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Quick identification of kuraridin, a noncytotoxic anti-MRSA (methicillin-resistant *Staphylococcus aureus*) agent from *Sophora flavescens* using high-speed counter-current chromatography

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ABSTRACT

Bacterial resistance to antibiotics has become a serious problem of public health that concerns almost all currently used antibacterial agents and that manifests in all fields of their application. To find more antibacterial agents from natural resources is all the time considered as an important strategy. *Sophora flavescens* is a popularly used antibacterial herb in Chinese Medicine, from which prenylated flavones were reported as the antibacterial ingredients but with a major concern of toxicity. In our screening on the antibacterial activities of various chemicals of this herb, 18 fractions were obtained from 8 g of 50% ethanol extract on a preparative high-speed counter-current chromatography (HSCCC, 1000 ml). The system of *n*-hexane/ethyl acetate/methanol/water (1:1:1:1) was used as the two-phase separation solvent. A chalcone named kuraridin was isolated from the best anti-MRSA fraction, together with sophoraflavanone G, a known active ingredient of *S. flavescens*. Their structures were elucidated by analysis of the NMR spectra. Both compounds exhibited significant anti-MRSA effects, compared to baicalein that is a well known anti-MRSA natural product. More important, kuraridin showed no toxicity on human peripheral blood mononuclear cells (PBMC) at the concentration up to 64 μg/ml while sophoraflavanone G inhibited over 50% of cellular activity at 4 μg/ml or higher concentration. These data suggested that opening of ring A of the prenylated flavones might decrease the toxicity and remain the anti-MRSA effect, from a viewpoint of structure–activity relationship.

1. Introduction

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) remain a major problem worldwide [1,2]. In Hong Kong, it accounts for 58.2% of *S. aureus* isolated from blood cultures and 69.8% of all *S. aureus* isolates in our public hospitals [3]. Notification of community associated (CA)-MRSA has been made mandatory in Hong Kong [4,5]. The pharmaceutical arsenal available to control MRSA is quite limited. Vancomycin is the mainstay of treatment of MRSA but overuse has generated fully resistant MRSA strains [6,7]. It is a great challenge for modern medicine to fight against the super bacteria. Screening of natural products for antibacterial agents attracts increasing attention in recent years [8,9]. For instance, reserpine potentiates tetracycline's activity against MRSA strains through inhibiting the Bmr efflux pump [10]. Some methoxylated flavones inhibit the MDR pump NorA in the presence of berberine and norfloxacin [11]. However, the toxicity is the major concern.

In a preliminary study, the antibacterial effects of 33 herbal medicines which are conventionally used in antibacterial treatments were tested against: (1) *S. aureus* (ATCC 25923), (2) methicllin-resistant *S. aureus* (MRSA) (ATCC BAA-43) and (3) *Escherichia coli* (ATCC 25922). The 90% ethanol extract of *Sophora*

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flavescens was found the most active against both SA and MRSA, compared to the water extract and the water/ethanol 5/95 extract [12]. Literature research demonstrated that the prenylated flavanones of this medicinal herb showed anti-MRSA activities, but with the concern of toxicity [13]. Beside flavanones, there are other types of chemicals in this herb, e.g. diterpene alkaloids [14]. We report here on the separation and screening by bioassay guided fractionation using high-speed counter-current chromatography of a *S. flavescens* extract in order to take a complete check on the comprehensive chemical profile of herb extract. The 50% ethanol extract which was believed to contain both water soluble and ethanol soluble components was selected.

As it is not easy to identify active ingredients from natural materials because they often contains a large number of chemical components, bioassay guided fractionation are usually used. Unfortunately, all the conventional separation methods are based on solid separation materials that will cause severe sample loss, and sometimes denaturation of samples, leading to an incomplete components inventory after fractionation. Such an unsatisfactory situation can be avoided by high-speed counter-current chromatography (HSCCC), a liquid-to-liquid separation technique based on solvent partition, which generally allows sample recovery with a good yield and without the use of any solid separation materials. This method has been proven successful in tracking out active components from natural products, within a quick and efficient mode [15,16].

Herein we report the fractionation of 50% ethanol extract of *S. flavescens* using HSCCC, the isolation of the fractions with higher antibacterial activity against MRSA, and the identification of their active compounds using NMR analysis. The toxicity of the isolated compounds has been tested on human peripheral blood mononuclear cells. Comparison of the bioactivities and structures of the active compounds led to an interesting suggestion regarding the structure–activity relationship.

2. Experimental

2.1. Apparatus

The preparative HSCCC instrument used in this study was TBE-1000A high-speed counter-current chromatography (Shanghai, Tauto Biotech, China) which had three polytetrafluoroethylene coils (total volume, 1000 ml). The β-value of the column varied from 0.59 at the internal layer to 0.75 at the external layer. This separation column was connected with a Buchi 615 MPLC series (middle pressure liquid chromatography, BUCHI Labortechnik AG, Switzerland) which provided pump, UV detector and sample collector. HSCCC fractions were analyzed using a Waters ACQUITY UPLC™ system (Waters Corp., MA, USA) equipped with a Waters Evaporative Light-scattering Detector (ELSD). Purification was performed using the MPLC coupled with a hand-made C_{18} column (40×2.5 cm). ^{13}C NMR spectra (100 MHz) were recorded on a Brucker DRX-400 spectrometer using Tetramethylsilane as internal standard. Mass spectra were obtained on a VGAuto Spec-3000 spectrometer.

2.2. Materials and reagents

The roots of *S. flavescens* were purchased from a herbal store in Hong Kong. The voucher specimen is deposited at the Institute of Chinese Medicine, the Chinese University of Hong Kong, with the voucher specimen number 2007-3067. Methanol, acetonitrile, *n*-hexane, ethyl acetate and acetic acid of HPLC grade were purchased from TEDIA company, Inc., USA. Distilled water was prepared using MILLI-Q SP reagent water system (Millipore, MA, USA). Ciprofloxacin was obtained from Bayer Healthcare (Leverkusen, Germany). Erythromycin, fusidic acid, gentamicin, kanamycin and oxacillin were purchased from Sigma Chemical Co. (St. Louis, USA). Mueller Hinton (MH) broth was obtained from Becton, Dickinson and company (USA).

2.3. Bacterial strains

Six laboratory *S. aureus* strains were used for the susceptibility tests. SA-ST239 [17], a representative strain of methicillin-resistant *S. aureus* (MRSA) is a healthcare-associated multidrug-resistant strain, which is prevalent in Asian countries. This clone has also been detected in South America and Eastern Europe, and variants of this strain corresponded to the UK epidemic MRSA 1,4,11, Brazilian and Hungarian clones, that disseminated widely in many continents [18]. *S. aureus* SA-1199B (harboring resistance to fluoroquinolones through overexpression of the NorA efflux pump) is ciprofloxacin resistant [19]. SA-RN4220-pUL5054 is resistant to 14- and 15-membered macrolides including erythromycin and contains the multicopies plasmid pUL5054 coding for MsrA, an efflux pump [20]. Three experimentally induced aminoglycosides resistant strains through methylation of specific nucleotides within the A-site of rRNA hampering the binding of aminoglycosides were also included in this study: (a) SA-APH2″-AAC6′ (aminoglycoside-6′-N-acetyltransferase/2″-O-phosphoryltransferase) is resistant to gentamicin, (b) SA-APH3′ (aminoglycoside-3′-O-phosphoryltransferase) is resistant to kanamycine, and (c) SA-ANT4′ (aminoglycoside-4′-O-phosphoryl transferase) is resistant to fusidic acid.

2.4. Preparation of two-phase solvent system and sample solution

The mostly used two-phase solvent system of hexane–ethyl acetate–methanol–water (HEMW, 1:1:1:1) was used here. The two-phase solvent system was prepared by adding the solvents to a separation funnel according to the volume ratios and fully equilibrated by shaking repeatedly at room temperature. The upper and lower phases were separated and degassed by sonication for 30 min before use.

The crushed roots (100 g) were allowed to soak in 1 L of 50% EtOH for 1 h followed by refluxing for another hour. The extract was collected and the extraction was repeated with another 1 L of 50% EtOH. The extracts were combined and centrifuged for 20 min (4400 rpm, 20 °C). The supernatant was collected and concentrated under reduced pressure at 50 °C. The concentrated extract was lyophilized to give a dried powder (20.37 g), 8 g out of which was dissolved in 80 mL of the two-phase solvent to make the sample solution.

2.5. HSCCC fractionation and subsequent HPLC examination and purification

The coil column was first entirely filled with the upper phase of the solvent system at a flow rate of 40 mL/min using a Buchi 615 MPLC pump (BUCHI Labortechnik AG, Switzerland). Then the apparatus was rotated at 500 rpm, and the lower phase was pumped into the column at the flow rate of 8 mL/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, the sample solution was injected through the injector. When the separation time reached 270 min, the rotation was stopped and all the solution was pushed out of the column by high-pressure gas. The separation temperature was controlled at 25 °C. The effluent from the outlet of the column was continuously monitored at 254 nm and was collected by 10 min/tube.

HSCCC fractions were analyzed using a Waters ACQUITY UPLC™ system (Waters Corp., MA, USA) equipped with a Waters ACQUITY

BEH C_{18} column (100 × 2.1 mm, 1.7 μm, Waters Corp., Ireland) and a Waters ELSD detector. The mobile phase ran at the flow rate of 0.3 mL/min in a gradient program: 40–100% acetonitrile in 0–5 min. The working conditions of the ELSD detector were as follows: drift tube temperature (75 °C), nebulizer temperature (48 °C), gain (300 °C), gas pressure (45 psi). Purification of the pool of active fractions (13–17) was performed using MPLC coupled with a hand-made C_{18} column (40 × 2.5 cm). The mobile phase was 60% acetonitrile at a flow rate of 10 mL/min, under UV detection at 254 nm.

2.6. Antibacterial assay

2.6.1. Determination of minimum inhibitory concentration (MIC)s

For antimicrobial susceptibility tests, cells (10^6 CFU/ml) were inoculated into MH broth and dispensed at 200 μL/well in 96-well microtiter plates. MICs were determined by serial 2-fold dilution of the test compound in MH broth. Stock solutions of baicalein were freshly prepared in DMSO and other agents were made in sterile deionised water. DMSO constituted less than 1% of the total test volume. The bacterial broth was incubated for 18 h at 37 °C. The bacterial growth in each well was measured at optical density (OD) of 620 nm using a spectrophotometer (Multimode Detector DTX 880, Beckman Coulter, USA) and was corrected by the background OD (obtained from the OD microorganism-free microtiter plates). The % growth inhibition was calculated by this formula:

$$\frac{(\text{OD of drug containing well} - \text{OD of the drug-free well})}{\text{OD of containing well}} \times 100$$

For the tested compounds, MIC was defined as the lowest antibacterial concentration, which resulted in ≥99% inhibition of growth compared to the drug-free control. MIC determination against *S. aureus* (ATCC 25923), a sensitive strain, was performed as a positive control.

A checkerboard assay was conducted to measure the synergy between the isolated compounds and antibiotics against a panel of MRSA resistant strains that had known resistant mechanisms. Serial 2-fold dilutions of the compounds and the corresponding antibiotics of the tested strain were mixed in each well of a 96-well microtiter plate so that each row (and column) contained a fixed amount of one agent and increasing amounts of the second agent. Stock solutions of the tested compounds were made in MH broth. The resulting plate presents a pattern in which every well contains a unique combination of concentrations between the two molecules. The concentrations of antibiotics ranged from 0 to 256 μg/ml, while the isolated compounds concentrations ranged from 0 to 16 μg/ml. Each plate contained a row and column in which a serial dilution of each agent was present alone. Each microtiter well was inoculated with approx. 10^5 CFU/ml of bacteria, and the plates were incubated at 37 °C for 24 h under aerobic conditions. MIC values obtained for a given combination were used to evaluate the effects of combination between the isolated compounds and antibiotics by calculating the Fractional Inhibitory Concentration Index (FICI) using fractional inhibitory concentration index (FIC) and the following formula: FIC of the isolated compounds = MIC of the isolated compounds in combination/MIC of the isolated compounds alone; FIC of drug B = MIC of drug B in combination/MIC of drug B alone; hence FICI = FIC of the isolated compounds + FIC of drug B. Off-scale MICs were converted to the next highest or next lowest doubling concentration. "Synergy" was defined when FIC index was less than or equal to 0.5; while "additive" in which the FIC index was greater than 0.5 and less than or equal to 1.0; whereas "indifferent" when the FIC index was greater than 1.0 and less than or equal to 2.0; and "antagonistic" in cases which the FIC index was greater than 2.0 [21].

2.6.2. Cellular toxicity test

The cytotoxicity of the isolated compounds was determined by XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) assay using buffy coat purified human peripheral mononuclear cells (PBMC) collected from Hong Kong Red Cross. PBMC were plated in 96-well plates at 10^5 cells/well [22]. Serial dilutions of the isolated compounds were added to the wells. The plates were maintained in a 37 °C incubator. After 48 h, 50 ml of XTT/PMS (phenazine methosulphate) solution (20 μM) was added to each well. Then, the plate was incubated at 37 °C for 48 h. The OD was determined by a spectrophotometer at 450 nm. The toxicity represents the ratio of OD of a well in the presence of tested compounds with the OD of control wells in the presence of medium containing DMSO. The cellular viability of at least 90% was considered to indicate a non-toxic compound.

2.6.3. Statistical analysis

Data were expressed as mean ± standard deviation. Statistical analyses and significance, as measured by the Student's *t*-test for paired samples were performed using Prism software version 4.0 (GraphPad Software, CA, USA). In all comparisons, $p < 0.05$ was considered statistically significant.

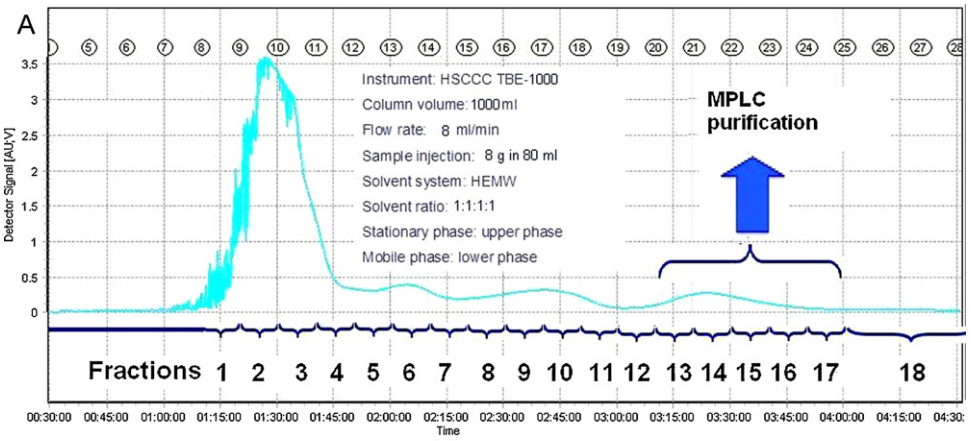
3. Results and discussion

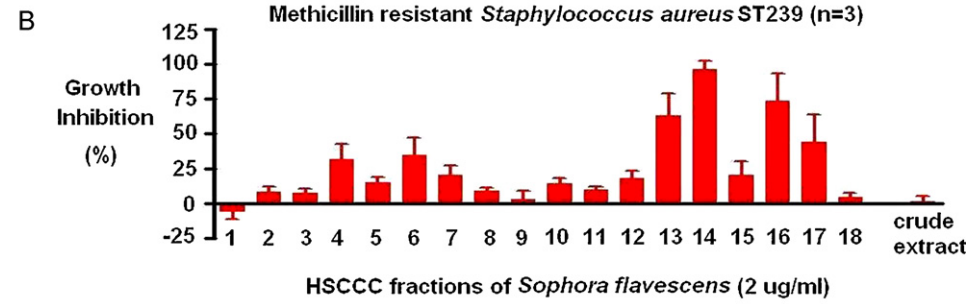
3.1. HSCCC fractionation

The 50% ethanol extract of *S. flavescens* (yield around 20%) was selected for fractionation because it contained the larger number of compounds compared with the water and 95% ethanol extracts, as shown from thin layer chromatography because it contained both water soluble and ethanol soluble components. This extract was used here for HSCCC fractionation in order to screen as many as possible components and take a complete check on the chemical profile of *S. flavescens*, for which conventional methods using solid separation materials showed some significant drawbacks. If silica gel was used, some water soluble chemicals could not be dissolved in the organic solvents; and if reverse phase material like C_{18} or C_8 was used, the sample loading would be quite limited due to poor solubility of the ethanol soluble chemicals. HSCCC using two-phase solvent system allows circumvent this problem, and therefore is suitable for samples containing compounds within a large polarity range. Using this method, 28 tubes were collected and combined into 18 fractions as shown in Fig. 1A. When the separation was stopped, there was still 1000 ml solution in the HSCCC column. The solution was pushed out by high pressure gas and combined with tubes no. 26–28 to make fraction no. 18, the last fraction. All the 18 fractions were dried and weighed. The results (Fig. 1C) showed the majority of the 50% ethanol extract of *S. flavescens* was in the first three fractions meaning polar chemicals. The nonpolar part was in the last fractions. The general yield was 7662.9 mg out of 8 g, giving the sample recovery up to 95.8% which ensured almost all the kinds of constituents of *S. flavescens* would be screened in the subsequent bioassay.

3.2. Antibacterial activity against MRSA ST239

All the HSCCC fractions were screened for their antibacterial effects against MRSA ST239. The sample concentration was first tested at 10 μg/ml, and almost all the fractions showed 100% inhibition. When the concentration was lowered to 2 μg/ml, as shown in Fig. 1B, these fractions showed significant differences. Significant growth inhibition against MRSA ST239 was observed in fractions no. 13–14 and no. 16–17. Fraction no. 14 exhibited the strongest antibacterial activity (96.9 ± 5.9%), while the crude extract of *S.*





C HSCCC fractions obtained from the crude extract of *S. flavescens* (8 g)

Fractions	Time (min)	Weight (mg)	Fractions	Time (min)	Weight (mg)
1	0-80	101.5	10	160-170	111.3
2	80-90	4520.1	11	170-180	32.3
3	90-100	2193.6	12	180-190	22.8
4	100-110	153.2	13	190-200	24.6
5	110-120	100.3	14	200-210	23.5
6	120-130	123.1	15	210-220	35.5
7	130-140	43.2	16	220-230	13.2
8	140-150	52.4	17	230-240	8.9
9	150-160	89.7	18	After 240	13.7
Total					7662.9
Recovery					95.8%

Fig. 1. HSCCC fractionation of the crude extract. (A) The HSCCC chromatogram (254 nm) of the crude extract; (B) effect of HSCCC fractions 1–18 and the crude 50% ethanolic extract of *S. flavescens* on growth inhibition of methicillin resistant *Staphylococcus aureus* ST239 ($n = 3$); (C) the resulted 18 fractions. The fractions (2 μg/ml) were incubated with ST239 for 24 h. The results were expressed as % growth inhibition ± standard deviation.

flavescens Ait at 2 μg/ml was ineffective. Fraction no. 15 showed low activity because in which an additional dissolvable white powder was found. In HPLC-ELSD analyses indicated that fraction no. 15 had a similar chemical profile like those of active fractions (no. 13–14 and no. 16–17), in which there were only two major components as illustrated in Fig. 2. These fractions were combined for further purification.

3.3. Identification of bioactive ingredients

As shown in Fig. 2, HPLC examination on the HSCCC fractions indicated there were two major peaks in the active fractions no. 13–17. ELSD detector was used here in order to exclude the influence of some impurity having strong UV absorption. These two major components were isolated using MPLC coupled with a preparative C_{18} column and subsequently measured for their MS and NMR spectra. By comparison with the published data, these two compounds were identified to be a prenylated dihydroflavanone sophoraflavanone G and a prenylated chalcone kuraridin [23,24].

Sophoraflavanone G: ^{13}C NMR (100 MHz, δ methanol-d_4 ppm): 17.8 (C-7a), 19.2 (C-10a), 25.8 (C-6a), 27.9 (C-1a), 32.3 (C-3a), 43.2 (C-3), 48.4 (C-2a), 75.7 (C-2), 96.2 (C-6), 103.2 (C-5′), 103.3 (C-10), 107.7 (C-3′), 108.6 (C-8), 111.2 (C-9a), 118.3 (C-1a), 124.8 (C-4a), 128.5 (C-20), 132.0 (C-5a), 149.7 (C-8a), 156.6 (C-6′), 159.5 (C-4′), 162.6 (C-5), 163.1 (C-9), 166.5 (C-7), 199.0 (C-4). Positive ESI-MS (m/z): 425 (M+H).

Kuraridin: ^{13}C NMR (100 MHz, δ methanol-d_4 ppm): 17.9 (C-7a), 19.0 (C-10a), 25.9 (C-6a), 28.2 (C-1a), 32.4 (C-3a), 48.0 (C-2a), 56.1 (C-5—OCH_3), 91.6 (C-6), 103.7 (C-5′), 107.0 (C-1), 108.9 (C-8), 108.9 (C-3′), 111.1 (C-9a), 116.3 (C-1′), 125.0 (C-4a), 125.5 (C-3), 131.6 (C-6′), 131.7 (C-5a), 139.8 (C-2), 149.9 (C-8a), 160.3 (C-2′), 162.2 (C-4′), 162.4 (C-5), 164.0 (C-7), 166.6 (C-9), 194.8 (C-4). Positive ESI-MS (m/z): 439 (M+H).

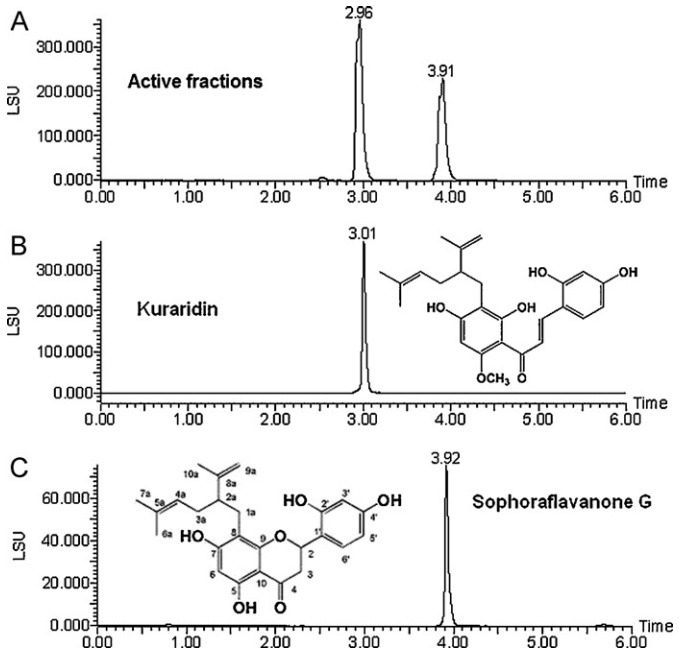


Fig. 2. HPLC-ELSD chromatograms of the active fractions (13–17) and isolated compounds. (A) Active fractions (13–17); (B) kuraridin; (C) sophoraflavanone G. HPLC analysis was carried out on HPLC analysis was carried out on a Waters ACQUITY UPLC™ system (Waters Corp., MA, USA) equipped with a Waters ACQUITY BEH C_{18} column (100 × 2.1 mm, 1.7 μm, Waters Corp., Ireland) and a Waters ELSD detector. The mobile phase ran at the flow rate of 0.3 mL/min in a gradient program: 40–100% CAN in 0–5 min. The working conditions of the ELSD detector were as follows: drift tube temperature (75 °C), nebulizer temperature (48 °C), gain (300 °C), gas pressure (45 psi).

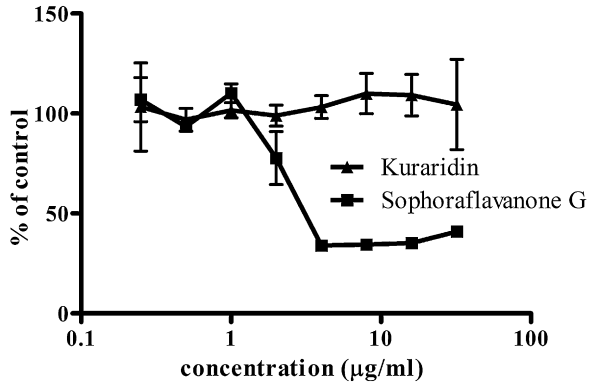


Fig. 3. Cellular toxicity (XTT assay) of the isolated compounds from *Sophora flavescens* Ait on human peripheral blood mononuclear cells (PBMC) ($n = 4$). The results were expressed as % growth of drug free control (mean of 4 values) ± standard deviation.

3.4. Effects of isolated compounds against different MRSA strains with known resistant mechanisms

S. aureus can exemplify the adaptive evolution of bacteria in the antibiotic era, better than any other human pathogen, as it has demonstrated a unique ability to quickly respond to each new antibiotic with the development of a resistance mechanism, starting with penicillin and methicillin, followed by linezolid and daptomycin [25]. New antibacterial agents against MRSA are therefore in urgent need. Compounds isolated from *S. flavescens* have been shown to possess antibacterial activities against MRSA and other bacteria strains as well [13,26]. To further investigate the therapeutic potential of the active ingredients of *S. flavescens* in MRSA treatment, we have chosen a panel of clinically relevant *S. aureus* strains with known resistance mechanisms in the current study. They included: SA-ST239, which is a representative strain of methicillin-resistant *S. aureus* (MRSA) and a healthcare-associated multidrug-resistant strain prevalent in Asian countries [17]; two bacterial efflux pump over expressed strains: SA-1199B, a multidrug-resistant strain that overexpresses the NorA efflux mechanism and confers a high level of resistance to certain fluoroquinolones [19], SA-RN4220-pUL5054 (a macrolide-resistant strain) which contains the multicopies plasmid pUL5054 coding for MsrA, an efflux pump [21]; and three experimentally induced aminoglycosides resistance strains SA-APH2″-AAC6′, SA-APH3′ and SA-ANT4′.

Sophoraflavanone G and kuraridin's effects against these six different MRSA strains with known resistant mechanisms were summarized in Table 1. Methicillin sensitive strain ATCC 25923 was used as positive control strain. These two compounds were effective against both methicillin sensitive and resistant *S. aureus* strains. The MICs of sophoraflavanone G against the MRSA were ranged from 2 to 4 μg/ml while the MICs of kuraridin were ranged from 8 to 16 μg/ml. Their activities were much stronger than that of baicalein which is a well known antibacterial natural product. Besides direct antibacterial actions, the synergistic activities of sophoraflavanone G and kuraridin with antibiotics against MRSA were evaluated by checkerboard assay and the results were expressed as fractional inhibitory concentration index (FICI) (Table 2). Effects were observed on most MRSA strains when sophoraflavanone G and kuraridin were used in combination with corresponding antibiotics. However, no synergistic antibacterial activity could be identified in sophoraflavanone G or kuraridin with corresponding antibiotics against the 6 MRSA strains.

Table 1
Minimum inhibitory concentrations of the isolated compounds from *Sophora flavescens* Ait against different methicillin sensitive and resistant strains ($n = 3$).

S. aureus strains	Minimum inhibitory concentration (MIC) μg/ml median (range)			
	Sophoraflavanone G	Kuraridin	Baicalein	Antibiotics
ATCC 25923	2 (2–2)	8 (8–8)	32 (32–64)	Oxacillin 0.13 (0.13–0.25)
APH2″AAC6	2 (2–4)	8 (8–8)	128 (128–128)	Gentamicin >128
APH3′	2 (1–4)	8 (8–8)	128 (64–128)	Kanamycin >128
ANT4′	4 (2–4)	8 (2–8)	128 (64–256)	Fusidic acid >128
RN4220	4 (4–4)	8 (8–16)	128 (64–128)	Erythromycin >128
ST239	4 (2–4)	8 (8–8)	128 (128–256)	Oxacillin >128
1199B	2 (1–4)	16 (4–16)	128 (64–128)	Ciprofloxacin 16 (8–16)

Table 2
Fractional inhibitory concentration index (FICI) of sophoraflavone G and kuraridin against 6-MRSA strains and methicillin sensitive strain ATCC25923. The FICI is the sum of the FICs of each of the drugs, which were defined as the MICs of each drug when used in combination divided by the MICs of each drug when used alone. The interaction of kuraridin or sophoraflavanone G with the tested antibiotics was defined as synergistic if the FICI was less than or equal to 0.5; additive if the FICI was greater than 0.5 and less than or equal 1.0; indifferent if the FIC index was greater than 1.0 and less than or equal to 2.0; and antagonistic if the FIC index was greater than 2.0 (n = 3).

S. aureus strains	Agents	FICI Median (range)	Outcome
1. 1199B	Sophoraflavanone G	0.75 (0.75–1.00)	Additive
Resistant to ciprofloxacin	Kuraridin	0.75 (0.50–1.25)	Additive
2. RN4220/pUL5054	Sophoraflavanone G	0.75 (0.56–0.75)	Additive
Resistant to erythromycin	Kuraridin	0.75 (0.63–1.00)	Additive
3. APH2″AAC6	Sophoraflavanone G	0.75 (0.75–1.00)	Additive
Resistant to gentamicin	Kuraridin	0.75 (0.75–1.00)	Additive
4. APH3′	Sophoraflavanone G	1.50 (1.50–1.50)	Indifferent
Resistant to kanamycin	Kuraridin	1.00 (0.63–1.00)	Additive
5. ANT4′	Sophoraflavanone G	1.00 (0.75–1.50)	Additive
Resistant to fusidic acid	Kuraridin	1.25 (1.00–1.50)	Indifferent
6. ST239	Sophoraflavanone G	1.50 (1.00–1.50)	Indifferent
Resistant to oxacillin	Kuraridin	1.00 (0.75–1.50)	Additive
7. ATCC25923	Sophoraflavanone G	0.63 (0.63–0.75)	Additive
Sensitive to oxacillin	Kuraridin	0.75 (0.38–0.75)	Additive

3.5. Cellular toxicity of the isolated compounds on human PBMC

The cellular toxicity of the isolated compounds on human PBMC was evaluated by XTT assay [27–29]. As shown in Fig. 3, after 48 h incubation with human PBMC, kuraridin was non-cytotoxic when compared with the drug free control in the range 0.25–64 μg/ml. By contrast, sophoraflavanone G was toxic from 4 to 64 μg/ml with more than 50% of cellular activity inhibition (Fig. 3). IC_{50} was determined as 3.2 ± 0.4 μg/ml.

4. Conclusion

HSCCC succeeded in discovery of active lead compound from medicinal natural resources by serving a high sample recovery. This time, two anti-MRSA compounds kuraridin and sophoraflavanone G were found in an efficient mode. They showed potent antibacterial activity against the tested MRSA strains with MIC values in the 1–16 μg/ml range. In checkerboard study, they also have additive effects with conventionally used antibiotics against the tested strains. These observations suggest that the antibacterial actions of both kuraridin and sophoraflavanone G are not directly related to inhibitions of bacterial efflux systems and aminoglycoside resistance as well. More important, in cytotoxicity test using human primary cells PBMC, sophoraflavanone G exhibited toxicity in its therapeutic concentration range but kuraridin was non-cytotoxic up to 64 μg/ml to human lymphocytes in the XTT assay. These two compounds contained very similar chemical structures, and kuraridin bearing a chalcone skeleton could be considered as one of the products of ring opening of sophoraflavanone G. Therefore, it was suggested that opening of ring A of the prenylated flavone might decrease the toxicity and remain the anti-MRSA effect, from a viewpoint of structure–activity relationship.

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