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Original Article

Evaluating the Potentiating Effect of Galbanic Acid from *Ferula szowitsiana* on Three Common Antibiotics Against Resistant Hospital Isolates of *Staphylococcus aureus*

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Abstract

The plant kingdom constitutes a source of new chemical compounds, which may be important due to their potential uses in medicine, or their other biological properties. In this study, the effects of Galbanic acid (GA), a sesquiterpene coumarin from roots of *Ferula szowitsiana*, were investigated as a modulator of antibiotic resistance in clinical isolates of *Staphylococcus aureus*. Isolates of *S. aureus* clinically resistant to methicillin, tetracycline and ciprofloxacin were isolated using disk diffusion method. The MICs of antibiotics were determined using broth macrotiter plate method against isolated bacteria. The inhibitory effects of GA alone and in combination with each antibiotic were investigated by macrotiter plate. None of the tested bacteria were affected by GA (up to 800 µg/mL). MICs of antibiotics against *S. aureus* (in µg/mL) were 10-80 for methicillin, 40->80 for tetracycline, and10-20 for ciprofloxacin. The MICs of antibiotics in presence of 400 µg/mL GA were less than 1.25 µg/mL for *S. aureus*, which is usually much less than the MICs against sensitive isolates. This study provides interesting results suggesting a potentiating activity of GA on antibiotics against the resistant strains of *S. aureus*.

Keywords: Antibiotics; Resistant bacterial isolates; Galbanic acid; Potentiation; *Staphylococcus aureus.*

Introduction

Staphylococcus aureus is an important community and major hospital-acquired pathogen (1, 2). This organism is of considerable concern due to its ability to acquire resistance to antibiotics. The problem of resistant Gram-positive bacteria highlights the urgent need for new drugs or combination therapies to treat the infections caused by resistant pathogens.

The plant kingdom is a rapidly recognized source of new chemical compounds, which may be important due to their potential uses in medicine, or their other biological properties. Galbanic acid (Figure 1), a major plant sesquiterpene coumarin presents in *Ferula szowitsiana*, has been reported to have

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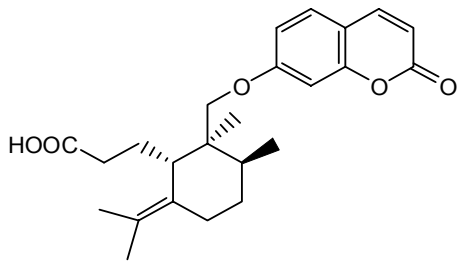

Figure 1. The structure of galbanic acid.

antibacterial enhancing effect on penicillin G and cephalexin against *S. aureus* (3). In addition, galbanic acid and its derivatives have been reported to possess anticoagulant and hepatoprotective activities (4).

In this paper, the effects of galbanic acid as a modulator of antibiotic resistance among clinical isolates of *S. aureus* are reported.

Experimental

Galbanic acid (GA)

This compound has been previously isolated from *F. szowitsiana* DC., a plant from Apiaceae family, and characterized (5). The plant was identified by Dr Akhani, Faculty of Sciences, University of Tehran, Tehran, Iran.

Confirmation of resistant isolates of S. aureus

Isolated samples of *S. aureus* (9 isolates) were obtained from the University Hospitals Imam Reza and Ghaem, Mashhad, Iran as resistant isolates. They were already subjected to disk diffusion method according to NCCLS (6) in the hospitals in order to obtain resistant strains. The antibiotics used for *S. aureus* susceptibility test were methicillin (30 µg), tetracycline (30 µg) and ciprofloxacin (5 µg). Antibiotic disks were purchased from Padtan Teb, Iran.

Determination of the minimum inhibitory concentration of antibiotics or GA

Broth macrodilution method was carried out on 24 well tissue culture plates (Orange Scientific, E. U.). In two fold broth dilution method, each 0.1 mL of each bacterial cell suspension (10^7 cfu/mL) was added to each well already containing 1 mL of different dilutions of either antibiotics or galbanic acid (GA) in Mueller-Hinton Broth (MHB) (Himedia, India), plus 1 mL of MHB and then was mixed. Antibiotics were tetracycline, ciprofloxacin and methicillin (Sigma, Poole, UK).

In each plate, wells of negative control (2 mL media) for sterility and positive control (2 mL media + 0.1 mL resistant/standard bacteria) were included. The Plates were incubated at 37°C for 18 h.

The presence or absence of growth of the microorganisms was assessed by MTT (2, 3, 5-tripheny tetrazolium chloride) assay (7). Briefly, 0.5 mL MTT (5 mg/mL) (Merck) was added to each well and the plates were incubated for 3 h at 37°C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration able to inhibit any red dye production. The experiments were repeated two times, each sample in triplicate. According to NCCLS (8) for ciprofloxacin, if the MIC is more than or equal to 4 µg/mL, the bacteria are considered resistant. This was 16 µg/mL for tetracycline and methicillin against *S. aureus.*

Interaction of antibiotics and GA

Interaction studies were performed by a broth checkerboard method (9). The concentrations ranged from 1.25 µg/mL to 80 µg/mL for all antibiotics and from 100 µg/mL to 400 µg/mL for GA. The final bacterial inoculums in each well was 5×10^4 cfu/mL. The plates were incubated at 37°C for 18 h. The growth of microorganisms was assessed by MTT assay as described above. The experiments were repeated two times, each sample in triplicate.

Results

From different *S. aureus* isolates tested, 9 isolates were resistant to at least one of the antibiotics (ciprofloxacin, methicillin or tetracycline), and they were considered for the continuation of the experiments. These are listed in Table 1 with their source sample and gender of the patients.

Table 1. Demographic data of patients and source samples.

Staphylococcus aureus					
No	source Sample	Gender	Susceptibility		
			Cip	Met	Tet
1	urine	M	+	+	+
2	urine	F	+	-	+
3	wound	M	+	-	+
4	urine	M	+	-	+
5	urine	M	+	-	-
6	wound	M	+	+	+
7	urine	M	-	+	-
8	neonate eye	M	-	+	-
9	urine	F	-	+	-

M: male, F: female, Cip: ciprofloxacin, Met: methicillin, Tet: tetracycline,
+: resistant, -: sensitive

All tested bacteria were resistant to GA (up to 800 µg/mL). MICs of antibiotics (µg/mL) against *S. aureus* were as follows: 10-80 for methicillin, 40->80 for tetracycline, and10-20 for ciprofloxacin (Table 2). The effective concentrations of ciprofloxacin in combination with 100, 200 and 400 µg/mL GA against *S. aureus* were 10-<1.25, 10-<1.25 and <1.25 µg/mL, respectively (Table 3). The effective concentrations of methicillin in combination with 100, 200 and 400 µg/mL GA against *S. aureus*, were 10, 5-<1.25 and <1.25 µg/mL, respectively (Table 4). The effective concentrations of tetracycline in combination with 100, 200 or 400 µg/mL GA against *S. aureus* were 80, <1.25 and <1.25 µg/mL, respectively (Table 5).

Table 2. The MICs of the compounds against standard or resistant hospital isolates of *S. aureus*.

Antibiotic or GA	MIC (µg/ml)	
	Standard*	Resistant
Methicillin	<2.5	10-80
Ciprofloxacin	<2.5	10-20
Tetracycline	<2.5	40->80
GA	>800	>800

* *Staphylococcus aureus* ATCC 6538p, GA: galbanic acid
The experiments were repeated two times, each sample in triplicate, and no MIC value variation was noted.

Discussion

To overcome the emerging resistance problem, studies on a combination of plant extracts and antibiotics against clinical isolates have been reported (10-12). The present study shows that GA, a new sesquiterpene coumarin isolated from the acetone extract of *F. szowitsiana* roots, reduces the MIC of the antibiotics ciprofloxacin, methicillin and tetracycline against resistant isolates of *S. aureus*. GA alone had no inhibitory effect on tested isolates in concenctrations up to 800 µg/mL (Table 2). Therefore, the observed effect in this case could be due to the antibiotic – GA combination. While GA at 100 µg/mL usually had no effect on MIC of antibiotics, increasing its concentration caused a decrease in MIC. This effect was concentration dependent.

As a group, coumarins have been found to stimulate macrophages, which could have an indirect effect on infections. Hydroxycinnamic

Table 3. MIC of ciprofloxacin in presence of GA at different levels against resistant hospital isolates of *S. aureus* using the checkerboard method.

Isolated CRSA	MIC (antibiotic) (µg/ml)	MIC (antibiotic + GA) (µg/ml)		
		GA concentration (µg/ml)		
		100	**200**	**400**
1	20	10	10	<1.25
2	20	<1.25	<1.25	<1.25
3	10	10	5	<1.25
4	20	<1.25	<1.25	<1.25
5	20	10	10	<1.25
6	10	10	<1.25	<1.25

CRSA: Ciprofloxacin-resistant *S. aureus*, GA: Galbanic acid
The experiments were repeated two times, each sample in triplicate, and no MIC value variation was noted.

Table 4. MIC of methicillin in presence of GA at different levels against resistant hospital isolates of *S. aureus* using checkerboard method.

Isolated MRSA	MIC (antibiotic) (µg/ml)	MIC (antibiotic + GA) (µg/ml)		
		GA concentration (µg/ml)		
		100	**200**	**400**
6	80	10	2.5	<1.25
7	20	10	5	<1.25
8	20	10	<1.25	<1.25

MRSA: methicillin-resistant *S. aureus*, GA: Galbanic acid
The experiments were repeated two times, each sample in triplicate, and no MIC value variation was noted.

acids, related to coumarins, seem to be inhibitory to Gram – positive bacteria. However, data about specific antibiotic properties of coumarins are scarce, although many reports give reason to believe that some utilities may reside in these phytochemicals (13).

Studies on antibiotic activity modulation effects of an extract or a crude fraction of a plant are usually performed using sub-inhibitory concentrations of the materials. The maximum concentration of GA used in this report for its potentiating effect was 400 µg/mL which is at least 3-fold lower than its MIC; as GA alone, even at 800 µg/mL, had no effect on these isolates (Table 2). However, it is believed that a concentration of 4-fold lower than the MIC is suitable when performing a potentiating assay (14).

GA having no inhibitory action on *S. aureus* at 400 µg/mL reduced the MIC of ciprofloxacin from 10-20 µg/mL to less than 1.25 µg/mL (8- to 16-fold reduction) which is close to the MIC against sensitive isolates (8). At 200 µg/mL of GA, 50% of isolates became sensitive to the action of ciprofloxacin. This effect was not considerable at 100 µg/mL of GA.

GA at 400 µg/mL reduced the MIC of methicillin from 10-80 µg/mL to less than 1.25 µg/mL which is close to the MIC against sensitive strains. At 200 µg/mL of GA, 50% of isolates became sensitive to the action of methicillin. This effect was not considerable at a concentration of 100 µg/mL.

At 200 µg/mL GA, the MIC of tetracycline against *S. aureus* was reduced from 40- 80 µg/mL to less than 1.25 µg/mL, i.e. 32- to 64-fold reduction in MIC.

This study shows that GA could act as a plant derived modulator of antibiotic activity on resistant isolates of *S. aureus*. The mechanism of this sensitization effect is not clear and merits investigation. A general role for sesquiterpenoid compounds as enhancers of nonspecific bacterial permeability to antibiotics and antimicrobials have been suggested (15). There is evidence on inhibition of efflux pump proteins by sesquiterpene coumarins (16). A coumarin derivative has been characterization

Table 5. MIC of tetracycline in presence of GA at different levels against resistant hospital isolates of *S. aureus* using checkerboard method.

Isolated TRSA	MIC (antibiotic) (µg/ml)	MIC (antibiotic + GA) (µg/ml)		
		GA concentration (µg/ml)		
		100	**200**	**400**
1	80	80	<1.25	<1.25
2	>80	80	<1.25	<1.25
3	>80	80	<1.25	<1.25
4	40	1.25	<1.25	<1.25
6	>80	80	<1.25	<1.25

TRSA: tetracycline-resistant *S. aureus*, GA: Galbanic acid
The experiments were repeated two times, each sample in triplicate, and no MIC value variation was noted.

after fractionation of grapefruit oil, which has been shown to enhance the activity of ethidium bromide and norfloxacin (17). This study provides interesting information on potentiating effect of GA on antibiotics against the resistant strains of *S. aureus*. Since all these three antibiotics can act as substrates for some MDR pumps of the pathogens (18), and GA as a sesquiterpene coumarin, reduced the MICs of these antibiotics against *S. aureus* isolates, this effect may be due to the inhibition of efflux pump proteins thus preventing extrusion of antibiotics. However, this hypothesis needs more investigation.

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