Antibacterial Alkaloids from Chelidonium majus Linn (Papaveraceae) against clinical isolates of methicillin-resistant Staphylococcus aureus

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ABSTRACT. Purpose. This study describes the antibacterial effect of extracts and compounds isolated from the aerial part of *Chelidonium majus* Linn. (Papaveraceae) acting against clinical strains of methicillin-resistant Staphylococcus aureus (MRSA). **Methods**. The activities were evaluated by using the macrobroth dilution method and reported as the MICs/MBCs (minimal inhibitory concentrations / minimal bactericidal concentrations). **Results**. Bioassay-guided fractionation of the most active extract from the aerial parts (EtOAc) led to the isolation of benzo[c]phenanthridine-type alkaloids 8-hydroxydihydrosanguinarine (hhS), 8-hydroxydihydrochelerythrine (hhC), which were potently active against MRSA strains with MICs/MBCs ranged from 0.49-15.63/1.95-62.50 µg/ml, respectively. **Conclusions**. The selective antibacterial activity reported in this paper for 8-hydroxylated benzo[c]phenanthridine-type alkaloids isolated from *C.majus* opens the possibility that they could be helpful for the developing of new antibacterial agents for treating the infection of MRSA which has created nosocomial problem worldwide.

Methicillin-resistant Staphylococcus aureus (MRSA) is a multidrug-resistant (MDR) bacterium. The resistance has increased relentlessly and well recognized as a global nosocomial problem in recent years. MRSA is resistant to β-lactams, aminoglycosides, fluoroquinolones and macrolide, only sensitive to vancomycin, but the isolate of vancomycin-resistant *S*. aureus (VRSA) has also been reported. It is resulted from the selective pressure of antibiotics currently used, leading to high morbidity and mortality [1]. Hence, there is an urgent need to develop novel active agents. As plants can produce antimicrobial compounds to protect themselves from biotic attack, searching for active agents from plant

sources might be an alternative clue [2].

Chelidonium majus Linn. (greater celandine, swallow-wort, and bai-qu-cai in Chinese) is the only species of the tribe Chelidonieae of the Papaveraceae family. It is rich in various types of isoquinoline alkaloids. The phytochemical and pharmacological properties, including antibacterial activity of *C. majus* have been reviewed, which is of great interest for its use in Chinese, Asian and European herbal medicines [3, 4]. *C. majus* has been traditionally used for treatment of gastric ulcer, astric cancer, oral infection, liver disease, and general pains. The extracts of *C. majus* are proven to be safe as components of veterinary and human phytopreparations, and of oral-hygiene agents [5]. Previous chemical studies of *C. majus* have reported the isolation of isoquinoline alkaloids such as chelidonine, chelerythrine, sanguinarine, berberine, coptisine, and *dl*-stylopine. Among them, chelindonine and protopine exhibited anti-tumor activity, and protoberberine showed antibacterial and antiviral activity, while sanguinarine and chelerythrine had anti-inflammatory activity. Recently, two benzo[c]phenanthridine type alkaloids 8-hydroxydihydrosanguinarine (hhS), 8-hydroxydihydrochelerythrine (hhC) were isolated and identified as acetylcholinesterase inhibitory compounds from ethanol extract of *C. majus* [6]. In the course of our screening for natural products that are active against MDR bacteria, we have paid special attention to the activity of alkaloids in traditional herbs used for combating the newly escalated pathogens. We have found that an alkaloid-containing extract from the aerial parts of *C. majus* contained inhibitory substances.

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Further activity-guided fractionation led to the isolation of four alkaloids hhS, hhC, dihydrosanguinarine (hS) and dihydrochelerythrine (hC). Here, we report the isolation of the four alkaloids from the extract and the assay of their activities against clinical strains of MRSA.

METHODS

Bacterial strains

MRSA strains (20 isolates) were obtained from the infectious samples of critically ill patients in Kunming General Hospital (KGH). Pathogen purification and identification were conducted in clinical microbiology laboratory of KGH following the conventional procedures [7] and the criteria of Clinical and Laboratory Standards Institute (CLSI) [8, 9]. The control strain ATCC 25923 (methicillin-sensitive *Staphylococcus aureus*, MSSA) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, China). Vancomycin (Eli Lilly Japan K. K., Seishin Laboratories) was used as the positive control agent.

Plant material and culture media

Crude extracts from *C. majus* aerial parts contained 10% of total alkaloids were purchased from a commercial supplier (Xian Hongsheng Biotech. Co., China). Standard Mueller–Hinton agar and broth (MHA and MHB, Tianhe Microbial Agents Co., Hangzhou, China) were used as bacterial culture media.

Screening for antibacterial fractions

The crude extracts (300 g) were firstly fractionated between H_2O and AcOEt (ethyl acetate). The AcOEt layer was concentrated *in vacuo* to afford residue (45.3g, EA). The residue was subjected to bioassay-guided fractionation by vacuum liquid chromatography (VLC, a column liquid chromatography method which is performed under reduced pressure) over SiO_2 (200-300 mesh, Qingdao, China) column and eluted with gradient systems of petroleum ether∕AcOEt∕EtOH (12:1:0–5:1:2). A total

of 23 fractions (EA1–23) thus collected, together with EA were then subjected to activity screening with MHA plate following the agar well diffusion method as described previously [10]. The fractions were prepared at concentrations of 3 mg/ml in 50% DMSO. Wells of 6 mm diameter were made in 20 ml MHA seeded with 20 µl of a suspension of test organisms containing 1.5×10^8 CFU/ml. 70 µl of extracts were fed into wells, incubated at 35°C for 24 h, after which time they were examined for zones of inhibition. Three fractions (EA1–3) that showed diameters of inhibitory zone ranged from 19.5 to 35.0 mm against MSSA and selected MRSA strains were determined (Table 1).

Isolation and identification of alkaloids from active fractions

The active fractions EA1–3 were further isolated by repeated vacuum liquid chromatography (VLC) over SiO_2 (500 mesh) to furnish pure alkaloids hhS (250 mg) and hhC (95 mg) from EA3 (eluting with petroleum ether∕AcOEt∕EtOH = 40:2:1–40:2:2), and hS (140 mg) from EA1 (eluting with petroleum ether∕AcOEt = 40:1) and hC (80 mg) from EA2 (eluting with petroleum ether∕AcOEt =25:1), respectively. The structures of the four compounds were identified by spectral analysis and direct comparison of their ^{1}H and ^{13}C NMR spectral data with those found and described in literatures [6, 11].

Antibacterial activity of the alkaloids

The minimal inhibitory concentrations (MICs) of hhS, hhC, hS and hC for the various isolates were determined following standard CLSI broth macrodilution methods [12]. Briefly, twofold dilutions were conducted in tubes containing an initial concentration of 1000 µg/ml of hhS and hhC and 3000 µg/ml of hS and hC, dissolved in 50% DMSO, respectively. Each tube contained an inoculum density of 5×10^5 CFU/ml of each of the test organisms. All organisms were grown in MHB. The tubes contained sampled alkaloids and isolates were incubated at 35°C for 20 h and were examined for growth in daylight. The growth of the microorganisms was determined by turbidity. Clear tubes indicated absence of bacterial growth. For every

experiment, a sterility check (50% DMSO and medium), negative control (50% DMSO, medium and inoculum) and positive control (50% DMSO, medium, inoculum and vancomycin or imipenem) were included. The MIC of the alkaloids was the lowest concentration in the medium that completely inhibited the visible growth. The solvent value was deducted accordingly to get the final results of activity. The minimal bactericidal concentrations (MBCs) were determined by inoculating the surfaces of MHA plates with 25 μl of samples taken from the clear tubes of the macrobroth susceptibility studies. After the bacterial suspensions had fully absorbed into the agar, the plates were further incubated at 35°C for 20 h and were examined for growth in daylight. The MBC was defined as the concentration of drug that resulted in > 99.9% killing of the bacterium relative to the concentration of bacterium that was present in test wells at 0 h [13]. All experiments were performed in triplicate (Table 2).

RESULTS

Bioassay-guided fractionation and isolation of the extracts from *C. majus* led to the characterization of four benzo[c]phenanthridine-type alkaloids hhS, hhC, hS and hC (Figure 1). The antibacterial activities of the fractions and pure compounds are shown in Table 1 and Table 2.

DISCUSSION

Compared with the few known types of chemical structures belonging to currently used antibiotics, the hardly limited structural types of novel plant antimicrobial compounds supply us with boundless and evergreen opportunities for developing alternative agents which are active against MDR microorganisms so as to meet the clinical problems of antibiotic resistance [2].

In the present study, the various activities of the four alkaloids were shown against both the MSSA strain and 20 isolates of MRSA (Table 2).

Results of the activity against clinical MDR isolates of MRSA are offered for the first time to the best of our knowledge, though another benzo[c]phenanthridine alkaloid from *Zanthoxylum clava-herculis* extracts, i.e. chelerythrine has been

proved to exhibit potent activity against strains of MRSA with MIC of 8-16 μg/ml [2]. Other previous reports of plant antibacterial benzo[c]phenanthridine type alkaloids have been dealt with the activity of hS and hC (from *Bocconia arborea*) against *S. aureus*, *E. coli* and *P. aeruginosa* [11]. The alkaloids 8-acetonyldihydronitidine and 8-acetonyl-dihydroavicine also showed significant growth inhibition of *S. aureus* with the MICs of 1.56 μg/ml and 3.12 μg/ml, respectively [14]. Nevertheless, no results of the antibacterial properties of the four compounds against MDR strains have been reported thus far.

1	R^1—R^2 = CH_2 ,	R^3=OH
2	R^1 = R^2 = CH_3 ,	R^3=OH
3	R^1—R^2 = CH_2 ,	R^3=H
4	R^1 = R^2 = CH_3 ,	R^3=H
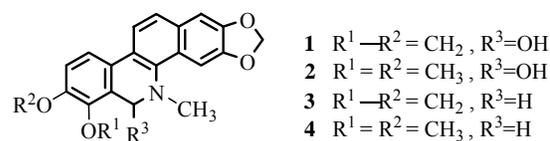
Figure 1. Structures of the four alkaloids

(**1**: hhS; **2**: hhC; **3**: hS; **4**: hC)

Table 1. Inhibition zone diameters of the crude alkaloids and active fractions against selected strains

Fractions	Zone diameters (mm) for strains:		
	MSSA	MRSA082	MRSA092
EA	19.5	18.0	19.0
EA1	21.0	19.5	19.0
EA2	26.5	26.0	20.0
EA3	35.0	33.5	32.5

It is interesting that the 8-hydroxylated benzo[c]phenanthridine alkaloids hhS and hhC showed potent in vitro inhibitory effects on both the MSSA and MRSA strains. The two compounds' least MICs/MBCs values against MRSA strains were as low as to 0.49/1.95 and 0.98/7.81μg/ml, respectively. The alkaloid hhS was demonstrated as the most potent of all tested antibacterial alkaloids and against all tested pathogens. Its 90% MICs (1.95 μg/ml) against MRSA were comparable to those of vancomycin (2.34 μg/ml). Correspondingly, the other two non-hydroxylated benzo[c]phenanthridine alkaloids hS and hC showed moderate to no inhibitory effects at the tested maximum concentration of 3000 μg/ml.

Table 2. MICs and MBCs (μg/ml) of the alkaloids and control agents for strains of MSSA and MRSA

Strain and agent	MICs/MBCs for MSSA (ATCC25923)	MICs/MBCs for MRSA (20 clinical isolates)		
		Range	50%	90%
hhS	0.49/	0.49-7.81/	0.49/	1.95/
	7.81	1.95-31.25	7.81	15.63
hhC	0.98/	0.98-15.63/	3.91/	7.81/
	15.63	7.81-62.50	31.25	62.50
hS	23.4/	93.8-750.0/	187.5/	750.0/
	375.0	375.0-1500.0	750.0	1500.0
hC	46.9/	375.0-1500.0/	750.0/	1500.0/
	750.0	375.0-1500.0	1500.0	3000.0
Vancomycin	1.17/	1.17-2.34/	1.17/	2.34/
	2.34	1.17-2.34	1.17	2.34

Since *S. aureus* is a major cause of hospital-acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices, and *S. aureus* rapidly develops as MRSA and resistance to many antimicrobial agents that cause therapeutic problems [1], new types of antimicrobial agents with chemical structures and mechanisms other than the currently used antibiotic are needed to develop for holding back the prevalence of this pathogenic microorganisms, for example the effects on bacterial efflux pump and DNA topoisomerase I [10, 15]. The potent anti-MRSA activity of hhS and hhC are two encouraging leads which are deserved for further investigation.

ACKNOWLEDGEMENTS

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