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Full Length Research Paper

Antibacterial and synergistic effects of *Nardostachytis rhizoma* extracts on methicillin-resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious clinical problem worldwide. Few new drugs are available against MRSA, because it has the ability to acquire resistance to most antibiotics which consequently increases the cost of medication. In the present study, the antibacterial activity of *Nardostachytis rhizoma* 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 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. Five clinical isolates (MRSA) were obtained from five different 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). Antibacterial activity (minimal inhibitory concentrations, MICs) was determined by broth dilution method, disc diffusion method, MTT test and checkerboard dilution test. Antimicrobial activity of *n*-hexane fraction of *N. rhizoma* was significant. Against the seven strains, the disc diffusion test was in the range of 14 to 18 mm and had a MICs ranging from 31.25 to 125 µg/ml. FICI values for *n*-hexane fraction (HFL) of *N. rhizome* + ampicillin (AM) and HFL + oxacillin (OX) were 0.1875 and 0.078125-0.09375, showing the increase of synergistic effect. When combined together, these antibiotic effects were dramatically increased. These effective combinations could be new promising agents in the management of MRSA and MSSA.

Key words: *Nardostachytis rhizoma*, synergism, antibacterial, methicillin-resistant *Staphylococcus aureus* (MRSA).

INTRODUCTION

Staphylococcus aureus is a bacterium that grows in the human nose and skin and is a major pathogen for the skin and soft-tissue infections. Methicillin antibiotics have been used against *S. aureus* however, since its detection in 1961, methicillin-resistant *S. aureus* (MRSA) has become the most problematic Gram-positive bacterium in

the public health field (Witte, 1999). This pathogen is associated with a variety of infectious diseases (Baltch et al., 2007) and has an average mortality rate of 36 to 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; Kastoris et al., 2010; Miranda-Novales et al., 2006). Furthermore, MRSA strains are resistant not only to beta-lactam antibiotics, but also to fluoroquinolones and other antibiotic families (Aqil et al., 2006). The primary purpose of this study was to investigate the *in vitro* effect on MRSA. *Nardostachytis rhizoma* is a perennial herb that grows in West and Northwest China. In traditional Chinese medicine, the roots and rhizomes of *Nardostachys chinensis Batal* are used for their stomachic and sedative effects (Lu, 1986). This plant is known to be rich in sesquiterpenoids, which were found to exhibit antimalarial, antinociceptive and cytotoxic activities (Itokawa et al., 1993) as well as have the ability to enhance neurite outgrowth, promoting activity (Lee et al., 2006). The chemical composition of the essential oils from roots and rhizomes of the *N. chinensis* obtained by hydrodistillation were analyzed by GC and GC-MS. Calarene (25.31%), aristolone (13.35%), α-selinene (7.32%) and β-maaliene (6.70%) were the major compounds of the 23 identified components which accounted for 92.76% of the total oil of *N. rhizoma* (Jihua et al., 2010).

MATERIALS AND METHODS

Plant material and sample preparation

N. rhizoma was purchased from on oriental drug store Well-being Hanyakkuk (Suncheon, korea), in April 2011. 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.1366). *N. rhizoma* was air-dried, and boiled in ethanol (2 L for 3 h). The ethanol extract of *N. rhizoma* (9.78% 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°C.

Equipment

An incubator (vision, Korea) was used.

Test microorganisms

Five clinical isolates (MRSA), namely DPS1 to DPS5, were obtained from five different 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). 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). Bacteria were suspended in Mueller-Hinton Broth and then incubated at 37°C for 24 h.

Antibiotics

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

Disc diffusion method

The disc diffusion method was described by the Clinical and Laboratory Standards Institute Standards and by using a modified agar-well diffusion method (CLSI, 2001). Bacterial strains grown on MHA at 37°C for 18 h were suspended in MHB and adjusted to a turbidity of 0.5 McFarland standard scale (approximately 1.5 x 10^8 CFU/ml). The MHA was poured into Petri dishes and inoculated with 100 µl of the suspension. Holes sterile paper discs (diameter 6 mm: Tokyo Roshi Kaihsa, Japan) were punched in the agar and filled with 500 and 250 µg extracts. The dissolution of the organic extracts was facilitated with the addition of 50% (v/v) DMSO Sigma, USA (50% DMSO was not active against all strains). AM and OX (DMSO, Sigma, USA) were used as positive controls, and the discs treated with DMSO were used as the negative control. The plates were placed in an incubator at 37°C for 18 h. The inhibition zone diameter around each of the discs was 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 bacterial suspension was prepared by growing microorganisms in broth for 24 h and the suspensions were adjusted to a 0.5 McFarland standard turbidity (approximately 1.5 x 10^8 CFU/ml). Final inoculums were adjusted to the 1.5 x 10^6 CFU/ml. These serially diluted extracts were then incubated together with inoculum at 37°C for 18 h. MIC was defined as the lowest concentration of AM, OX, *N. rhizoma* extracts and fractions (*n*-hexane, EtOAc, *n*-BuOH, aqueous) that inhibited the bacterial growth. At the end of the incubation period, the well plates were visually examined for turbidity. Cloudiness indicated that bacterial growth has not been inhibited by the concentration of antimicrobial agent contained in the medium.

Checkerboard dilution test

The synergistic combinations were investigated in the preliminary checkerboard method performed using the MRSA, MSSA and one clinical isolate strains via MIC determination, according to the published standards (Miranda-Novales et al., 2006; Odds, 2003). 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 to 0.75, partial synergy; 0.76 to 1.0, additive effect; > 1.0 to 4.0, indifference; and >4.0,

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Table 1. Antimicrobial activity of *N. rhizoma* ethanol extract against *S. aureus* strain ATCC 33591 and ATCC 25923, DPS1, DPS2, DPS3, DPS4, DPS5 under dark.

Parameter	Minimal inhibitory concentration (MIC) (µg/ml)						
S. aureus strain	ATCC33591	ATCC25923	DPS1[a]	DPS2	DPS3	DPS4	DPS5
Dark	500	250	1000	250	1000	1000	1000

DPS1[a] indicates *Staphylococcus* strains from the Department of Plastic Surgery, Wonkwang University Hospital; ND, no detected activity at this concentration.

Table 2. Antimicrobial activity of *N. rhizoma* ethanol extract, *n*-hexane, EtOAc, *n*-BuOH and water fractions against *S. aureus* strain ATCC33591, ATCC25923, DPS1, DPS2, DPS3, DPS4, DPS5 under dark.

***S. aureus* strain**	**Minimal inhibitory concentration (MIC) (µg/ml)**					
	***n*-hexane**	**EtOAc**	***n*-BuOH**	**H_2O**	**Ampicillin**	**Oxacillin**
ATCC33591	125	1000	ND	ND	500	500
ATCC25923	62.5	500	ND	ND	31.25	500
DPS1[a]	250	1000	ND	ND	31.25	1000
DPS2	31.25	125	ND	ND	1000	250
DPS3	62.5	1000	ND	ND	31.25	1000
DPS4	125	1000	ND	ND	31.25	1000
DPS5	125	1000	ND	ND	15.63	500

DPS1[a] indicates *Staphylococcus* strains from the Department of Plastic Surgery, Wonkwang University Hospital; ND, no detected activity at this concentration.

antagonism. Finally, the varying rates of synergy between the two agents were determined (Mazumdor et al., 2005). 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 (Abate et al., 1998; Scheuber et al., 1983; Shi et al., 2008). Briefly, a stock solution of 5 mg/ml MTT (Sigma) was prepared in phosphate-buffered saline and kept at -70°C. A final concentration of 1 mg/ml of MTT was used in the assay. After 24 h of incubation at 37°C, 20 µl of the yellow MTT was added to the 96-well microtiter plate (0.3 ml volume) and incubated for an additional 20 min. The presence of a blue color indicates the presence of bacteria.

RESULTS AND DISCUSSION

Ethanol extract had a MIC of 500 µg/ml against *S. aureus* ATCC 33591 under dark condition, and had a MIC of 250 µg/ml against *S. aureus* ATCC 25923 in the same condition (Table 1). Table 2 shows that the antimicrobial activity of *n*-hexane fraction was remarkable, and had a MIC of 31.25 to 250 µg/ml against *S. aureus* strains. Antimicrobial activity of *n*-hexane fraction was remarkable. The *n*-hexane fraction of *N. rhizoma* showed antimicrobial activity inhibition zone of 14 to 18 mm against *S. aureus* strains (Table 3). *n*-hexane fraction of *N. rhizoma* lowered the MICs against the MRSA strain and MSSA but FICI values for HFN + AM and HFN + OX were 0.1875 and 0.078125 to 0.09375, showing the increase of synergistic effect (Table 4).

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 antimicrobial drugs for the treatment of infectious diseases. 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 used for preventing the emergence of resistant mutants (Drago et al., 2007). When combined together, these antibiotic effects were dramatically increased. Different drug combinations are reported to treat infections caused by pathogens (Drago et al., 2007; Liu et al., 2000; Miranda-Novales et al., 2006). Antimicrobial activity of *n*-hexane fraction was remarkable, and had a MICs ranging from 31.25 to 250 µg/ml and Checkerboard dilution test was performed to determine the action of HFN alone as well as its synergistic action with AM, or OX against the two strains. When tested against ATCC 33591, our data indicate that HFN alone only had moderate inhibitory effect on the growth of MRSA. However, in the presence of a non-growth inhibitory dose of HFN (125 µg/ml) or AM (500 µg/ml), HFN together with AM was highly effective with a FICI of 0.1875. Similar effects were also observed in MSSA strain. These results show that HFN in combination with these antibiotics could effectively inhibit MRSA growth. It may be partly due to the fact that they

Table 3. Antimicrobial activity of *N. rhizoma* ethanol extract and *n*-hexane fractions (µg/ml) against *S. aureus* strain ATCC33591, ATCC25923, DPS1, DPS2 DPS3, DPS4, DPS5 under dark.

S. aureus strain	Zone of inhibitory (mm)							
	ethanol extract		*n*-hexane fractions		AM		OX	
	500	250	500	250	500	250	500	250
ATCC33591	8	ND	15	13	12	9	ND	ND
ATCC25923	10	8	17	14	10	8	ND	ND
DPS1[a]	7	ND	14	11	16	11	ND	ND
DPS2	12	9	18	15	21	19	20	12
DPS3	9	ND	16	12	19	11	ND	ND
DPS4	8	ND	15	12	16	13	ND	ND
DPS5	8	ND	14	11	20	15	ND	ND

DPS1[a] indicates *Staphylococcus* strains from the Department of Plastic Surgery, Wonkwang University Hospital; ND, no detected activity at this concentration.

Table 4. Interpreted FICI[b] response for HFN[c] + AM and HFN + OX combination against a standard MRSA strain and a standard MSSA strain under dark.

Strains	MIC of HFN + AM (µg/ml)					MIC of HFN + OX (µg/ml)				
	HFN		AM			HFN		OX		
	Alone	With AM	Alone	With HFN	FICI	Alone	With OX	Alone	With HFN	FICI
ATCC 33591	125	15.625	500	31.25	0.1875	125	7.8	500	15.625	0.09375
ATCC 25923	62.5	7.8	31.25	1.59	0.1875	62.5	3.9	500	7.8	0.078125

FICI[b] fractional inhibitory concentration index, HFN[c] *n*-hexane fraction of *N. rhizome*, FIC fractional inhibitory concentration.

had abundant monoterpenoids and sesquiterpenoids 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 *N. rhizoma* extracts and *n*-hexane fraction have an antibacterial effect on MRSA and MSSA, showing the increase of synergistic effect.

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