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Antibacterial Activity of *Hylomecon hylomeconoides* Against Methicillin-Resistant *Staphylococcus aureus*

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Abstract Methicillin-resistant *Staphylococcus aureus* (MRSA) is serious clinical urgent problems worldwide. In the present study, the antibacterial activity of *Hylomecon hylomeconoides* was investigated. The EtOH extract and its fraction (*n*-hexane, CH_2Cl_2, EtOAc, and H_2O) were investigated against MRSA. The most active extract (CH_2Cl_2) led to the isolation of 6-methoxydihydrosanguinarine (6-MS), 6-acetonylhydrosanguinarine, and dihydrosanguinarine. These compounds were very active against MRSA strains with minimum inhibitory concentrations (MICs) ranging from 1.95 to 250 μg/ml. Our study did however focus on 6-MS as it appeared to be the most active with MICs in the range of 1.9 to 3.9 μg/ml. These results encourage us to think that 6-MS can be used as a natural antibacterial agent.

Keywords Antibacterial · MRSA · *Hylomecon hylomeconoides* · EtOH extract
6-Methoxydihydrosanguinarine

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a problem since the 1960s as its infection is associated with higher mortality and increase cost in the hospitals [1]. It

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becomes more and more evident that bacteria, when faced with a new developed drug, respond with clever mechanisms of resistance [2]. Today, with this emergence of antibiotic-resistant pathogens like methicillin-resistant *S. aureus* (MRSA), a new approach to natural products must be taken. These natural products are more and more in demand by their non-side effect benefit [3]. Therefore, our ongoing efforts to find bioactive natural products have led us to study the antibacterial activity of *Hylomecon hylomeconoides*.

They are two species of *Hylomecon* growing in Korea: *H. hylomeconoides* and *Hylomecon vernale* [4]. *H. hylomeconoides* belongs to Papaveraceae family, reported to produce lots of benzophenanthridine alkaloids [5]. *H. hylomeconoides* are benzophenanthridine alkaloids and alkaloids have shown antibacterial activity [6]. Sanguinarine derivatives, for example, are one of the compounds isolated from *H. hylomeconoides* [5]. Thus, we verified the antibacterial activity of *H. hylomeconoides* through extraction. *H. hylomeconoides* leaf, stem, and roots were screen-tested for antimicrobial activity using microdilution broth method against two strains of ATCC 33591 and ATCC 25923. The fractionation of the most active extract (CH_2Cl_2) led to the isolation of 6-methoxydihydrosanguinarine (6-MS), 6-acetonylhydrosanguinarine (6-AS), and dihydrosanguinarine (DS). These fractions were thereafter tested for their antimicrobial potential against two strains of *S. aureus* and six other clinical isolates from the Wonkwang University Hospital.

This study shows the isolation of active compound from *H. hylomeconoides* tested against MRSA in comparison to a commercial antibiotic.

Materials and Methods

Plant Materials and Sample Preparation

H. hylomeconoides were collected from Hwasun, Korea, in July, 2008. They were identified by Dr. D.Y. Kwon. A voucher specimen (No. 08-01) was deposited in the Laboratory of Herbalogy, College of Pharmacy, Wonkwang University, Iksan, Korea. *H. hylomeconoides* is air-dried roots, stems, and leaves (10 g), which were then boiled in 30 ml of ethanol for 3 h. The EtOH extract yields were calculated on a dry weight basis as 1.70% for root, 0.42% for stem, and 0.60% for leaf. The extract was filtered (pore size, 0.45 m), lyophilized, and kept at 4 °C and were screen-tested for antimicrobial activity. We partitioned 100 g of *H. hylomeconoides* roots with EtOH and it yields 10.7%. The other extracts partitioned with organic solvents of different polarities yield *n*-hexane, CH_2Cl_2, EtOAc, and H_2O (1.25%, 1.71%, 0.53%, and 12%, respectively).

Isolation of Sanguinarine Derivatives

6-MS, 6-AS, and DS were isolated from roots of *H. hylomeconoides* by repeated column chromatography as reported previously [7, 8]. Their structures were determined by spectroscopic analysis, especially ^1H and ^{13}C NMR and ESI-MS spectra.

6-methoxydihydrosanguinarine (Fig. 1); white amorphous powder, mp 201.9–203.1 °C, ESI-MS 332 $[M\text{-}OCH_3]^+$. ^1H NMR (300 MHz, $CDCl_3$) δ, 2.78 (3H, s), 3.46 (3H, s), 6.11 (2H, s), 6.93 (1H, d, J=8.2 Hz), 7.11 (1H, s), 7.40 (1H, d, J=8.2 Hz), 7.47 (1H, d, J=8.6 Hz), 7.69(1H, s), 7.75 (1H, d, J=8.6 Hz). ^{13}C NMR (75MHz, $CDCl_3$) δ, 104.6 (C-1), 147.4 (C-2), 148.0 (C-3), 100.6 (C-4), 126.8 (C-4a), 138.1 (C-4b), 85.9 (C-6), 113.1 (C-6a), 145.2 (C-7), 147.2 (C-8), 108.8 (C-9), 116.3 (C-10), 125.7 (C-10a), 122.8

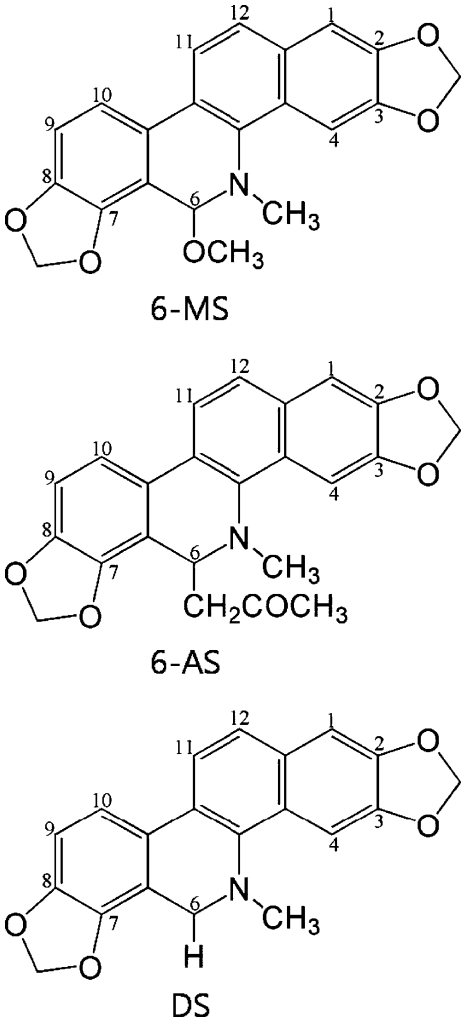

Fig. 1 The chemical structure of 6-methoxydihydrosanguinarine (*6-MS*), 6 -acetonylhydrosanguinarine (*6-AS*), and dihydrosanguinarine (*DS*)

(C-10b), 120.2 (C-11), 123.7 (C-12), 131.0 (C-12a), 101.0 (2,3-OCH_2O), 101.7 (7,8-OCH_2O), 40.8 (NMe), 54.1 (OMe).

6-acetonyldihydrosanguinarine (Fig. 1); white needle crystal, mp 191.4–191.9 °C, ESI-MS 390 $[M+H]^+$. ^1H NMR (300 MHz, $CDCl_3$) δ, 2.24(3H, s), 2.34 (2H, s), 2.82 (3H, s), 5.05 (1H, dd, J=0.5, 4.2 Hz), 6.20 (2H, s), 6.21 (2H, s), 7.03 (1H, d, J=8.2 Hz), 7.27 (1H, s), 7.50 (1H, d, J=8.2 Hz), 7.65 (1H, d, J=8.6 Hz), 7.71(1H, s), 7.86 (1H, d, J=8.6 Hz). ^{13}C NMR (75MHz, $CDCl_3$) δ, 104.3 (C-1), 147.5 (C-2), 148.2 (C-3), 100.5 (C-4), 127.4 (C-4a), 139.2 (C-4b), 54.4 (C-6), 115.9 (C-6a), 144.2 (C-7), 147.1 (C-8), 107.5 (C-9), 116.4 (C-10), 125.6 (C-10a), 123.4 (C-10b), 119.9 (C-11), 123.9 (C-12), 130.9 (C-12a), 101.0 (2,3-OCH_2O), 101.5 (7,8-OCH_2O), 42.9 (NMe), 46.6 (C-1′), 207.0 (C-2′), 30.8 (C-3′).

Dihydrosanguinarine (Fig. 1); colorless crystal, mp 191.6–193.7 °C, ESI-MS 334 $[M+H]^+$, ^1H NMR (300 MHz, $CDCl_3$) δ, 2.79 (3H, s), 4.37 (2H, s), 6.20 (2H, s), 6.21

(2H, s), 7.01 (1H, d, J=8.1 Hz), 7.27 (1H, s), 7.43 (1H, d, J=8.1 Hz), 7.64 (1H, d, J=8.6 Hz), 7.85 (1H, s), 7.85 (1H, d, J=8.6 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ, 104.3 (C-1), 147.5 (C-2), 148.2 (C-3), 100.7 (C-4), 127.2 (C-4a), 142.5 (C-4b), 48.5 (C-6), 113.6 (C-6a), 144.6 (C-7), 147.1 (C-8), 107.2 (C-9), 116.2 (C-10), 126.5 (C-10a), 124.4 (C-10b), 120.3 (C-11), 124.0 (C-12), 130.8 (C-12a), 101.0 (2,3-OCH_2O), 101.3 (7,8-OCH_2O), 41.6 (NMe).

The purities were over 99% by HPLC analysis [nova-Pak C_{18} (3.9×150 mm) column (Waters, CA, USA) with the eluent of acetonitrile–phosphate buffer (50 mM, pH 7.0; 50:50, v/v), flow rate; 1.0 ml/min, detection; UV 280 and 320 nm simultaneously] [5].

Bacterial Strains and Growth Conditions

Among the eight *S. aureus* strains used in this study (Table 1), six clinical isolates (MRSA) were obtained from six unique patients at Wonkwang University Hospital (Iksan, South Korea). The other two strains were *S. aureus* ATCC 33591 (methicillin-resistant strain) and *S. aureus* ATCC 25923 (methicillin-susceptible strain). ATCC 25923 (American Type Culture Collection, Manassas, VA, USA) and ATCC 33591 were commercially purchased. Before use, all bacteria were stored in 30% glycerol and frozen at −70 °C. The bacteria were cultured in Mueller–Hinton broth (MHB) and Mueller–Hinton agar (MHA; Difco Laboratories, Baltimore, MD, USA).

Note that *S. aureus* and MRSA strains were selected as test microorganisms as for two decades; therapeutic options have been very limited. In the case of MRSA, it is resistant not only to β-lactams but also to other types of antibiotics [9]. Also, bacteria were suspended in Mueller–Hinton broth and then incubated at 37 °C for 24 h.

Determination of the mecA Gene

Detection of the mecA gene in the MRSA strains was performed by polymerase chain reaction (PCR) amplification. Prior to the DNA extraction, frozen bacteria were subcultured twice onto Mueller–Hinton agar plates (MHA plates). For rapid extraction, one to five bacterial colonies were suspended in 300 μl of cell lysis buffer and heated at 100 °C for 20 min. After centrifugation at 12,000 rpm for 10 min, 2 μl of the supernatant was used for the DNA extraction. PCR reactions were performed using a MRSA Primer Mix Kit (Genotek Co, Daejeon). The PCR amplification consisted of 30 cycles (94 °C, 60 s; 55 °C, 60 s; 72 °C, 60 s). The primers used in this study were as follows: mecA—forward primer: 5′-ATGAGATTAGGCATCGTTTC-3′ reverse primer: 5′-TGGATGACAGTACCTGAGCC-3′. The final PCR products were separated on 2% agarose gel.

Table 1 Minimum inhibition concentration (MIC) values (microgram per milliliter) of bacterial growth in the presence of extracts from *H. hylomeconoides*.

S. aureus strain	MIC (μg/ml)			
	Leaf	Stem	Root	Ampicillin
ATCC 25923	>500	>500	125	0.06
ATCC 33591	>500	500	125	31.25

Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute [10] guideline. Briefly, a preparation of the microorganisms inocula was done on 24-h broth cultures, and the suspensions were adjusted to a 0.5 McFarland standard turbidity (approximately 10^8 CFU/ml). Final inoculums were adjusted to the 10^4 CFU/ml. The MHB was supplemented with serial ampicillin and *H. hylomeconoides* root CH_2Cl_2 fraction (HHCF), 6-MS, 6-AS, and DS concentrations. The MIC was defined as the lowest concentration in which there is no visible growth after 24 h of incubation at 37 °C.

Disk Diffusion Method

The antibacterial activities of HHCF and 6-MS were tested against *S. aureus* ATCC 25923 (MSSA), *S. aureus* ATCC 33591(MRSA), and DPS-1 (clinical MRSA) and ampicillin was used as positive control. The disk diffusion method was as described by the Clinical and Laboratory Standards Institute Standards and by using a modified agar-well diffusion method [11]. Briefly, sterile paper disks (6 mm; Toyo Roshi Kaihsa, Japan) were loaded with 20 μl of HHCF, 6-MS, and ampicillin (AM; varying concentrations 5 and 10 μg) dissolved in 50% dimethyl sulfoxide (DMSO, Sigma, USA) and then left to dry for 18 h at 37 °C in a sterile room. The bacterial suspensions were then diluted to a turbidity of approximately 0.5 McFarland (approximately 1.5×10^8 CFU/ml) and then further diluted to obtain the final inoculum. Next, the MHA was poured into Petri dishes and inoculated with 100 μl of the suspension containing 1×10^5 CFU/ml of bacteria. Ampicillin (Sigma Chemical Co, St. Louis, USA) was used as the positive control, and disks treated with 50% DMSO were used as the negative control. The plates were then placed in an incubator (Vision Co, Seoul, Korea) at 37 °C for 24 h, after which the diameter of the zone of inhibition around each of the disks was measured and recorded. Each experiment was performed in triplicate.

Results and Discussion

Table 1 shows that the bioactivity of *H. hylomeconoides* is mostly seen in roots compared to the leaf and stem. That can be explained by the presence of alkaloids in *H. hylomeconoides* roots [5]. The bioactivity of alkaloids has been published [12], and their antimicrobial effect demonstrated in some bacteria [13].

H. hylomeconoides roots were then fractioned into EtOH, *n*-hexane, CH_2Cl_2, EtOAc, and H_2O. The extracts along with ampicillin were used for MIC determination using the microdilution broth method. The results were recorded as MIC in Table 2. The CH_2Cl_2 fraction of *H. hylomeconoides* roots EtOH extract showed good antibacterial effects for the two strains of *S. aureus*. The CH_2Cl_2 MIC for ATCC33591 was 7.8 μg/ml compared to ampicillin at 31.25 μg/ml. This is an encouraging result in regards to the ability of MRSA to be resistant to most antibiotics.

The CH_2Cl_2 fraction was then fractioned as shown in Fig. 1 into 6-MS, 6-AS and DS. The fractioned compounds after were tested against different strains of *S. aureus* as shown in Table 3. From the fractions obtained, 6-MS appears to be the sole compound showing

Table 2 Antimicrobial activity of *H. hylomeconoides* root EtOH extract and *n*-hexane, CH_2Cl_2, EtOAc, and H_2O fractions against *S. aureus* (ATCC 33591, ATCC 25923) strains.

S. aureus strain	MIC (μg/ml)					
	EtOH	Hexane	CH_2Cl_2	EtOAc	H_2O	Ampicillin
ATCC 25923	125	125	7.8	>500	>500	0.06
ATCC 33591	125	62.5	7.8	>500	>500	31.25

antibacterial activity with MICs ranging from 1.9 to 3.9 μg/ml. As already reported, 6-MS is a benzophenanthridine alkaloid (Fig. 1) with anti-platelet aggregation activity [14]. From the structure of sanguinarine derivatives isolated from *H. hylomeconoides*, 6-MS like the other two are different in their C6-R group (OCH_3), 6-AS (CH_2COCH_3), and DS (H). 6-MS seems, by its different biological properties, to be an object of extensive research projects to make better the health of human kind. Its antimicrobial activity is remarkable especially compared to often-used antibiotics such as ampicillin. The antibacterial activity of *H. hylomeconoides* can safely be attributed to 6-MS as 6-AS and DS are not showing any significant activity to be of an interest for further investigation at least against *S. aureus* strains. To better elucidate this assumption, a disk diffusion test was done, measuring the zone of inhibition against *S. aureus* ATCC 25923(MSSA) [A], *S. aureus* ATCC 33591 (MRSA) [B], and DPS-1 (clinical MRSA) [C], in the presence of *H. hylomeconoides* root CH_2Cl_2 fraction (HHCF), 6-MS, and AM used individually at 5 and 10 μg (Fig. 2). While the results agree with the logic of dose dependency for all the drugs used, the zone of inhibition of all strains in presence of 6-MS is much larger than ampicillin when exposed to

Table 3 The MICs of *H. hylomeconoides* is root CH_2Cl_2 fraction (HHCF), 6-methoxydihydrosanguinarine (6-MS), 6-acetonylhydrosanguinarine (6-AS), dihydrosanguinarine (DS), and ampicillin (AM) against *S. aureus* strain.

	Class	mecA gene	MIC (μg/ml)				
			HHCF	6-MS	6-AS	DS	AM
S. aureus strain							
ATCC 25923	MSSA	−	7.8	1.95	>250	>250	0.06
ATCC 33591	MRSA	+	7.8	3.9	>250	>250	31.25
Clinical isolates							
DPS 1[a]	MRSA	+	15.6	1.95	>250	>250	31.25
DPS 2	MRSA	+	15.6	3.9	>250	>250	62.5
DPS 3	MRSA	+	7.8	1.95	>250	>250	15.6
DPS 4	MRSA	+	7.8	3.9	>250	>250	62.5
DPS 5	MRSA	+	7.8	3.9	>250	>250	31.25
DPS 6	MRSA	+	7.8	1.95	>250	>250	31.25

(+) positive, (−) negative

[a] DPS indicates staphylococcal strains from the Department of Plastic Surgery, Wonkwang University Hospital

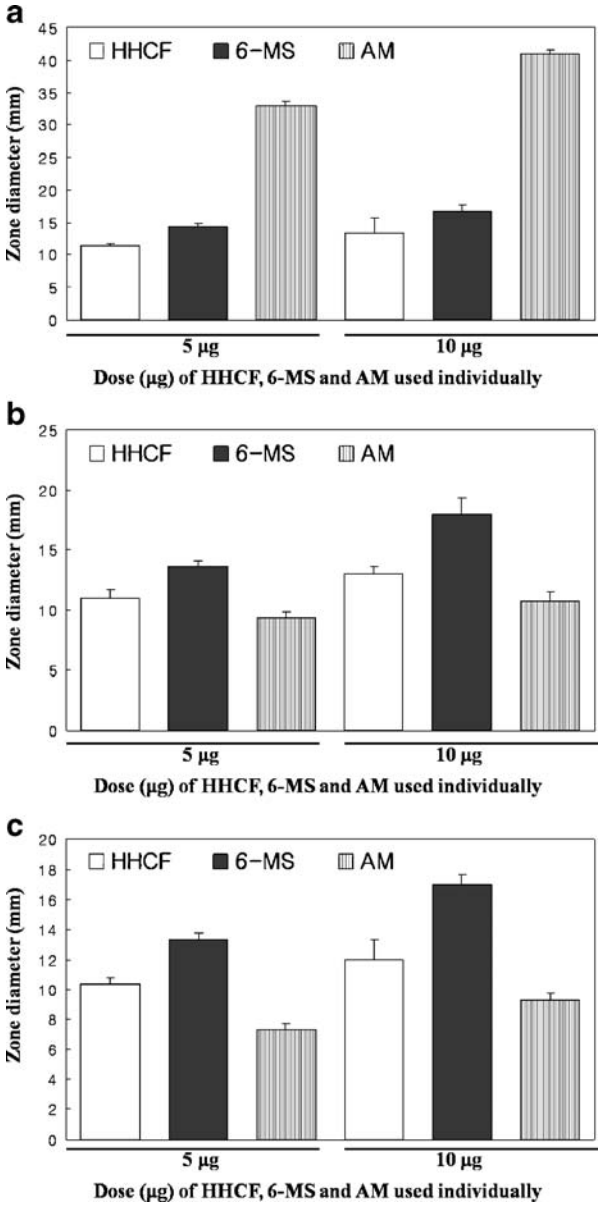

Fig. 2 Zone of inhibition against *S. aureus* ATCC 25923 (MSSA; **a**), *S. aureus* ATCC 33591 (MRSA; **b**), and DPS-1 (clinical MRSA; **c**) in the presence of *H. hylomeconoides* root CH_2Cl_2 fraction (*HHCF*), 6-methoxydihydrosanguinarine (*6-MS*), and ampicillin (*AM*) used individually at 5 and 10 µg. The results represent means of triplicate determinations undertaken on two separate occasions

MRSA. Note that zone of inhibition obtained from CH_2Cl_2 is not much of consideration as stated earlier that its antibacterial activity is likely due do 6-MS.

While the antimicrobial mechanism action is not yet well understood, 6-MS appears to be a very good natural product that can be used against multi-drug-resistant bacteria in the likeness of MRSA awaiting further testing.

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