Galbanic Acid from *Ferula szowitsiana* Enhanced the Antibacterial Activity of Penicillin G and Cephalexin against *Staphylococcus aureus*

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In this study the enhancement effect of *Ferula szowitsiana* roots' acetone extract on the antibacterial activity of penicillin G and cephalexin was evaluated against *Staphylococcus aureus*. Disk diffusion and broth dilution methods were used to determine the antibacterial activity of these antibiotics in the absence and presence of plant extract and its various fractions separated by TLC plate. The active component of plant extract involved in enhancement of penicillin G's and cephalexin's activities had *Rf*=0.336 on a TLC plate. The spectral data (^{1}H-, ^{13}C-NMR) of this compound revealed that this compound was 7-[6-(β-carboxyethyl)-5-isopropylidene-1,2-dimethylcyclo-hexylmethoxy]coumarin (galbanic acid), previously isolated from *Ferula assa-foetida*. In the presence of sub-inhibitory concentration of galbanic acid (100 μg/ml) the MIC of penicillin G for *S. aureus* decreased from 64 to 1 (a sixteen four-fold decrease) and for cephalexin from 128 to 1 μg/ml (a one hundred twenty eight-fold decrease). The highest fold decrease in MIC was observed for cephalexin in combination of galbanic acid against test strain. These results signify that the low concentration of galbanic acid (100 μg/ml) potentiates the antimicrobial action of penicillin G and cephalexin suggesting a possible utilization of these compounds in combination therapy against *S. aureus*.

Key words antibacterial activity; *Ferula szowitsiana*; penicillin G; cephalexin; *Staphylococcus aureus*; synergism

Most of the progress in 20th century modern medicine in surgery, cancer chemotherapy and organ transplantation is attributed to the use of antibiotics.[1)] The emergence of bacterial resistance to antibiotics and its dissemination, however, are major health problems, leading to treatment drawbacks for a large number of drugs.[1,2)] Consequently there has been increasing interest in the use of inhibitors of antibiotic resistance for combination therapy.[3)] This approach co-administers antimicrobial agents with an inhibitor that deactivates the resistant bacteria's resistance mechanism and increases the antimicrobial agents' effectiveness. This approach has the advantage of extending the usefulness of antibiotics with known pharmacological, toxicological and treatment properties.[4,5)] In this regard, interest has increased in plant-based natural products to combat infectious diseases.[6—8)] The natural product reserpine is known to inhibit the multidrug transporter NorA and to enhance the activity of fluoroquinolone antibiotic norfloxacin.[9)] In our program's search we have been screening various plants for their ability to decrease bacterial resistance to penicillin G and cephalexin, which are extensively used to treat infections caused by bacteria. An extract prepared from the *Ferula szowitsiana* roots was selected for further investigation. This extract's enhancing effects and its active component (galbanic acid) on the antimicrobial activity of mentioned antibiotics were evaluated against *Staphylococcus aureus*.

MATERIALS AND METHODS

General Experimental Procedures Melting points were determined on an Electrothermal 9100 apparatus and are uncorrected. IR spectra were recorded as KBr pellets or films on a Bomen MB-154 Fourier Transform. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker 400 MHz spectrometer in $CDCl_3$ and CD_3OD at 400 MHz (^{1}H) and 100 MHz (^{13}C). EI-MS were obtained on a Varian Match 7A mass spectrometer. Column chromatography was conducted using silica gel 230—400 mesh (Merck) as absorbing phase. Preparative TLC was performed on silica gel 60 GF_{254} plates (Merck) and observation of plates was carried out under UV CAMAG spectrometer (254 nm).

Preparation of Extract and Chromatography The roots of *F. szowitsiana* were collected in July 2004, from Qotour valley, Khoy, in north-west of Iran (frontier of Iran–Turkey). A voucher specimen (No. 6653-TEH) was deposited at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences. The roots of the plant were air-dried at room temperature and pulverized (250 g). The acetone extract was prepared by macerating the powder for 48 h with three changes of solution at room temperature. The combined solvent extracts were evaporated to yield a brownish, viscous residue (8% yield). The residue was fractionated by thin layer chromatography (TLC) on silica gel (60F 254 Merck) using petroleum ether/ethyl acetate (2 : 1) as the solvent system. The fractions were visualized under UV light at 254 nm and were eluted using acetone (Merck). Furthermore, active constituent was abundantly purified using following method.[10)] Part of the extract (15 g) was subjected to column chromatography on silica gel (5×50 cm) using petroleum ether with increasing volumes of acetone [petroleum ether (100), petroleum ether–acetone (95 : 5), (90 : 10), (85 : 15), (80 : 20), (75 : 25), (70 : 30), (60 : 40), (50 : 50) and acetone

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Table 1. Enhancement of Antimicrobial Activity of Penicillin G and Cephalexin against *Staphylococcus aureus* by Acetone Extract of *Ferula szowitsiana*

Samples	Mean diameter of inhibition zones (mm)						Increase in fold area[c,d]			
	No antibiotic added to the medium (A)		20 μg/ml of penicillin G added to the medium (B)[a]		20 μg/ml of cephalexin added to the medium (C)[a]		Penicillin G		Cephalexin	
	100[b]	1000	100	1000	100	1000	100	1000	100	1000
Crude extract	—	10±0.5	8±0.5	14±1	8±1	15±0.5	0.31	0.96	0.31	1.25
TLC fraction No. 3	—[e]	10±0.5	18±1	27±1	17±0.5	28±1	5.61	6.30	4.90	6.84
Other TLC fractions (negative controls)	—	—	—	—	—	—	—	—	—	—

a) Plates contained sub-inhibitory concentrations of tested antibiotics (20 μg/ml). *b*) Amount of (μg) of the samples per disks. *c*) Mean surface area of the inhibition zone (mm^2) was calculated for each tested antibiotic from the mean diameter. Fold increase for penicillin G in each dose was calculated as $(b^2-a^2)/a^2$, where *a* and *b* are the areas of inhibition zones for A and B, respectively. In the same way $(c^2-a^2)/a^2$ was used for cephalexin (C). *d*) In the absence of bacterial growth inhibition zones, the disks' diameters (7 mm) were used to calculate the fold increase in columns 5 and 6. *e*) (—) No clear zones of inhibition.

(100)]. The fractions were compared by TLC (silica gel using petroleum ether–EtOAc as solvent), and those giving similar coumarin spots were combined and further purified on preparative TLC to give auraptene (**1**, 90 mg), umbelliprenin (**2**, 346 mg), methyl galbanate (**3**, 50 mg), farnesiferol B (**4**, 20 mg), Farnesiferol C (**5**, 43 mg), galbanic acid (**6**, 6.716 g) and persicasulfide A (**7**, 16 mg). The melting point, ^{1}H- and ^{13}C-NMR data of the obtained isolated known compounds was confirmed by previous literatures.[11,12]

Antimicrobial Activities of the Acetone Extract of *F. szowitsiana* Roots and Its TLC Fractions A disk diffusion method was used to assay the acetone extract of *F. szowitsiana* roots and its TLC fractions for bactericidal activity against test strains on Müeller–Hinton Agar (MHA) plates. To assay the enhancement of antimicrobial activities, a sub-inhibitory concentration (20 μg/ml) of penicillin G and cephalexin was separately added to the plates. A single colony of test strain was grown overnight in Müeller–Hinton Broth (MHB) on a rotary shaker (200 rpm) at 35 °C. Resistance clinical isolate of *S. aureus* from our collection were used. The inocula were prepared by diluting the overnight cultures with 0.9% NaCl to a 0.5 McFarland standard and were applied to the plates along with the disks containing the acetone extract of *F. szowitsiana* roots and/or its different TLC fractions. After incubation at 35 °C for 18 h, the inhibition zones were measured. The assays were performed in triplicate.

Determination of the Minimum Inhibitory Concentration of Active Compound Susceptibility tests were carried out by the standard broth micro dilution method in accordance with the NCCLS guidelines (2000) in MHB with an inoculum of approximately 10^3 colony-forming units (CFU)/ml.[13] The MHB was supplemented with serial antibiotics concentrations ranging from 0.125 to 128 μg/ml, and galbanic acid at concentrations from 60 to 960 μg/ml. The data were reported as MICs, the lowest concentration of antibiotic and active TLC fraction (galbanic acid) inhibiting visible growth after 24 h of incubation at 35 °C. To evaluate active component's effect in combination with antibiotics, increasing concentrations (with a twofold step, *i.e.*, 0.125, 0.250, … , 128 μg/ml) of penicillin G and cephalexin were added to MHB containing galbanic acid at sub-MIC concentration (100 μg/ml). Tubes containing an identical amount of MHB, but free from antibiotics and galbanic acid, and tubes separately containing the antibiotic or galbanic acid were included in each assay as a growth control. After 24 h of incubation at 35 °C, the lowest antibiotic concentration in combination with galbanic acid that prevented the development of turbidity was regarded as the MIC*.

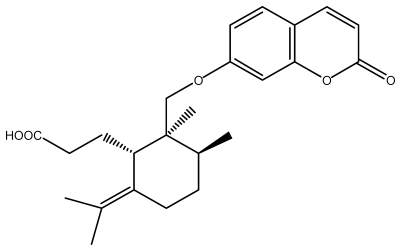

Fig. 1. Chemical Structure of Galbanic Acid

RESULTS

TLC analysis of the acetone extract of *F. szowitsiana* roots showed at least six distinct fractions, which were visualized UV at 254 nm. The antimicrobial activities of *F. szowitsiana* roots extract and each of the fractions were tested against test strains by a disk diffusion method. Neither the acetone extract of *F. szowitsiana* roots nor any of the six fractions eluted from the preparative TLC plates showed any antimicrobial activity against test strains on MHA plates at a content of 100 μg/disk (Table 1). On the plate containing sub-inhibitory concentrations of antibiotics, however, zones of inhibition were observed with the acetone extract of *F. szowitsiana* roots and fraction No. 3 (Table 1). The acetone extract of *F. szowitsiana* roots showed intrinsic antibacterial activity at the highest concentration tested (1000 μg/disk), but the inhibition zone increased in the presence of sub-inhibitory concentrations of drugs for acetone extract of *F. szowitsiana* roots (Table 1). The active component of the acetone extract of *F. szowitsiana* roots involved in enhancement of penicillin G's and cephalexin's activities had Rf=0.336 on TLC plates. The structure of the compound as white crystals was confirmed by ^{1}H-, ^{13}C-NMR spectra and melting point data with those previously described in the literature.[11,12] This data revealed that this compound was 7-[6-(β-carboxyethyl)-5-isopropylidene-1,2-dimethylcyclo-hexylmethoxy]coumarin (galbanic acid), previously isolated from *Ferula assa-foetida* (Fig. 1).[14]

The effect of the galbanic acid on the enhancement of the

Table 2. Susceptibility of Test Strains to Galbanic Acid (MIC), Antibiotics (MIC) and the Combination of Galbanic Acid and Antibiotics (MIC*), as Well as Their MIC Reduction Folds[a,b]

Test strains	Galbanic acid	Penicillin G			Cephalexin		
	MIC	MIC	MIC*	MIC reduction fold	MIC	MIC*	MIC reduction fold
S. aureus	>120	64	1	64	128	1	128

a) All media were supplemented with 100 μg/ml of active constituent (galbanic acid) selected during bioassay guided fractionation. *b*) MICs values are represented in μg/ml. Standard deviations in all experiments were negligible.

tested antibiotics' antimicrobial activity was also investigated against test strain (Table 2). Penicillin G's potency against test strain was increased sixteen four-fold when tested with a sub-toxic concentration of galbanic acid (Table 2). Also, galbanic acid increased cephalexin's bactericidal activity. In the presence of 100 μg/ml of galbanic acid the MIC of cephalexin for test strain decreased from 128 to 1 μg/ml (a one hundred twenty eight-fold decrease).

DISCUSSION

These results indicate that the antibacterial effect of penicillin G and cephalexin is enhanced by acetone extract of *F. szowitsiana* roots. The active component of this extract involved in enhancing the tested antibiotics was isolated using column chromatography and was identified as galbanic acid previously isolated from *Ferula assa-foetida*.[14] Our results confirmed that this component's inhibitory effect on the test strains could not be observed at a concentration <120 μg/ml (Table 2). This component's combination effect with penicillin G and cephalexin was investigated against a resistance clinical isolates using the broth dilution method. It should be pointed out that the galbanic acid concentration of 100 μg/ml was chosen to guarantee that the effect produced was due to the combination and not to the effect of the galbanic acid itself. So the effect observed in this condition could be due to the antibiotic–galbanic acid combination. At the concentration tested, galbanic acid significantly improved antibiotic efficacy against *S. aureus* when combined with penicillin G and cephalexin (Table 2). The reason for this difference is not known and merits investigation. To overcome the emerging resistance problem, studies on a combination of plant extracts with antibiotics against clinical test strains have been reported.[15—17] This is the first report of the enhancement of penicillin G and cephalexin activities with an extract prepared from *F. szowitsiana* roots and its active constituent.

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