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Original Article

Evaluation of the effects of galbanic acid from *Ferula szowitsiana* and conferol from *F. badrakema*, as modulators of multi-drug resistance in clinical isolates of *Escherichia coli* and *Staphylococcus aureus*

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Abstract

Galbanic acid, a sesquiterpene coumarin from *Ferula szowitsiana*, and conferol, another sesquiterpene coumarin from *F. badrakema*, were evaluated for their effects on the reversal of multi-drug resistance in clinical isolates of *Staphylococcus aureus* and *Escherichia coli*, respectively. Neither galbanic acid (up to 1000 μg/ml) nor conferol (up to 400 μg/ml) by itself shows any antibacterial activities against tested strains. The minimum inhibitory concentrations (MICs) of ciprofloxacin and tetracycline were determined using macrodilution technique in the presence and absence of sub-inhibitory concentrations of galbanic acid (31.25-1000 μg/ml) or conferol (50-400 μg/ml), however they caused no change in MICs of the antibiotics. Galbanic acid did not show any inhibitory effect on efflux phenomenon of *E. coli*. This can be related to the outer membrane of gram-negative bacteria which is impermeable to lipophilic compounds or another mechanism rather than efflux responsible for resistance in tested *E. coli* strains. An inhibitory effect of conferol on the efflux was compared with verapamil as a positive control. Because efflux is the only known mechanism of resistance to ethidium bromide (model efflux substrate) and verapamil reduced MIC of ethidium bromide, efflux mechanism can be considered as one of the resistance mechanisms in tested *S. aureus* strains. Conferol, however, did not enhance the antibiotic efficacy mediated by inhibiting efflux pumps in bacteria.

Keywords: Conferol; *Escherichia coli*; *Ferula*; Galbanic acid; Multi-drug resistance; *Staphylococcus aureus*

INTRODUCTION

Bacterial infections are becoming more challenging to treat, due to the emergence of multi-drug resistant (MDR) pathogenic bacteria (1). One of the resistance mechanisms is reduced drug uptake into the bacterial cell. There are several mechanisms through which the uptake of a drug into a cell can be reduced: changes in the structure of the cell membrane; loss or mutations of porins in the cell membranes; and active efflux of the drug from the cells (2). Efflux of antibiotics is a clinically important and common resistance mechanism for bacteria often endowing organisms with MDR phenotypes (3).

S. aureus is an important community- and major hospital-acquired pathogen. This organism is a considerable concern, due to its ability to acquire resistance towards the newest antibacterial drugs (4). An analysis of the genome sequence of methicillin-resistant *S. aureus* N315 indicates that there are more than 20 open reading frames capable of encoding antibiotic efflux pumps (3). To date, more than 10 efflux pumps have been described for *S. aureus*, most of them are capable of extruding compounds of different chemical classes (5).

E. coli is recognized as a commensal organism and also the most common cause of urinary tract infections and diarrhea. In children and the immunocompromised patients, it can cause more serious infections associated with higher morbidity and mortality (6). For gram-negative bacteria, the resistance nodulation and cell division (RND) efflux

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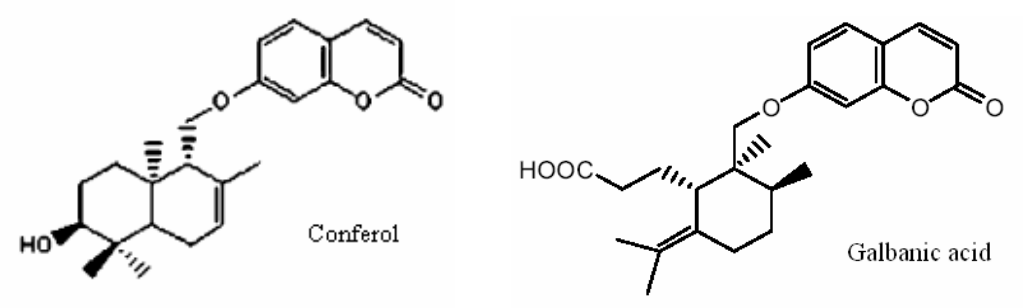

Fig. 1. Chemical structure of conferol, a sesquiterpene-coumarin isolated from *Ferula badrakema* and galbanic acid a sesquiterpene-coumarin isolated from *Ferula szowitsiana.*

systems are major contributors to resistance such as AcrAB-TolC which is involved in the resistance of *E. coli* (1).

Continued research in existing classes of antibiotics to identify derivatives that efflux minimally, and development of efflux pump inhibitors that could be used in combination with existing antibiotics to increase their potency, are strategies to combat efflux-mediated resistance (7).

The exclusively old world genus *Ferula* belongs to the family Umbelliferae distributed throughout the Mediterranean area and central Asia, especially in the former USSR and neighboring countries such as Iran. This genus is well documented as a good source of biologically active compounds such as sesquiterpene derivatives (8). Some sesquiterpenoid compounds act as enhancers of the nonspecific bacterial permeability to antibiotics resulting from disruption of the cytoplasmic membrane. There are also some evidence on the reversal of MDR in tumor cells via inhibition of P-glycoprotein by these compounds (9,10). In this study, the possible effects of two sesquiterpene coumarin compounds, galbanic acid and conferol, on improvement of the antibiotics activity in resistant strains of *E. coli* and *S. aureus*, respectively, were investigated.

MATERIALS AND METHODS

Galbanic acid and conferol

Galbanic acid and conferol were isolated from *F. szowitsiana* and *F. badrakema* as described previously (11,12).

Bacterial strains

Six isolated samples of *S. aureus* and seven isolated samples of *E. coli* were obtained from Imam Reza and Ghaem University Hospitals, Mashhad, Iran, as resistant isolates. They were already subjected to disk diffusion method in the hospitals to obtain resistant strains. The antibiotic disks used were methicillin (30 μg), tetracycline (30 μg) and ciprofloxacin (5 μg) purchased from Padtan Teb, Iran. *S. aureus* ATCC 29737 and *E. coli* ATCC 8739 were used as standard strains.

Determination of the MICs of antibiotics and galbanic acid for E.coli strains, and antibiotics and, conferol, ethidium bromide and verapamil for S. aureus strains

MICs were determined by macrodilution technique using 24 well plates, in triplicate. Using two-fold broth dilution method, 0.1 ml aliquots of the bacterial cell suspension (10^6cfu/ml) was added into each well containing 1 ml of serial two-fold dilutions of tetracycline (Ningxia Qiyuan, China), ciprofloxacin (Temad, Iran), galbanic acid, ethidium bromide (CinnaGen, Iran), verapamil (Recordati, Italy) or conferol in MHB (Mueller-Hinton Broth) (Himedia, India). Galbanic acid was dissolved in ethanol before dilution into MHB, at final concentration of 5% which had no antibacterial effect on its own. Also conferol was dissolved in dimethyl sulfoxide (Merck, Germany) at final concentration of 2% (13), and diluted in MHB supplemented with 0.5-1% Tween 80 (Merck, Germany) (14). In each plate, inoculated and uninoculated wells of tested-material-free

broth were included (the first well controls the adequacy of the broth to support the growth of the organism and the second is a check of sterility). The plates were incubated overnight at 37 °C (15).

To indicate bacterial growth, 0.5 ml of 2,3,5-triphenyltetrazolium chloride (TTC) 5 mg/ml (Sigma, UK) were added to the wells and incubated at 37 °C for 10-30 min (16).

Combination effects of galbanic acid or conferol with antibiotics

Combination studies were performed using the broth chequerboard assay; sub-inhibitory concentrations of galbanic acid (31.25-1000 µg/ml) were added to serial two-fold dilutions of ciprofloxacin or tetracycline; and sub-inhibitory concentrations of conferol (50-400 µg/ml) or verapamil as the positive control (25-200 µg/ml) were added to serial two-fold dilutions of ciprofloxacin, tetracycline or ethidium bromide as a test substrate. After inoculating the wells with 0.1 ml of the bacterial cell suspension (10^6 cfu/ml), the plates were incubated overnight at 37 °C. The growth of microorganisms was assessed by TTC assay as described above (15,17).

RESULTS

The MICs of ciprofloxacin and tetracycline against *E. coli* were ≥40 and ≥80 µg/ml, respectively. MICs of antibiotics against *S. aureus* were 10-80 µg/ml for ciprofloxacin and 80-160 µg/ml for tetracycline. *S. aureus* and *E. coli* isolates were considered resistant to ciprofloxacin and tetracycline when the MICs (µg/ml) were ≥4 and ≥16, respectively, according to National Committee on Clinical Laboratory Standards breakpoint criteria (18).

Galbanic acid alone had no inhibitory effect on *E. coli* isolates (up to 1000 µg/ml) and did not change the MICs of the antibiotics against *E. coli* (Tables 1 and 2).

Table 1. MIC of ciprofloxacin in the absence and the presence of galbanic acid against standard and resistant hospital isolates of *E. coli*.

***E. coli* strains**	**MIC ciprofloxacin (µg/ml)**	**MIC (ciprofloxacin + galbanic acid) (µg/ml)**					
		Galbanic acid concentration (µg/ml)					
		31.25	62.5	125	250	500	1000
1	>40	>40	>40	>40	>40	>40	>40
2	>40	>40	>40	>40	>40	>40	>40
3	>40	>40	>40	>40	>40	>40	>40
4	>40	>40	>40	>40	>40	>40	>40
5	40	40	40	40	40	40	40
6	>40	>40	>40	>40	>40	>40	>40
7	>40	>40	>40	>40	>40	>40	>40
ATCC8739	5	5	5	5	5	5	5

Table 2. MIC of tetracycline in the absence and the presence of galbanic acid against standard and resistant hospital isolates of *E. coli*.

***E. coli* strains**	**MIC tetracycline (µg/ml)**	**MIC (tetracycline + galbanic acid) (µg/ml)**					
		Galbanic acid concentration (µg/ml)					
		31.25	62.5	125	250	500	1000
1	>80	>80	>80	>80	>80	>80	>80
2	80	80	80	80	80	80	80
3	>80	>80	>80	>80	>80	>80	>80
4	>80	>80	>80	>80	>80	>80	>80
5	>80	>80	>80	>80	>80	>80	>80
6	>80	>80	>80	>80	>80	>80	>80
7	>80	>80	>80	>80	>80	>80	>80
ATCC8739	20	20	20	20	20	20	20

Conferol and verapamil (up to 400 µg/ml) by itself did not show any antibacterial activities against *S. aureus*. A synergistic study between verapamil and antibiotics (or ethidium bromide) led to a 2- to 8-fold reduction in MICs in clinical strains of *S. aureus*. The MICs of the ciprofloxacin, tetracycline and ethidium bromide in the presence of verapamil at the concentrations of 50, 100 and 200 µg/ml were reduced 2- and 4-fold in almost all *S. aureus* strains, and 8-fold in 50% of *S. aureus* strains. Verapamil exposure resulted in 2- to 4-fold reduction in MICs, against susceptible standard strains of *S. aureus* (Tables 3-5). However conferol caused no changes in MICs of antibiotics or ethidium bromide against any strains of *S. aureus* studied (Tables 6-8).

Table 3. MIC of ciprofloxacin in the absence and the presence of verapamil against standard and resistant hospital isolates of *S. aureus*.

***S. aureus* strains**	**MIC ciprofloxacin (µg/ml)**	**MIC (ciprofloxacin + verapamil) (µg/ml)**			
		Verapamil concentration (µg/ml)			
		25	50	100	200
1	10	10	5	2.5	1.2≤
2	80	80	40	20	10
3	20	20	10	5	5
4	40	40	40	20	10
5	10	10	5	2.5	2.5
6	10	10	5	2.5	1.2≤
ATCC 29737	0.3	0.3	0.15	0.15	0.07

Table 4. MIC of tetracycline in the absence and the presence of verapamil against standard and resistant hospital isolates of *S. aureus.*

***S .aureus* strains**	**MIC tetracycline (µg/ml)**	**MIC (tetracycline + verapamil) (µg/ml)**			
		Verapamil concentration (µg/ml)			
		25	50	100	200
1	160	160	80	40	20
2	2.5	2.5	1.2	0.6	0.6
3	160	160	80	40	40
4	80	80	40	20	10
5	160	160	80	40	20
6	160	160	80	40	40
ATCC 29737	0.62	0.62	0.62	0.3	0.3

Table 5. MIC of ethidium bromide in the absence and the presence of verapamil against standard and resistant hospital isolates of *S. aureus.*

***S. aureus* strains**	**MIC EtBr (µg/ml)**	**MIC (EtBr + verapamil) (µg/ml)**			
		Verapamil concentration (µg/ml)			
		25	50	100	200
1	16	16	8	4	2
2	16>	16>	16	8	4
3	8	8	4	2	1
4	4	4	2	1	0.5≤
5	2	2	1	1	0.5≤
6	4	4	2	1	1
ATCC 29737	2	2	1	0.5≤	0.5≤

EtBr: ethidium bromide

Table 6. MIC of ciprofloxacin in the absence and the presence of conferol against standard and resistant hospital isolates of *S. aureus.*

***S. aureus* strains**	**MIC ciprofloxacin (µg/ml)**	**MIC (ciprofloxacin + conferol) (µg/ml)**			
		Conferol concentration (µg/ml)			
		50	100	200	400
1	10	10	10	10	10
2	80	80	80	80	80
3	20	20	20	20	20
4	40	40	40	40	40
5	10	10	10	10	10
6	10	10	10	10	10
ATCC 29737	0.3	0.3	0.3	0.3	0.3

Table 7. MIC of tetracycline in the absence and the presence of conferol against standard and resistant hospital isolates of *S. aureus.*

***S. aureus* strains**	**MIC tetracycline (µg/ml)**	**MIC (tetracycline + conferol) (µg/ml)**			
		Conferol concentration (µg/ml)			
		50	**100**	200	400
1	160	160	160	160	160
2	2.5	2.5	2.5	2.5	2.5
3	160	160	160	160	160
4	80	80	80	80	80
5	160	160	160	160	160
6	160	160	160	160	160
ATCC 29737	0.62	0.62	0.62	0.62	0.62

Table 8. MIC of ethidium bromide in the absence and the presence of conferol against standard and resistant hospital isolates of *S. aureus.*

***S. aureus* strains**	**MIC EtBr (µg/ml)**	**MIC (EtBr + conferol) (µg/ml)**			
		Conferol concentration (µg/ml)			
		50	100	200	400
1	16	16	16	16	16
2	16>	16>	16>	16>	16
3	8	8	8	8	8
4	4	4	4	4	4
5	2	2	2	2	2
6	4	4	4	4	4
ATCC 29737	2	2	2	2	2

EtBr: ethidium bromide

DISCUSSION

Multi-drug efflux is an increasingly reported phenomenon and has been described for many organisms, including bacteria, fungi and protozoa, and as a mechanism of resistance in mammalian tumor cells (19,20). One natural role of efflux pumps in prokaryotic and eukaryotic cells is to remove toxins from the interior of the cell. This protective function enables bacterial cells to survive in the presence of antibiotics during the treatment of infections. Many academic and pharmaceutical programs have focused on identifying inhibitors of gram-negative and gram-positive efflux systems that could potentially be used in combination with antibiotics to improve efficacy and to suppress resistance (3).

It has been suggested that plants could provide a rich source of MDR efflux pump inhibitors (6). Compounds such as sesquiterpene-coumarin ether and driportlandin have been identified as promising modulators of MDR in mammalian cells (10). Galbanic acid a sesquiterpene coumarin isolated from *F. szowitsiana*, enhances the activity of penicillin G, cephalexin (21), ciprofloxacin and tetracycline against *S. aureus* possibly by inhibiting the efflux mechanism (22,23). In this study, the effect of galbanic acid on the reversal of resistance in gram-negative bacteria, *E. coli* was investigated. The results show that various concentrations of this compound did not have any modulating activity. Such effect can be explained by the following reasons:

-In gram-negative bacteria the outer membrane significantly slows down the entry of both lipophilic and hydrophilic agents. The former, are hindered by the lipopolysaccharide components of the outer leaflet of the outer membrane bilayer. Hydrophilic agents cross the outer membrane through water-filled porins whose size prevents rapid diffusion (7). Outer membrane as an additional permeability barrier, explains the greater resistance observed by gram-negative bacteria as opposed to gram-positive organisms (4). Galbanic acid, probably could not cross the outer membrane to inhibit efflux proteins because of its hydrophobic structure.

-Another mechanism other than efflux might be responsible for resistance in tested *E. coli* isolates.

-It is also possible that highly resistant strains have acquired too many target-based mutations, making it difficult, even with an ideal efflux inhibitor, to achieve sufficient intracellular antibiotic concentrations to overcome the reduction in target binding affinity (3).

-In *E. coli*, MDR pumps that confer resistance to fluoroquinolones and tetracycline belong to the RND (Resistance Nodulation Cell Division) family. In *S. aureus*, by contrast, resistance to the aforementioned compounds is mainly due to the activity of pumps belonging to MFS (Major Facilitator Superfamily). It is therefore less likely that a single inhibitor will potentiate fluoroquinolones and tetracycline in both *E. coli* and *S. aureus* (7).

Conferol is a sesquiterpene coumarin isolated from *F. badrakema*. There is a report that conferol can enhance the cytotoxicity of chemotherapeutic agents in tumor cells (12). While there is limited structural homology between bacterial and mammalian efflux systems, there is significant substrate overlap. Therefore, it is not surprising that many mammalian MDR inhibitors also affect bacterial efflux (3). On the other hand, conferol is structurally similar to galbanic acid, ineffective on *E. coli* strains, but acting as modulator of MDR in *S. aureus* strains. Thus, in other part of this study, the possible effect of conferol on the improvement of antibiotic activity in resistant strains of *S. aureus* was examined. The calcium channel antagonist verapamil, a known inhibitor of efflux pumps, was used as positive control (1,24).

Conferol and verapamil (up to 400 µg/ml) by itself did not show any antibacterial activity against any of the *S. aureus* strains studied (both clinical resistant isolates and standard). A synergistic study between verapamil and antibiotics (or ethidium bromide) led to a 2- to 8-fold reduction in MICs in resistant clinical isolates and 2- to 4-fold reductions in MICs in standard strain of *S. aureus*.

Ethidium bromide is a model efflux substrate

that the only known mechanism of resistance to it is via efflux (3). Since verapamil caused a 2- to 8-fold reduction in ethidium bromide MIC, efflux mechanism can be considered as one of the resistance mechanisms in tested *S. aureus* isolates (1,6). The fact that verapamil also reduced the MICs of antibiotics and ethidium bromide in *S. aureus* ATCC 29737 suggests basal intrinsic efflux activity that confers a baseline low level of intrinsic resistance to structurally unrelated compounds considered toxic to the bacterial cell (5,24).

Conferol can significantly enhance the cytotoxicity of chemotherapeutic drugs in tumor cells by interaction with the trans-membrane domains of P-glycoprotein (12); however according to the results obtained in the synergistic studies in bacteria, it did not inhibit bacterial efflux proteins to enhance antibiotics efficacy. This could be related to structural differences between bacterial and mammalian efflux systems; the mammalian P-glycoprotein whose over expression confers resistance to cytotoxic compounds belongs to ABC transporters (ATP-binding cassette), whereas in *S. aureus*, MDR is mainly conferred by MFS efflux systems such as NorA (1).

CONCLUSION

Galbanic acid, a sesquiterpene coumarin from *F. szowitsiana*, and conferol, a sesqui-terpene coumarin from *F. badrakema*, did not show any effect as modulators of antibiotic resistance on clinical isolates of *E. coli* or *S. aureus*, respectively.

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