition of drug efflux mechanisms, is a promising research area. In this regard, extensive studies have been performed in order to obtain new effective resistance modifiers from plants (11). The use of these natural bacterial resistance modifiers, including EPIs can facilitate the re-introduction of therapeutically ineffective antibiotics back into clinical use; such as ciprofloxacin, and might even suppress the emergence of MDR strains (19).

There is a report that driportlandin (Fig. 1. B), a sesquiterpene coumarin ether compound, showed a significant effect in inhibiting the efflux pump activity mediated by p-glycoprotein as compared with that of verapamil, a known resistance inhibitor (11). In the present study, we described the effect of galbanic acid, a major plant sesquiterpene coumarin present in *Ferula szowitsiana* on antimicrobial activity of antibiotics against *S. aureus* (18), as a modulator of antibiotic resistance on clinical isolates of *S. aureus*, and its possible inhibitory effect on MDR pumps.

MATERIALS AND METHODS

Materials and bacterial strains

Galbanic acid has been previously isolated and characterized by our group from the roots of *Ferula szowitsiana* DC., a plant of Apiaceae family (Fig. 1. A) (8). This plant was identified by a member of Herbarium of Faculty of Sciences, University of Tehran, Tehran, Iran. Verapamil and ethidium bromide (10 mg/ml solution) were obtained from Recordati (Italy), and Sina Gen (Iran) respectively.

Six isolates of *S. aureus* were received from University Hospital of Imam Reza, in Mashhad, Iran, as multidrug resistant isolates (resistant to cloxacillin, oxacillin, penicillin, erythromycin, chloramfenicol, ampicillin, ciprofloxacin, gentamicin, and tetracycline, sensitive to vancomycin, resistant to cefotaxime and intermediate to ceftazidime). They were subjected again to disk diffusion method for confirmation of their resistance. *S. aureus* ATCC 6538P (American Type Culture Collection) was used as a control. The used antibiotic disks were methicillin (30 µg/ml), ciprofloxacin (5 µg/ml) and tetracycline (30 µg/ml) [Pad Tan Teb, Iran].

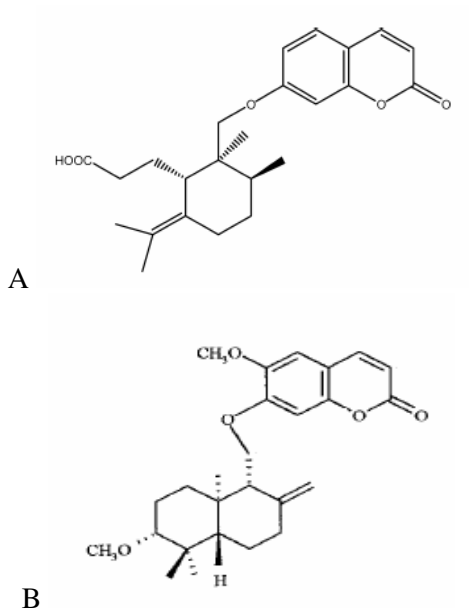

Figure 1. Chemical structure of galbanic acid (A), driportlandin (B)

Susceptibility testing

MICs of ciprofloxacin (Temad, Iran), tetracycline (Ningxia Qiyuan, China), ethidium bromide and galbanic acid, were determined by macrodilution technique according to the NCCLS guidelines (14), using *S. aureus* ATCC 6538P as the control strain. In two fold broth dilution method, Muller Hinton Broth (MHB) (Difco, USA), with an inoculum size of approximately 10^6 CFU/ml, was supplemented with serial

concentrations of either antibiotics (2.5-80 µg/ml), ethidium bromide (0.5-16 µg/ml), or galbanic acid (25-800 µg/ml, dissolved in dimethyl sulfoxide [DMSO] at final concentration of 1-2% v/v DMSO, with no antibacterial effect on its own). Plates were incubated at 37ºC for 18 h.

Potentiation of antibiotic activity by galbanic acid

Potentiation studies were performed by a broth checkerboard method (5). Final bacterial inoculum size in each well was 10^6 CFU/ml. Galbanic acid was tested at serial two fold sub-MIC concentrations (37.5-300 µg/ml), in combination with either antibiotics (2.5-80 µg /ml) or ethidium bromide (0.5-16 µg /ml). The plates were incubated at 37ºC for 18 h. As the positive control, verapamil, a known resistance inhibitor was tested in the same manner at serial two fold dilutions (12.5- 100 µg /ml).

Accumulation and efflux of ethidium bromide

Measurement of the level of ethidium bromide accumulation and efflux in 6 clinical multidrug resistant isolates of *S. aureus* and *S. aureus* ATCC 6538P was based on previously described method (3, 15, 16). Briefly, for measurement of the level of accumulation, bacterial suspension with an optical density of 0.2 at 550 nm was prepared in uptake buffer (NaCl, 110 mM; KCl, 7 mM; NH4Cl, 5 mM; Na2HPO4, 0.4 mM; Tris base, 52 mM; glucose 0.2 % adjusted to pH 7.5 with HCl) and was then exposed to ethidium bromide at a concentration of 2 µg/ml. The increase in fluorescence as ethidium bromide entered the cells was recorded fluorometrically with Shimadzu RF- 540 spectrofluorimeter (λem = 600 nm, λex = 530 nm) at room temperature. The effect of verapamil and galbanic acid on the level of accumulation was determined in a similar way, after addition of either verapamil (100 µg/ml) or galbanic acid (300 µg/ml) to the uptake buffer. To determine ethidium bromide loss, bacterial suspension was exposed to ethidium bromide (2 µg/ml) for 30 min at 37ºC. The cells were then pelleted by centrifugation and re-suspended in fresh uptake buffer. This process was repeated again in the presence of either verapamil (100 µg /ml) or galbanic acid (300 µg /ml). The loss of ethidium bromide from the cells was measured as a decrease in fluorescence.

RESULTS

Susceptibility testing

Galbanic acid had no inhibitory effect on the bacterial isolates tested (up to 800 µg/ml). The MICs of ciprofloxacin, tetracycline and ethidium bromide, against *S. aureus* were 10-80, 10-80 and 4-16 µg/ml, respectively. The MEC (minimum effective concentration) for inhibition of ethidium bromide efflux was defined as the lowest amount of either galbanic acid (300 µg/ml) or verapamil (100 µg/ml) which produced the maximum reduction in the MIC. The MICs of ciprofloxacin, tetracycline and ethidium bromide were reduced to less than 2.5-5, 2.5-5 and 0.5-2 µg/ml, respectively, in the presence of either galbanic acid (300 µg/ml) or verapamil (100 µg/ml) (Table 1).

Accumulation and efflux of ethidium bromide

Figure 2 compares the levels of ethidium bromide accumulation in *S. aureus* ATCC 6538P and isolates of *S. aureus*. As shown in Fig. 2, the rate and amount of accumulation in isolates of *S. aureus* was slower and lower than *S. aureus* ATCC 6538P. Fig. 3 compares the efflux of ethidium bromide in *S. aureus* ATCC 6538P and clinical isolates of *S. aureus*. The rate of ethidium bromide loss from isolates of *S. aureus* is higher than that of *S. aureus* ATCC 6538P. The rate of ethidium bromide (2 µg /ml) accumulation in clinical isolates was enhanced with galbanic acid (300 µg/ml) (Fig. 2). A decrease in loss of ethidium bromide from bacteria was observed in the presence of galbanic acid (Fig. 3). Similar results were obtained when verapamil (100 µg/ml) was used as an efflux pump inhibitor.

Table 1. Effect of verapamil (efflux pump- inhibitor) and galbanic acid on susceptibility of S. aureus to ciprofloxacin and tetracycline as well as ethidium bromide.

	MIC (µg/ml) of		
Isolated *S. aureus*	**ciprofloxacin (µg/ml)**	**ciprofloxacin+galbanic acid (300 µg/ml)**	**ciprofloxacin+verapamil (100 µg/ml)**
1	40	5	≤ 2.5
2	10	≤ 2.5	≤ 2.5
3	80	≤ 2.5	≤ 2.5
4	40	≤ 2.5	≤ 2.5
5	20	≤ 2.5	5
6	5	≤ 2.5	≤ 2.5
S. aureus ATCC 6538P	5	≤ 2.5	≤ 2.5
Isolated *S. aureus*	**tetracycline (µg/ml)**	**tetracycline+galbanic acid (300 µg/ml)**	**tetracycline+verapamil (100 µg/ml)**
1	80	≤ 2.5	≤ 2.5
2	40	5	5
3	40	≤ 2.5	≤ 2.5
4	10	≤ 2.5	≤ 2.5
5	40	5	≤ 2.5
6	80	5	≤ 2.5
S. aureus ATCC 6538P	≤ 2.5	≤ 2.5	≤ 2.5
Isolated *S. aureus*	**ethidiumbromide (µg/ml)**	**ethidium bromide+ galbanic acid (300 µg/ml)**	**ethidiumbromide+verapamil (100 µg/ml)**
1	8	2	2
2	4	1	2
3	16	≤ 0.5	≤ 0.5
4	8	≤ 0.5	≤ 0.5
5	8	≤ 0.5	≤ 0.5
6	2	≤ 0.5	≤ 0.5
S. aureus ATCC 6538P	1	≤1	≤1

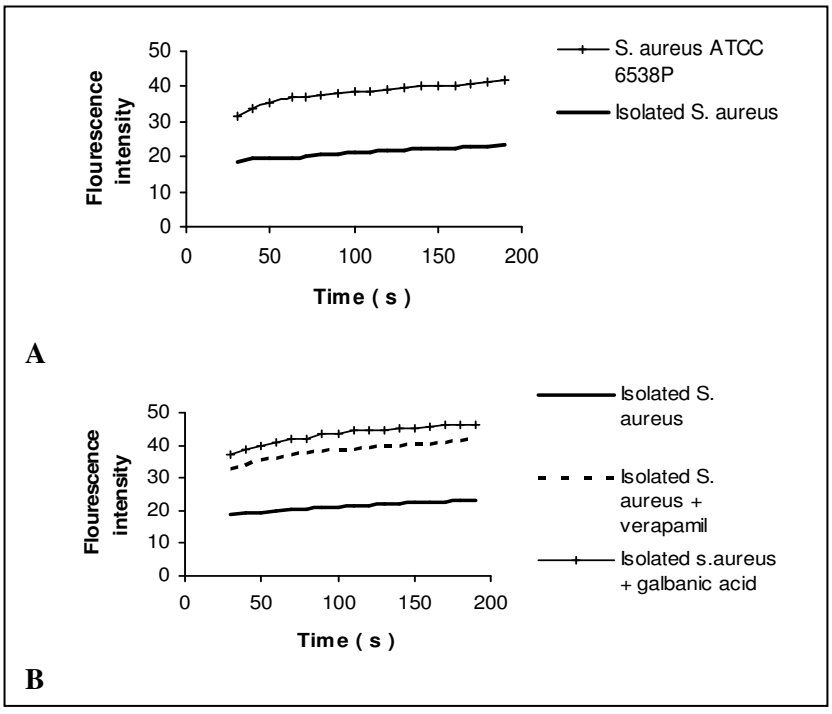

Figure 2. The levels of accumulation of ethidium bromide in *S. aureus* ATCC 6538P and clinical isolates of *S. aureus*, alone or in the presence of galbanic acid or verapamil

A: Accumulation of ethidium bromide by the *S. aureus* ATCC 6538P, and clinical isolates. Data from time 0 to 30 second was not shown.

B: Accumulation of ethidium bromide by the clinical isolates of *S. aureus,* alone and in the presence of galbanic acid or verapamil. Data from time 0 to 30 second was not shown.

Each point is the mean of at least three experiments. Data for clinical isolates are the mean of all 6 isolates.

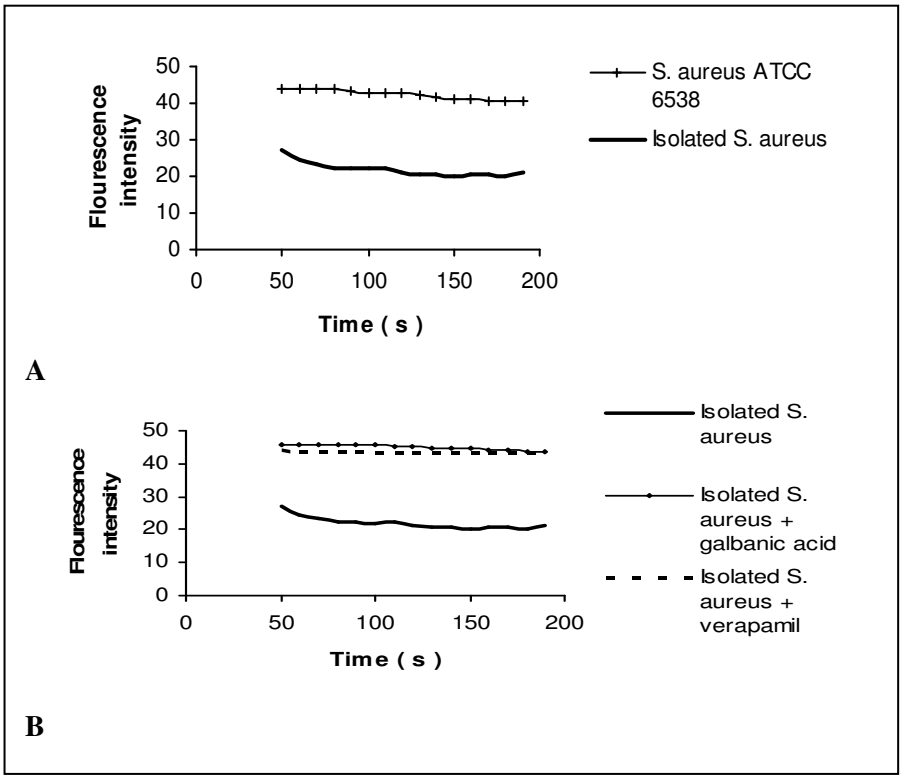

Figure 3. The efflux of ethidium bromide from *S. aureus* ATCC 6538P and clinical isolates of *S. aureus*, alone or in the presence of galbanic acid or verapamil

A: Efflux of ethidium bromide from the *S. aureus* ATCC 6538P, and clinical isolates. Data from time 0 to 50 second was not shown.

B: Efflux of ethidium bromide from clinical isolates *S. aureus,* alone and in presence of galbanic acid or verapamil. Data from time 0 to 50 second was not shown.

Each point is the mean of at least three experiments. Data for clinical isolates are the mean of all 6 isolates.

DISCUSSION

The rapid spread of bacteria expressing MDR has necessitated the research and discovery of new antibacterial and resistance-modifying agents (19). A search for compounds that interact with efflux pump proteins and can restore antimicrobial susceptibility has been ongoing for over a decade, initially focusing on Gram-positive bacteria, especially *S. aureus*. Several efflux pump genes are present on the genome of this bacterium, such as NorA and NorB of which NorA confers MDR to chloramphenicol, dyes, fluoroquinolones, antiseptics and disinfectants (17). Over-expression of NorB confers decreased susceptibility to tetracycline, fluoroquinolones, disinfectants, and dyes (17). There are numerous potentially beneficial consequences of inhibition of efflux pumps such as improving the clinical performance of various antibiotics, and several research laboratories and pharmaceutical companies have initiated programs to discover and develop compounds with efflux pump inhibitory action (10).These findings suggest that plants could provide a rich source of MDR efflux pump inhibitors and restore the activity of MDR efflux pump substrates.

There is evidence on inhibition of p-glycoprotein of mammalians cells by a sesquiterpene coumarin compound, similar to positive control, verapamil (11). While there is limited structural homology between bacterial and mammalian efflux systems, there is significant substrate overlap (16). Therefore, it is not surprising that many mammalian MDR inhibitors, also effect bacterial efflux (1, 2, 7, 13, 16). In this study, galbanic acid -a sesquiterpene coumarin from *F. szowitsiana*- acted as a potentiator of ciprofloxacin and tetracycline activity against 6 resistant clinical isolates of *S. aureus,* with the results similar to verapamil (an efflux pump inhibitor). There was also a decrease in MIC of ethidium bromide in the presence of either verapamil or galbanic acid (at concentrations with no inhibitory effect) (Table 1). Ethidium bromide is a substrate for many Gram-positive multidrug resistance pumps, including NorA or NorB (9, 20). To validate

the presence of efflux mechanism, the use of a mutant over-expressing the efflux pumps is necessary. Since efflux is the only known mechanism for ethidium bromide resistance (9), the reversal of its MIC for the clinical isolates of *S. aureus,* in the presence of verapamil or galbanic acid, could be an indication of the efflux mechanisms of resistance and the role of galbanic acid as an efflux inhibitor. The efficiency of the efflux pumps, for which the ethidium bromide is a substrate, can be assessed fluorometrically by the loss of fluorescence over time from cells loaded with ethidium bromide (7, 9). The increased loss and decreased accumulation of ethidium bromide shown in clinical isolates (Figs 2A and 3A), suggest the presence of efflux mechanism of resistance. There was a difference in the amount of ethidium bromide between isolated *S. aureus* and isolated *S. aureus* + galbanic acid or verapamil at 50 s (Figs 2B and 3B). This shows that in the presence of verapamil or galbanic acid (at MEC) there was an increase in the level of ethidium bromide accumulation and a decrease in loss of ethidium bromide in clinical isolates. This could suggest that galbanic acid inhibits the efflux of ethidium bromide in the same manner as verapamil does. Further work is required to either establish the genetic basis of efflux pump resistance in clinical isolates used or to work on isolates with over-production of efflux pumps.

ACKNOWLEDGMENTS

This work was supported financially by the Vice Chancellor for Research of Mashhad University of Medical Sciences, Mashhad, Iran with approval number of 86538.

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