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Antimicrobial activity and synergism of Sami-Hyanglyun-Hwan with ciprofloxacin against methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Objective: To investigate the antibacterial activity of SHH extracted with either water or ethanol against methicillin-resistant *Staphylococcus aureus* (MRSA) and combinatory antimicrobial effect with ciprofloxacin (CIP) by time kill assay and checkerboard dilution test.

Methods: The antibacterial activity determined by broth dilution method indicated that the antibacterial activity of Sami-Hyanglyun-Hwan (SHH) water extract (SHHW) and SHH ethanol extract (SHHE) ranged from 250 to 2000 μg/mL and 125 to 1000 μg/mL against MRSA, respectively.

Results: In the checkerboard method, the combinations of SHHE with CIP had a partial synergistic or synergistic effect against MRSA. The time-kill curves showed that a combined SHHE and CIP treatment reduced the bacterial counts dramatically after 24 h.

Conclusions: The present study demonstrates the therapeutic ability of SHHE against MRSA infections.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a commensal of the human skin, gastrointestinal tract and nares. It causes skin and soft tissue infections, invasive disease, sepsis, and endocarditis [1]. The treatment of *S. aureus* infections has been evolved by the antibiotic-resistant strains, called methicillin-resistant *S. aureus* (MRSA), that have increased resistance feature against infectious diseases therapeutics [2]. MRSA is a human pathogen and a cause of hospital-acquired and community-acquired infections. It is a major global health concern resulting in nearly 20000 deaths in the United States alone [3]. MRSA isolates are resistant to all possible penicillin and other β-lactam [4]. Antibiotic resistance in MRSA has resulted in limited treatment options. Thus, there is a critical need for the discovery of new antibiotics in development against MRSA and treatment strategies to circumvent this growing public health concern.

Studies have demonstrated that combination drug therapy is a more effective alternative to slow down or stop development of drug resistance against bacteria [5] and is recommended treatment for bacterial infections such as MRSA [6].

Sami-Hyanglyun-Hwan (SHH), is a traditional Korean medicine (TKM) prescription that has been used for several hundred years by the Korean community. This classical botanical formulation consists of four Korean herbs that include the *Coptidis rhizoma* (*C. rhizoma*), *Rhei rhizoma* (*R. rhizoma*), *Aucklandiae radix* (*A. radix*) and *Arecae semen* (*A. semen*).

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Korean herbs are potential sources of useful medicinal plants. Recently, research in herbs prescribed in TKM has attracted great attention as many of them have been shown to exhibit numerous biological activities including anti-virus [7], anti-inflammatory effects [8,9] and anti-cancer [10,11]. Ciprofloxacin (CIP) a well-known broad range antibiotic working against both gram positive and gram negative bacteria and widely used for common pathogen causing infections [8,9]. In an effort to discover novel antibacterial agents or antiviral formulations from TKM, SHH, one of the most frequently used Korean prescriptions, was investigated for its *in vitro* antibacterial activity. We herein report also, the promising anti-MRSA synergy of CIP combined with SHH extracted with ethanol.

2. Materials and methods

2.1. Plant materials

C. rhizoma, *R. rhizoma*, *A. radix* and *A. semen* were purchased from the Oriental drug store Daehak Hanyakkuk (Iksan, Korea), and authenticated by Dr. D.Y. Kwon. All voucher specimens were deposited in the Laboratory of Herbalogy, College of Pharmacy, Wonkwang University, Iksan, Korea.

2.2. Preparation of the SHH water or ethanol extracts

A total of 500 mL of de-ionized distilled water (dd water) or 70% EtOH were added to 26 g of SHH prescription, which 15 g of *C. rhizoma*, 6 g of *R. rhizoma*, 3 g of *A. radix* and 2 g of *A. semen* was heated until the preparation boiled. After 2 h the decoction was then percolated to obtain filtrate, and drugs were re-boiled with fresh 500 mL of dd water or 70% EtOH. After two more rounds of percolation and filtration, collected filtrates were then poured together, concentrated under reduced pressure, lyophilized stored at −20 °C until use. The yield of the SHH water extract was 17.4% (4.53 g) and EtOH extract was 14.2%.

2.3. Bacterial strains and culture medium

Among the 8 strains of *S. aureus* used in this study, 2 clinical MRSA isolates were obtained from 2 different patients at Wonkwang University Hospital and 2 strains were commercially purchased *S. aureus* ATCC 33591 (methicillin-resistant strain) and *S. aureus* ATCC 25923 [methicillin-susceptible strain (MSSA)] (American Type Culture Collection, Manassas, VA). The remaining 4 strains were obtained from Culture Collection of Antimicrobial Resistant Microbes. All bacteria were stored as 30% glycerol stocks and frozen at −70 °C until use. The bacterial strains were suspended in Mueller-Hinton broth (MHB) and incubated at 37 °C for 24 h.

2.4. Antimicrobial reagents

MHB and Mueller-Hinton agar (MHA) (Difco Laboratories, Baltimore, MD, USA). Ampicillin, oxacillin, CIP, erythromycin, and solvents were purchased from Sigma Aldrich (St. Louis, USA).

2.5. Antimicrobial resistance testing

Detection of the *mec*A gene in MRSA strains was performed by polymerase chain reaction (PCR) amplification (Table 1).

Table 1
Determination of the *mec*A gene of the *S. aureus* strains used in the experiment.

Strains	Serotypes	Class	*mec*A gene	β-Lactamase activity	Antibiotic resistance pattern
WK-1	ATCC 25923	MSSA	−	−	−
WK-2	ATCC 33591	MRSA	+	+	AM, OX
WK-3	[a]DPS-1	MRSA	+	+	AM, OX
WK-4	DPS-5	MRSA	+	−	AM, OX
WK-5	CCARM 3090	MRSA	+	+	AM, OX, CIP
Wk-6	CCARM 3091	MRSA	+	+	AM, OX, CIP
WK-7	CCARM 3095	MRSA	+	+	AM, OX, CIP
WK-8	CCARM 3102	MRSA	+	+	AM, OX, CIP

[a] DPS indicates staphylococcal strains from the Department of Plastic Surgery, Wonkwang University Hospital. AM, ampicillin; OX, oxacillin; CIP, ciprofloxacin.

Prior to DNA extraction, bacteria stock cultures were subcultured twice on to 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 12000 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, Daejeon, Republic of Korea). The PCR amplification was consisted of 30 cycles (94 °C, 60 s; 55 °C, 60 s; 72 °C, 60 s). The final PCR products were separated on a 2% agarose gel.

2.6. Disc diffusion

The paper disc diffusion method was used to determine antibacterial activity [10]. Sterile paper discs (6 mm; Toyo Roshi Kaihsa, Japan) were loaded with 20 μL of SHHW or SHHE (varying concentrations: 50, 100, and 200 μg) dissolved in 10% dimethyl sulfoxide (DMSO, Sigma, USA), and were left to dry for 12 h at 37 °C under sterile conditions. The bacterial suspensions were diluted to match the 0.5 McFarland standard scale (approximately 1.5×10^8 CFU/mL), and were further diluted to obtain the final inoculum. The MHA was poured into petri dishes and inoculated with 100 μL of the suspension containing 1×10^5 CFU of bacteria. The inhibition zone diameter around each of the discs was measured and recorded at the end of the incubation period. Ampicillin was included as positive control and 10% DMSO served as negative controls.

2.7. Determination of the minimum inhibitory concentrations (MICs)

The MIC determinations were performed using the broth microdilution method described by the Clinical and Laboratory Standard Institute guidelines [11]. Serial 2-fold dilutions of SHHW or SHHE in MHB were prepared in sterile 96-well microplates and microtubes. The MRSA inocula were adjusted to the 0.5 McFarland standard [approximately colony-forming units (CFU)/mL] in MHB. The final inocula were adjusted to CFU/spot. The MIC was defined as the lowest concentration of SHHE that permits microorganism growth after prior incubation at 37 °C for 24 h.

2.8. Checkerboard dilution test

The checkerboard method was used to identify the interactions between SHHE and antibiotics [12]. The antimicrobial