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Anti-MRSA action of *Papenfussiella kuromo*

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Abstract – *Papenfussiella kuromo* (PK) is a marine plant and an abundant ecological resource for the future; it is found in almost 80% of the terrestrial biosphere. The aim of this study was to investigate the antibacterial activity of PK against methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant pathogen. The minimum inhibitory concentrations (MICs) of PK hexane fraction (PKH) against 7 strains of MRSA ranged from 1.0 to 2.0 mg/mL. In the checkerboard dilution method, a synergistic effect of the PKH and the antibiotics (oxacillin and norfloxacin) was seen. PKH markedly reduced the MIC of each of the 4 antibiotics against MRSA. The time-kill assay showed that the synergistic activity of PKH and an antibiotic reduced the bacterial counts below the lowest detectable limit after 24 h. These findings suggest that PKH has antibacterial activity, and may be important baseline data in future extensive studies of living marine resources as a source of compounds active against MRSA.
Keywords – *Papenfussiella kuromo* (PK), MRSA, Antibacterial activity, Synergistic effect.

Introduction

Papenfussiella kuromo (Yendo) Inagaki (PK), a seaweed and an abundant ecological resource, is a brown algae belonging to the family Chordariaceae. It is distributed widely throughout the west coast of north pacific, Dokdo in Korea, and Japan. PK has been reported to possess anti-inflammatory effect in Peptidoglycan (PGN)- and lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells activation.[1] α-Glucosidase inhibitory effects of macroalgal species, including PK, have been studied.[2] But, the antimicrobial activity of PK has not yet been reported. Currently, there is a tendency to extensively use land resources, which could lead to them being exhausted; therefore, it is worthwhile to consider marine macroalgae as resources.

In this study, we investigated the antimicrobial activity of *Papenfussiella kuromo* hexane fraction (PKH) against methicillin-resistant *Staphylococcus aureus* (MRSA). *S. aureus* is a major human pathogen and can cause a variety of disorders, including bacteremia, endocarditis, sepsis, hospital-acquired pneumonia, vertebral osteomyelitis, abscess, and surgical wound infections.[3,4] Resistance to methicillin, a β-lactam antibiotic, develops with the acquisition of genes that encode a penicillin-binding protein (PBP2' and PBP2a) with decreased affinity for β-lactams, and leads to the catalysis of effective cell wall synthesis in the presence of penicillins, including cephalosporins and carbapenems. This resistance is attributed to the expression of the mecA gene.[5] MRSA is generated when methicillin-susceptible *S. aureus* (MSSA) acquires this mecA gene, which is carried on a mobile genetic element known as SCCmec. *Staphylococcus* cassette chromosome (SCC) is DNA fragments can transfer, and for that reason, it can have various resistance genes.

In the present study, we investigated the minimum inhibitory concentrations (MICs) and the synergistic effects of PKH when used with different antibiotics.

Experimental

Preparation of PK – PK collected by Dr. H.G. Choi was air-dried in the dark at room temperature and then ground to a powder by using a mechanical grinder. Approximately 500 g of the powdered material was then extracted in 1,500 ml of ethanol for 7 days at room

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temperature. The extract was filtered (pore size, 0.45 μm), lyophilized, and stored at 4 ºC. The yield of the extracted material was approximately 40 g. The EtOH extract was fractionated into hexane (Hex), methylene chloride (MC), ethyl acetate (EA), and n-Butyl alcohol (BuOH) layers. Each partition layer was evaporated, and freeze-dried. The dried residue was then dissolved in dimethyl sulfoxide (DMSO) for further use. A *papenfussiella kuromo* sample was deposited at the Herbarium of the College of Pharmacy, Wonkwang University, Iksan.

Reagents and instruments – The antibiotics oxacillin (OXI) and norfloxacin (NOR), as well as the reagent MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg/ml) were purchased from Sigma-Aldrich Co. Difco Mueller Hinton broth (MHB) and BD Mueller Hinton agar (MHA) were used as nutrient media.

Bacterial strains and growth conditions – Among the 7 strains of MRSA used in this study, 2 were clinical isolates of MRSA obtained from 2 unique patients at Wonkwang University Hospital (Iksan, Korea). *S. aureus* ATCC 33591 (methicillin-resistant strain), was purchased from the American Type Culture Collection (Manassas, VA). Methicillin-resistant bacteria CCARM 3090, CCARM 3091, CCARM 3095, and CCARM 3102 were provided by the Culture Collection of Antimicrobial Resistant Microbes (CCARM, Republic of Korea). These bacterial cultures were suspended in MHB and incubated at 37 ºC for 24 h. MHA was used in the agar diffusion method.

Disc diffusion method – The disc diffusion method, as described by the Clinical and Laboratory Standards Institute, and a modified agar-well diffusion technique were used to determine antibacterial activity.[6] Sterile paper discs (6 mm; Toyo Roshi Kaisha, Tokyo) were loaded with 20 μL of PK fractions to yield a concentration of 0.1 mg/disc. These were dissolved in DMSO and left to dry at 37 ºC in a sterile room for 12 h. The stock solution of DMSO was freshly diluted with distilled water to obtain a 50% solution. 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 a bacterial concentration of 1.5×10^5 CFU/mL. The plates were placed in an incubator (Vision, Bucheon City, Korea) at 37 ºC for 24 h. The diameter of the inhibition zone around each of the discs was measured and recorded at the end of the incubation period. The experiment was carried out in triplicate.

Determination of minimum inhibitory concentrations (MICs) – The MIC determinations were performed using the broth micro-dilution method described by the Clinical and Laboratory Standard Institute guidelines.[7] The microdilution method was used to determine the MICs of OXI, NOR, and PKH. Two-fold serial dilutions of PKH and the antibiotics in MHB were prepared in sterile 96-well microplates and microtubes. The concentrations of the MRSA suspensions were adjusted, according to the 0.5 McFarland standards, to approximately 1.5×10^8 CFU/mL. The concentrations of the final inocula were adjusted to 1.5×10^5 CFU/mL. MHB was supplemented with serial concentrations of PKH and the antibiotics. The MIC was defined as the least concentration after 18 h of incubation at 37 ºC at which no visible growth inhibition was observed (CLSI 2000).[7] At the end of the incubation period, the plates were visually examined for turbidity. Cloudiness indicated that bacterial growth had not been inhibited by the concentration of the antimicrobial agent contained in the medium.

Checkerboard dilution test – The antimicrobial effects resulting from combined inhibition by 2 antimicrobial agents were assessed in the checkerboard test.[8,9] The combinations assayed were PKH in comibination with OXI and NOR. Serial dilutions of the 2 test antimicrobial agents were mixed in cation-supplemented MHB. The inocula were prepared from colonies that had been grown overnight on MHA. The final bacterial concentration after inoculation was 1.5×10^5 CFU/mL. After 24 h of incubation at 37 ºC, the MIC was determined as the least concentration at which no visible growth was observed. For each reference strain, the combinations were tested in triplicate. *In vitro* interaction was quantified by determining the fractional inhibitory concentration (FIC). The fractional inhibitory concentration index (FICI) is the sum of the FICs of each of the two drugs in the combination, which in turn is defined as the MIC of each drug when used in combination divided by the MIC of the drug when used alone. The FIC index was determined using the following formula:

$$\text{FIC index} = FIC_A + FIC_B = [A] / MIC_A + [B] / MIC_B$$

where [A] is the concentration of drug A, MIC_A is its MIC, and FIC_A is the FIC of drug A for the organism, and [B], MIC_B, and FIC_B are the corresponding values for drug B. The FIC index thus obtained was interpreted as follows: < 0.5, synergy; 0.5 - 0.75, partial synergy; 0.76 - 1, additive effect; > 1 - 4, indifference; and > 4, antagonism.[10,11]

Time-kill assay – To evaluate the combined effects of the two antimicrobial agent for 24 h, a time-kill assay of the MRSA strains was carried out, in which the colonies of the living or viable units remaining in 100 μL of the

Table 1. Diameter of zone of inhibition (mm) of *Papenfussiella kuromo* against *Staphylococcus aureus*

Strains	Class	Diameter of zone of inhibition (mm) of Fractions			
		Hex fr.[1)]	MC fr.[2)]	EA fr.[3)]	BtOH fr.[4)]
		0.1 mg/disc			
ATCC 25923	MSSA	12.3 ± 0.58	16	ND[5)]	ND
ATCC 33591	MRSA	13	10.6 ± 0.58	ND	ND
DPS-1	MRSA	22	20	ND	ND
DPS-3	MRSA	18.3 ± 0.58	17	ND	ND
CCARM 3091	MRSA	17	11	ND	ND

[1)]Hex hexane, [2)]MC methylene chloride, [3)]EA ethyl acetate, [4)]BtOH butanol, [5)]ND No detected activity at this concentration.

culture medium were counted.[12] A cation-supplemented MHB that included either a single antimicrobial agent or a combination of two was used. The bacterial suspensions were diluted to a concentration of approximately 1.5×10^5 CFU/mL in fresh MHB, and an antimicrobial agent was added to a concentrations of 1/2 MIC of each two agent and the sum of two agents (1/2 MIC_A + 1/2 MIC_B). A test plate containing only MHB was inoculated as a control. Viable colony counts were performed at intervals of 0, 4, 8, 16, and 24 h by finding the colonies arising from 100 μL aliquots of 10-fold serially diluted specimens plated on MHA at 37 ºC for 24 h. The number of viable cells on a drug-free MHA plate after incubation for 24 h was 30 to 300 colonies. The lower limit of sensitivity of the colony counts was 100 CFU/mL. Antimicrobials were considered bactericidal at the lowest concentration that reduced the original inoculum by 3 log10 CFU/mL (99.9%) for each of the time periods, and they were considered bacteriostatic at concentrations that reduced it by less than 3 log10 CFU/mL. Time-kill assays for all the experiments were performed at least 3 times to confirm the results, and the data are presented as the mean ± standard deviation.

Results and Discussion

The study did show the PK was effective in treating MRSA infection. The result of antimicrobial efficacy of PK frantions against *S. aureus* strains was evaluated by examining surrounding inhibition zones in the paper disc diffusion method. The growth of each of the tested *S. aureus* strains was inhibited at a concentration of 0.1 mg/disc. As shown in Table 1, PK hexane (Hex) and methylene chloride (MC) fractions showed antibacterial activity against *S. aureus* strains. The zone of inhibition increased in a concentration-dependent manner (this table has not shown). The antimicrobial susceptibility tests of the PKH against 7 strains of MRSA were performed using the broth dilution method, and the results are presented in Table 2. All the MRSA isolates were sensitive to the PKH. The MIC of PKH against the MRSA strains ranged from 1.0 to 2.0 mg/mL (Table 2). Accordingly, the PKH screened show a valid effect against the 7 strains tested.

Table 2. Minimum inhibitory concentrations of PKH, OXI, and NOR against MRSA

Strains	MIC (mg/mL)		
	PKH	OXI[1)]	NOR[2)]
ATCC 33591	1.0	0.5	0.25
DPS-1	2.0	0.5	0.031
DPS-3	2.0	0.5	0.5
CCARM 3090	1.0	1.0	0.5
CCARM 3091	2.0	1.0	0.031
CCARM 3095	2.0	0.125	1.0
CCARM 3102	2.0	0.125	0.5

[1)]OX oxacillin, [2)]NOR norfloxacin

The combined effects of PKH and the different antibiotics were tested on 7 strains of MRSA (Table 3). Combination with PKH markedly lowered the MICs of OXI and NOR against the 7 strains. The fractional inhibitory concentration indexes (FICI) calculated from the results of the checkerboard titer assays are listed in Table 3. Of the 14 combinations of organisms with antibiotics and PKH, 11 showed evidence of synergy, 1 of partial synergy, and 2 of an additive effect. When OXI and NOR were separately evaluated in combination with PKH, a significant decrease in the MIC of the antibacterial drugs against the MRSA strains was observed. For example, the MIC of OXI against DPS-1 decreased from 0.5, when used as a single challenge, to 0.063 mg/mL in the presence of PKH. Furthermore, the fact that the MIC of the PKH alone also decreased from 2.0 to 0.125 mg/mL constitutes evidence of an increase in the synergistic effect of the antibiotics and PKH. The FICIs were calculated as described above.

Table 3. The effects of PKH and antibiotics (OXI and NOR), alone and in combination, against MRSA

Strains	MIC (mg/mL)											
	PKH		OXI		FICI[1)]	Outcome	PKH		NOR		FICI	Outcome
	Alone	+ AMP	Alone	+ PKH			Alone	+ OXI	Alone	+ PKH		
ATCC 33591	1.0	0.125	0.5	0.25	0.62	PS[2)]	1.0	0.25	0.25	0.063	0.5	synergy
DPS-1	2.0	0.125	0.5	0.063	0.18	synergy	2.0	0.125	0.031	0.001	0.09	synergy
DPS-2	2.0	0.031	0.5	0.001	0.02	synergy	2.0	0.25	0.5	0.063	0.24	synergy
CCARM 3090	1.0	0.063	1.0	0.25	0.31	synergy	1.0	0.25	0.5	0.063	0.5	synergy
CCARM 3091	2.0	0.031	1.0	0.5	0.26	synergy	2.0	1.0	0.031	0	0.5	synergy
CCARM 3095	2.0	2.0	0.125	0.125	2.0	AE[3)]	2.0	0.5	1.0	0.125	0.37	synergy
CCARM 3102	2.0	2.0	0.125	0.125	2.0	AE	2.0	0.5	0.5	0.063	0.37	synergy

[1)]FICI,fractional inhibitory concentration index, [2)]PS partial synergy, [3)]AE additive effect

The time-dependent effect of PKH in combination with selected antibiotics (OXI and NOR) at various concentrations is shown in Fig. 1. The number of surviving colonies detected on the MHA plates treated with combinations of antibiotics and PKH were markedly lower than that on the plates treated with the antibiotics and PKH separately. Fig. 1 showed the inhibition in the growth of MRSA with concentrations of 1/2 MIC PKH + 1/2 MIC antibiotic and complete inhibition of growth after 24 h of incubation with PKH and an antibiotic.

Since MRSA was discovered in 1961, a number of clones have spread widely and quickly around the world.[13] Known multidrug-resistant bacteria include vancomycin-resistant *S. aureus* (VRSA), MRSA, vancomycin-resistant *enterococci* (VRE), carbapenem-resistant *enterococci* (CRE), multidrug-resistant *Pseudomonas aeruginosa* (MRPA), and multidrug-resistant *Acinetobacter baumannii* (MRAB).[14,15] The widespread emergence of multidrug-resistant bacteria has interfered with the prevention and cure of infectious diseases, and the extensive use of antibiotics itself leads to drug resistance and new bacterial pathogens.[16] Therefore, strategies to overcome antibiotic resistance are necessary. We investigated the antimicrobial activity of PKH and of combinations of PKH and 2 conventional antibacterial agents (OXI and NOR) against gram-positive bacteria, especially MRSA.

Hex and MC fractions of PK showed antibacterial activity against MRSA in the antibacterial susceptibility test. When used in combination with the antibiotics (OXI and NOR), PKH exhibited a synergistic effect as well as antimicrobial activity against all the strains of MRSA tested. A substantial amount of research has been conducted on the synergistic antimicrobial activity of plant-derived extracts or compounds and antibiotics in minimizing antibiotic resistance against multidrug-resistant bacteria, including MRSA.[17-19] In this study, we examined

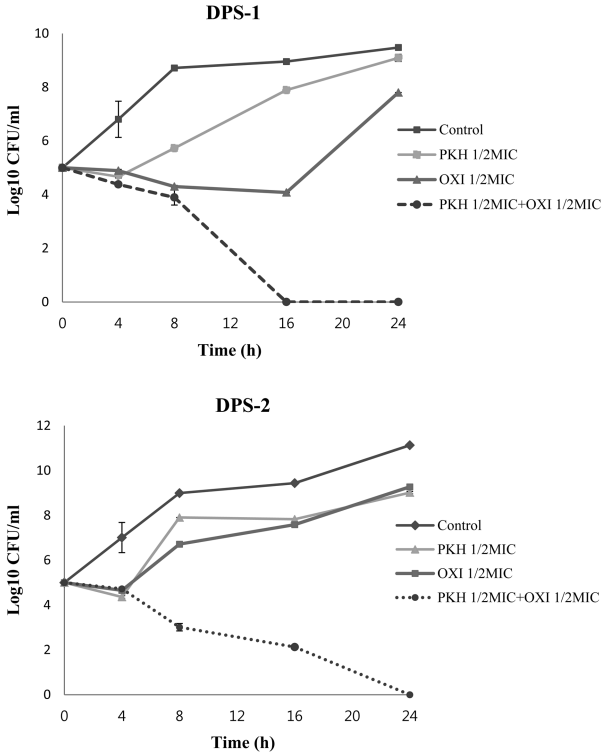

Fig. 1. Time-kill curves of 2 strains of MRSA obtained on using PKH and OXI.

the synergistic effects of PKH with conventional antibiotics OXI and NOR. OXI, both β-lactam antibiotic, inhibit cell wall synthesis.[19] PKH in combination with the β-lactam antibiotics demonstrated significantly reinforced activity *in vitro* against MRSA. NOR, quinolone antibiotic, inhibit nucleic acid metabolism and DNA synthesis.[20] PKH, when used in combination with the quinolone antibiotics, markedly reduced the MICs against MRSA. These synergistic activities have been verified in the time-kill assay (Fig. 1).

Our investigations revealed that the antimicrobial

activity of PKH was potentiated when it was used in combination with the antibiotics OXI and NOR. Furthermore, PKH, an abundant macrophyte, has the potential, as a natural anti-MRSA drug, to reduce multi-drug resistance caused by the excessive use of antibiotics.

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