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Original Research Article

Antibacterial Activity and Synergism of Hydnocarpi Semen Extracts with Ampicillin or Oxacillin against Methicillin-resistant *Staphylococcus aureus*

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Abstract - Methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious clinical and an urgent problem worldwide. Few new drugs are available against MRSA, because MRSA has the ability to acquire resistance to most antibiotics, which consequently increases the cost of medication. In the present study, the antibacterial activity of Hydnocarpi Semen was investigated. The most effective method is to develop antibiotics from the natural products without having any toxic or side effects. Therefore, there is a need to develop alternative antibacterial drugs for the treatment of infectious diseases. Five Clinical isolates (MRSA) were obtained from five different patients at Wonkwang University Hospital (Iksan, South Korea). The Other 2 strains were ATCC 33591 (Methicillin-resistant strain) and ATCC 25923 (Methicillin-susceptible strain). Antibacterial activity (Minimal Inhibitory Concentrations, MICs) was determined by broth dilution method, disk diffusion method, MTT test, and checkerboard dilution test. Antibacterial activity of n-hexane fraction was remarkable, and had a MICs ranging from 31.25-125 ㎍/㎖. FICI values for HFH+AM and HFH+OX were 0.13-0.19 and 0.04-0.29, showing the increase of synergistic effect. When combined together, these antibacterial effects were dramatically increased.

Key words - Hydnocarpi Semen, synergism, Antibacterial, Methicillin-resistant *Staphylococcus aureus* (MRSA)

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium that grows in the human nose and skin and is a major pathogen for skin and soft-tissue infections. Methicillin antibiotics have been used against *S. aureus*; however, since its detection in 1961, MRSA has become the most problematic gram-positive bacterium in the public health arena (Joung *et al*., 2012). MRSA is an organism that represents a worldwide threat owing its ability to acquire resistance to most antibiotics (Gibbons, 2004; Aqil *et al*., 2006). This pathogen is associated with a variety of infectious diseases (Shin *et al*., 2012; Baltch *et al*., 2007) and has an average mortality rate of 36%−50% (Dancer, 2008). With increasing antimicrobial resistance to various drugs, combination therapy appears to be a useful option, particularly in developing countries where the availability of drugs is limited (Aqil *et al*., 2006; Miranda-Novales *et al*., 2006). Further, MRSA strains are resistant not only to beta-lactam antibiotics but also to fluoroquinolones and other families of antibiotics (Aqil *et al*., 2006). Hydnocarpi Semen has been used for the treatment of leprosy (Hansen's disease) which is a chronic disease caused by Mycobacterium leprae and ethylacetate (EtOAc) fraction from Hydnocarpi Semen extract were evaluated for their wound healing activity by using an *in vitro* acute inflammation model (Lee *et al*., 2012; Oommen *et al*., 1999; Oommen, 2000). However, antimicrobial capacity of Hydnocarpi Semen against *Staphylococcus aureus* remains unknown. Therefore, we investigated antibacterial activities of Hydnocarpi Semen alone and of Hydnocarpi Semen in conjunction with antibiotics widely used in clinical settings and we concluded that Hydnocarpi Semen exhibits considerable antibacterial activity against MRSA.

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Materials and methods

Plant materials

Plant material and sample preparation Hydnocarpi Semen purchased from an Oriental drug store Well-Being Hanyakkuk (Suncheon, Korea), in April, 2015 samples were identified by Prof. Dong-Young Shin of the Department of Development in Plant Resources, a voucher specimen was deposited in the Laboratory of Oriental Pharmacology (N.1364). Hydnocarpi Semen was air-dried to 200 g, which were then boiled in 2 L of ethanol for 3 hr. The ethanol extract of Hydnocarpi Semen (4.26% w/w) was partitioned with organic solvents of different polarities to yield n-hexane, EtOAc, n-BuOH and water fractions, in sequence. The samples were stored at 4℃.

Test Microorganisms

Five Clinical isolates (MRSA) were obtained from five different patients at Wonkwang University Hospital (Iksan, South Korea) The Other 2 strains were *S. aureus* ATCC 33591 (Methicillin-resistant strain) and *S. aureus* ATCC 25923 (Methicillin-susceptible strain). Before use, all of the bacteria were stored in 30% glycerol and frozen at -70℃. The bacteria were cultured in Mueller-Hinton Broth (MHB) and Mueller-Hinton Agar (MHA) (Difco Laboratories, Baltimore, MD, USA). The bacteria were suspended in Mueller-Hinton Broth and then incubated at 37℃ for 24 hr.

Antibiotics

Ampicillin (AM) and Oxacillin (OX) (Sigma Chemical Co. St. Louis, M0, USA) were used

Disk Diffusion Method

The disk diffusion method was as described by the clinical and Laboratory standards Institute standards and by using a modified agar-well diffusion method (National Committee for Clinical Laboratory Standards (CLSI), 2006). Bacterial strains grown on MHA at 37℃ for 18 hr were suspended in MHB and adjusted to a turbidity of 0.5 McFarland standard scale (approximately 1.5×10^8 CFU/㎖). The MHA was poured into petri dishes and inoculated with 100 ㎕ of the suspension sterile paper disks (diameter 6 ㎜: Tokyo Roshi Kaihsa, Japan) were punched in the agar and filled with 500 ㎍ and 250 ㎍. The dissolution of the organic extracts were facilitated with the addition of 50% (v/v) DMSO (50% DMSO was not active against all strains). AM and OX were used as positive controls, and the disks treated with DMSO were used as the negative control. The plates were incubated at 37℃ for 18 hr. The inhibition zone diameter around each of the disks were measured and recorded at the end of the incubation period.

Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) was determined using the broth microdilution method according to the clinical and Laboratory standards Institute guideline (CLSI., 2000). Briefly, a preparation of the microorganisms inoculated were done on 24 hr Broth cultures, and the suspensions were adjusted to a 0.5 McFarland standard turbidity (approximately 1.5×10^8 CFU/㎖). Final inoculums were adjusted to the 1.5×10^6 CFU/㎖. These serially diluted cultures were then incubated at 37℃ for 18 hr. MIC was defined at the lowest concentration of AM, OX, Hydnocarpi Semen extracts, Fractions (n-hexane, EtOAc, n-BuOH, H_2O). At the end of the incubation period, the well plates were visually examined for turbidity. Cloudiness indicates that bacterial growth has not been inhibited by the concentration of antimicrobial agents contained in the medium. A colorimetric assay for rapid detection of the presence of bacteria was also performed (see below, Colorimetric assay using 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide [MTT] test).

Checkerboard dilution test

The synergistic combinations were investigated in the preliminary checkerboard method performed using the MRSA, MSSA and the five isolate strains came from five patients via MIC determination, according to the CLSI guidelines (Mazumdar *et al*., 2005). The MIC was defined as the lowest concentration of drug alone or in combination that inhibited the visible growth. The in vitro interaction was quantified by determining the fractional inhibitory concentration (FIC). The FIC index was calculated as follows: FIC = (MIC of drug A in combination/MIC of

drug A alone) + (MIC of drug B in combination/MIC of drug B alone). FIC indices (FICI) were interpreted as follows: <0.5, synergy; 0.5-0.75, partial synergy; 0.76-1.0, additive effect; >1.0-4.0, indifference; and >4.0, antagonism. All experiments were independently repeated three times.

Colorimetric assay using MTT test

A colorimetric assay based on MTT for rapid detection of the presence of bacteria was performed as previously described (Luis *et al*., 2014; Joung *et al*., 2015; Shi *et al*., 2008). Briefly, a stock solution of 5mg/㎖ MTT (Sigma) was prepared in phosphate-buffered saline and kept at -70℃. A final concentration of 1mg/mL of MTT was used in the assay. After 24hrs of incubation a 37℃, 20 ㎕ of the yellow MTT was added to the 96-well microtiter plate and incubated for an additional 20 min. The presence of a blue color indicates the presence of bacteria.

Results

The (Methicillin-resistant) of 6 MRSA strains and *Staphylococcus aureus* (*S. aureus*) ATCC 25923 (Methicillin-susceptible strain) to the tested antibiotics are shown in Table 1 to 3. antibacterial activity of n-hexane fraction was remarkable, and had a MICs ranging from 31.25- 125 ㎍/㎖. Table 4: This test was performed to determine the

Table 1. Antimicrobial activity of Hydnocarpi Semen hexane fraction against *S. aureus strains*

S. aureus strain	Zone of Inhibitory (㎜)					
	n-hexane fractions (㎍/㎖)		AM (㎍/㎖)		OX (㎍/㎖)	
	500 ㎍	250 ㎍	500 ㎍	250 ㎍	500 ㎍	250 ㎍
ATCC 33591	15	13	12	9	ND[z]	ND
ATCC 25923	18	15	10	8	ND	ND
DPS-1	16	14	16	11	ND	ND
DPS-2	20	16	21	19	20	12
DPS-3	15	13	19	11	ND	ND
DPS-4	16	12	16	13	ND	ND
DPS-5	17	14	20	15	ND	ND

[z]ND; no detected activity at this concentration.

Table 2. Antimicrobial activity of Hydnocarpi Semen extract and n-hexane, EtOAc, n-BuOH, water fractions against *S. aureus* ATCC33591 strains

S. aureus strains	Minimal Inhibitory Concentration (MIC) (㎍/㎖)						
	Extract	Frction				Antibiotics	
ATCC	EtOH	n-hexane	EtOAc	n-BuOH	water	AM	OX
33591	125	125	1000	ND[z]	ND	500	500

[z]ND; no detected activity at this concentration.

Table 3. Antimicrobial activity of Hydnocarpi Semen hexane fraction against *S. aureus* strains

S. aureus strains	Minimal Inhibitory Concentration (MIC) (㎍/㎖)			
	Extrct		Antibiotics	
	EtOH	n-hexane	AM	OX
ATCC 33591	125	125	500	500
ATCC 25923	62.5	31.25	500	250
DPS-1[a]	125	125	250	250
DPS-2	125	62.5	250	500
DPS-3	62.5	62.5	125	250
DPS-4	125	125	250	250
DPS-5	125	125	250	250

Table 4. Interpreted FICI response for HFH +AM and HFH +OX combination against a standard MRSA strain and a standard MSSA strain under dark conditions

Strains	MIC of HFH + AM)(㎍/㎖)					MIC of HFH+ OX)(㎍/㎖)				
	HFH[z]		AM			HFH		OX		
	Alone	With AM	Alone	With HFH	FICI[y]	Alone	With OX	Alone	With HFH	FICI
ATCC 33591	125	15.625	500	31.25	0.19	125	31.25	500	15.625	0.28
ATCC 25923	31.25	1.95	500	31.25	0.13	31.25	0.975	250	0.975	0.04

[z]HFH; n-hexane fraction of Hydnocarpi Semen.
[y]FICI; fractional inhibitory concentration index.

action of HFH alone, as well as its synergistic action with AM, or OX against the 2 strains. When tested against ATCC 33591, our data indicated that HFH alone only had moderate inhibitory effect on the growth of MRSA. However, in the presence of a nongrowth inhibitory dose of HFH (125 ㎍/㎖) or AM (500 ㎍/㎖), HFH together with AM were highly effective with a FICI of 0.19. Similar effects were also observed using MSSA strain. These results showed that HFH in combination with these antibiotics could effectively inhibit MRSA growth.

Discussion

Due to the recent appearance of MRSA and the "Super Bacteria" showing the resistance to multiple antibiotics, the development of new antibiotics is urgently required, which is even tendered as a social issue. The ability of MRSA to acquire resistance to most antibiotics has significantly increased the worldwide mortality caused by the MRSA infection (Gibbons, 2004; Dancer, 2008). The most effective method is to develop antibiotics from natural products without having any toxic or side effects. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. The use of two drugs in combination is a good alternative to slow the process of developing drug resistance and to restore the effectiveness of drugs that are no longer prescribed. Combination therapy is the most commonly recommended empirical treatment for bacterial infections in intensive care units, where monotherapy may not be effective against all potential pathogens, and for preventing the emergence of resistant mutants (Drago *et al*., 2007). When combined together, these antibiotic effects were dramatically increased. These effective combinations could be new promising agents in the management of MRSA. For antimicrobial drugs, the clinical application of a combination of inhibitory agents often begins with in vitro tests that show positive interactions for inhibiting the growth of target microorganisms (Mohammad *et al*., 2015). Different drug combinations are reported to treat infections caused by pathogens (Slifierz *et al*., 2015; Vidaillac *et al*., 2010). It may be partly due to the fact that they had abundant hydnocarpic acid, chaulmoogric acid, gorlic caid, palmatin, apigenin which contributed to their antimicrobial activity and should be further studied. Antimicrobial and antioxidant mechanisms of the essential oils, as well as their active components need to be further studied and clarified. In conclusion, we found that Hydnocarpi Semen extracts and n-hexane fraction have an antibacterial effect on MRSA and MSSA, and showing the increase of synergistic effect.

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