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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