Sophoraflavanone G from *Sophora pachycarpa* Enhanced the Antibacterial Activity of Gentamycin against *Staphylococcus aureus*

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In this study the enhancement effect of *Sophora pachycarpa* roots' acetone extract on the antibacterial activity of gentamycin was evaluated against *Staphylococcus aureus.* Disc diffusion and broth dilution methods were used to determine the antibacterial activity of gentamycin in the absence and presence of plant extract and its various fractions separated by TLC. A clinical isolate of *S. aureus* was used as test strain. The active component of the plant extract involved in enhancement of gentamycin's activity had $R_f = 0.72$ on a TLC plate. The spectral data (^{1}H NMR, ^{13}C NMR) of this compound revealed that this compound was 5,7,2′,4′-tetrahydroxy-8-lavandulylflavanone (sophoraflavanone G), previously isolated from *Sophora exigua*. In the presence of 0.03 μg/mL of sophoraflavanone G the MIC value of gentamycin for *S. aureus* decreased from 32 to 8 μg/mL (a four-fold decrease). These results signify that the ultra-low concentration of sophoraflavanone G potentiates the antimicrobial action of gentamycin suggesting a possible utilization of this compound in combination therapy against *Staphylococcus aureus.*

Key words: Antibacterial Activity, Sophoraflavanone G, Synergism

Introduction

Most of the progress during the 20th century in surgery of modern medicine, cancer chemotherapy and organ transplantation is attributed to the use of antibiotics (Braga *et al.,* 2005). 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 (Braga *et al.*, 2005; Schito, 2006). Consequently there has been increasing interest in the use of inhibitors of antibiotic resistance for combination therapy (Wright, 2005). 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 (Renau *et al.*, 1998; Wright, 2000). In this regard, interest has increased in plant-based natural products to combat infectious diseases (Cowan, 1999; Liu *et al.*, 2001; Oumzil *et al.,* 2002; Mimica-Dukic *et al.,* 2003; Shahverdi *et al.,* 2004). The natural product reserpine is known to inhibit the multidrug transporter NorA and to enhance the activity of the fluoroquinolone antibiotic norfloxacin (Markham and Neyfakh, 1996). In our program's search we screened various plants for their ability to decrease bacterial resistance to gentamycin, which is extensively used to treat infections caused by bacteria. An extract prepared from *Sophora pachycarpa* roots was selected for further investigation. In this investigation the enhancing effects of this extract and its active component (sophoraflavanone G) on the antimicrobial activity of gentamycin were evaluated against *Staphylococcus aureus.*

Materials and Methods

Plant materials

Sophora pachycarpa (Fabaceae) was collected in May 2005 in the north of Khorasan Razavi province, Mashhad (Ferdowsi University Campus), Iran. The plant was identified in the Department of Pharmacognosy, Faculty of Pharmacy, Mashhad University of Medical Sciences (MUMS), Mashhad, Iran. A voucher specimen (No. 06-019-016) has been deposited at the Herbarium of the Faculty of Pharmacy, MUMS.

Preparation of extract and chromatography

The roots of the plant were air-dried at room temperature and pulverized (150 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 (4.3% yield). The residue was fractionated by thin

layer chromatography (TLC) on silica gel (60F 254; Merck) using petroleum ether/acetone (50:50) 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 the following method (Amin-Ar-Ramimeh, 2005). The plant extract (6.5 g) was subjected to column chromatography (300 g silica gel). Elution with petroleum ether/acetone (100:0, 1 L; 95:5, 1 L; 85:15, 2 L; 75:25, 2 L; 70:30, 1 L; 65:35, 1 L; 60:40, 1 L; 55:45, 1 L; 50:50, 1 L; 0:100, 1 L) gave several fractions. The purification of fraction 2 (95:5, 1 L) using preparative TLC yielded 50 mg of active compound.

Antimicrobial activities of the acetone extract of S. pachycarpa roots and its TLC fractions

A disc diffusion method was used to assay the acetone extract of *S. pachycarpa* roots and its TLC fractions for bactericidal activity against test strains on Müller-Hinton Agar (MHA) plates. To assay the enhancement of antimicrobial activities, a sub-inhibitory concentration of gentamycin (8 μg/mL) was separately added to the plates. A single colony of test strains was grown overnight in Müller-Hinton Broth (MHB) on a rotary shaker (200 rpm) at 35 °C. A clinical isolate of *S. aureus* from our collection was 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 discs containing the acetone extract of *S. pachycarpa* 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.

Spectroscopy

^{1}H and ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY, HSQC and HMBC spectra were recorded on a Bruker Avance DRX 500 spectrometer. ^{1}H NMR and ^{13}C NMR spectra were measured in CD_3COCD_3.

Determination of the minimum inhibitory concentration of the active constituent

Susceptibility tests were carried out by the standard broth dilution method in accordance with the NCCLS guidelines (National Committee for Clinical Laboratory Standards, 2000) in MHB with an inoculum of approximately 10^5 colony-forming units (CFU)/mL. The MHB was supplemented with serial antibiotic concentrations ranging from 0.125 to 16 μg/mL, and sophoraflavanone G at concentrations from 0.00625 to 1.6 μg/mL. The data were reported as MIC values, the lowest concentration of antibiotic and active TLC fraction (sophoraflavanone G) inhibiting the visible growth after 24 h of incubation at 37 °C. To evaluate active TLC fraction's effect in combination with antibiotic, increasing concentrations (with a two-fold step, *i.e.*, 0.0156, 0.0312, ..., 16 μg/mL) of gentamycin were added to MHB containing a sub-inhibitory concentration of sophoraflavanone G (0.03 μg/mL). Tubes containing an identical amount of MHB, but were free from antibiotics and sophoraflavanone G, and tubes separately containing the antibiotic or sophoraflavanone G were included in each assay as a growth control. After 24 h of incubation at 37 °C, the lowest antibiotic concentration in combination with sophoraflavanone G that prevented the development of turbidity was regarded as the MIC* value.

Results and Discussion

TLC analysis of the acetone extract of *S. pachycarpa* roots showed at least 6 distinct fractions, which were visualized at 254 nm. The antimicrobial activities of the *S. pachycarpa* acetone extract and each of the fractions were tested against test strains by a disc diffusion method. Bioactivity-guided fractionation of this extract led to the isolation of a compound shown in Fig. 1. Either the acetone extract of *S. pachycarpa* roots or fraction

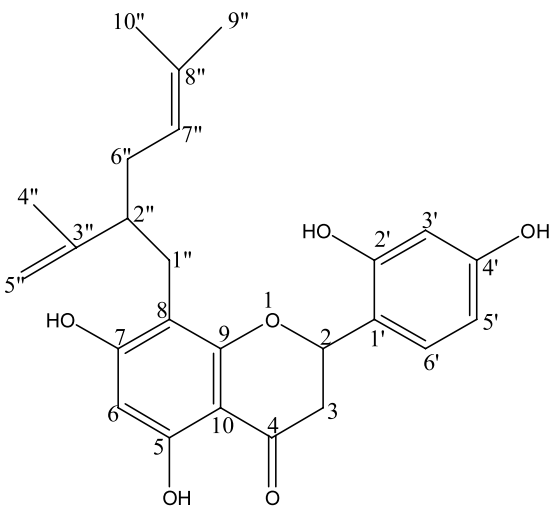

Fig. 1. Chemical structure of sophoraflavanone G.

Table I. Enhancement of antimicrobial activity of gentamycin against *Staphylococcus aureus* by *S. pachycarpa* acetone extract (100 μg/disc) and TLC fraction 3 (10 μg/disc).

Sample	Mean diameter of inhibition zone [mm]		Increase in fold area[b]
	No antibiotic added to the medium (A)	8 μg/mL of gentamycin added to the medium (B)[a]	
Crude extract	15.0 ± 1	22.0 ± 0.5	1.15
TLC fraction	14.5 ± 0.5	22.0 ± 1	1.3

[a] Plates contained sub-inhibitory concentrations of tested antibiotic (8 μg/mL).
[b] Mean surface area of the inhibition zone (mm^2) was calculated for the tested antibiotic from the mean diameter. Fold increase for gentamycin was calculated as $(b^2 - a^2)/a^2$, where a and b are the areas of inhibition zones for A and B, respectively.

Table II. Susceptibility of test strains to sophoraflavanone G (MIC), gentamycin (MIC) and the combination of sophoraflavanone G and antibiotic (MIC*)[a], as well as their MIC reduction folds.

Test strain	Sophoraflavanone G	Gentamycin		
	MIC[b]	MIC	MIC*	MIC reduction
S. aureus	0.05	32	8	4-fold

[a] All media were supplemented with 0.03 μg/mL of sophoraflavanone G selected during bioassay-guided fractionation.
[b] MIC values are represented in μg/mL. Standard deviations in all experiments were negligible.

3 eluted from the preparative TLC plates showed good antimicrobial activity against the test strain on MHA plates at tested concentrations. On the plate containing sub-inhibitory concentrations of antibiotics, however, larger zones of inhibition were observed with the acetone extract of *S. pachycarpa* roots and fraction 3. The acetone extract of *S. pachycarpa* roots and fraction 3 showed intrinsic antibacterial activity at the tested concentrations, but the inhibition zone increased in the presence of sub-inhibitory concentrations of gentamycin for the acetone extract of *S. pachycarpa* roots and TLC fraction 3 (Table I). The active component of the plant extract involved in the enhancement effect of gentamycin had R_f = 0.72 on TLC plates. The structure of the yellow crystalline compound was confirmed by ^{1}H NMR, ^{13}C NMR spectra and its melting point (Iinuma *et al*., 1995). This data revealed that this compound was 5,7,2′,4′-tetrahydroxy-8-lavandulylflavanone (sophoraflavanone G), previously isolated from *Sophora exigua* (Tsuchiya and Iinuma, 2000).

The effect of sophoraflavanone G on the enhancement of the tested antibiotic's antimicrobial activity was also investigated against *S. aureus* (Table II). Gentamycin's potency against the test strain was increased four-fold when tested with a sub-toxic concentration of sophoraflavanone G (Table II). On the other hand in the presence of ultra-low concentration of sophoraflavanone G (0.03 μg/mL) the MIC value of gentamycin for *S. aureus* decreased from 32 to 8 μg/mL (a four-fold decrease). These results indicate that the antibacterial effect of gentamycin is enhanced by the acetone extract of *S. pachycarpa* roots. The active component of this extract involved in enhancing the tested antibiotics was isolated using the TLC technique and was identified. This component's combination effect with gentamycin was investigated against *S. aureus* using the broth dilution method. It should be pointed out that the sophoraflavanone G concentration of 0.03 μg/mL was chosen to guarantee that the effect produced was due to the combination and not to the effect of sophoraflavanone G itself. So the effect observed under this condition could be due to the antibiotic-sophoraflavanone G combination. At the concentration tested, sophoraflavanone G significantly improved the antibiotic efficacy against *S. aureus* when combined with gentamycin (Table II). Furthermore, our preliminary investigation showed that sophoraflavanone G can increase the antibac-

terial activity of some other antibiotics such as amoxicillin, tetracycline, and ciprofloxacin (data not shown). At this time the reason for this enhancement 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 (Shahverdi *et al*., 2004; Shin and Pyun, 2004; Gibbons, 2005). Sophoraflavanone G has been reported to exhibit anti-*S. aureus* acitivity (Sohn *et al.,* 2004). To the best of our knowledge, this is the first finding on the combination effect of this compound on gentamycin activity against *S. aureus.*

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