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Full Length Research Paper

Antibacterial and synergistic effects of *Kochia scoparia* extracts against methicillin-resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is serious clinical urgent problems worldwide. Few new drugs are available against methicillin-resistant *S. aureus* (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 *Kochia scoparia* was investigated. The antibacterial activities of EtOH extract of *K. scoparia* and its *n*-hexane, EtOAc, *n*-BuOH and water fractions were evaluated against 15 strains of methicillin-resistant *S. aureus* (MRSA) and 1 standard methicillin-susceptible *S. aureus* (MSSA) strain by using the disc diffusion method, minimal inhibitory concentrations (MICs) assay, colorimetric assay using MTT test and checkerboard dilution test under dark. Antimicrobial activity of *n*-hexane fraction of *K. scoparia* was remarkable. Against the 16 strains, the disc diffusion test was in the range of 15-18 mm, the minimum inhibitory concentrations (MICs) were in the range of 7.8 to 31.25 µg/ml and FICI values for *n*-hexane fraction of *Kochia scoparia*+AM and *n*-hexane fraction of *K. scoparia*+OX were 0.31 to 0.75 and 0.12 to 0.37 showing the increase of synergistic effect.

Key words: *Kochia scoparia*, 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 arena (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; Miranda-Novales et al., 2006; Kastoris et al., 2010). Furthermore, MRSA strains are resistant not only to beta-lactam antibiotics but also to fluoroquinolones and other families of antibiotics (Aqil et al., 2006). The primary purpose of this study was to investigate the *in vitro* effect against MRSA. *Kochia scoparia* is used as a traditional oriental medicine (Yoshikawa et al., 1997a, b). *K. scoparia* has been used as a tonic, diuretic, analgesic, and antidote and for the treatment of cutaneous pruritus in traditional Korean

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preparations. It has been mentioned as a treatment for thermal skin diseases (Matsuda et al., 1997a; M1997b) and liver disorders and used in traditional medicine for the alleviation effect of jaundice and edema (Jin, 1984). It has been reported that *K. scoparia* contain several of oleanolic acid glycosides (Wen et al., 1995; Yoshikawa et al., 1997). Momordin Ic is an active principle of antinociceptive, anti-inflammatory (Matsuda et al., 1997a) and antiallergic activities (Matsuda et al., 1997b).

MATERIALS AND METHODS

Plant material and sample preparation

K. scoparia were collected from Sunchon, southern Republic of Korea, in June, 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.1369). Kochiae fructus was air-dried, and boiled in ethanol (2 L for 3 h). The ethanol extract of *K. scoparia* Kochiae (8.67% 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).

Test microorganisms

14 Clinical isolates (MRSA) were obtained from fourteen 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 bacteria were stored in 30% glycerol and frozen at -70℃. The bacteria were cultured in Mueller-H inton Broth (MHB) and Mueller-Hinton Agar (MHA) (Difco Laboratories, Baltimore, MD, USA). Bacteria were suspended in Mueller-Hinton Broth and then incubated at 37℃ 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℃ for 18 h were suspended in MHB and adjus ted to a turbidity of 0.5 McFarland standard scale (approximately 1.5×10^8 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). (DMSO, Sigma, USA) AM and OX 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℃ for 18 h. T he 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 microorganism's suspension was prepared by growing microorganism in broth for 24 h and the suspensions were adjusted to a 0.5 McFarland standard turbidity (approximately $1.5\text{x}10^8$ CFU/ml). Final inoculums were adjusted to the 1.5×10^6 CFU/ml. These serially diluted extracts were then incubated along with inoculum at 37℃ for 18 h. MIC was defined at t he lowest concentration of AM, OX, Kochiae Fructus extracts, Fractions (*n*-hexane, EtOAc, *n*-BuOH, aqueous). 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. 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, 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 (Scheuber et al., 1983; Abate et al., 1998; Shi et al., 2008)). Briefly, a stock solution of 5 mg/ml MTT (Sigma) was prepared in phosphate-buffered saline and kept at -70℃. A final concentration of 1 mg/ml of MTT was used in the assay. After 24 h of incubation a 37℃, 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

The *S. aureus* strains were used in the experiments (Table 1). Ethanol extract had a MIC of 1000 µg/ml against *S. aureus* ATCC 33591 under dark, and had a MIC of 250 µg/ml against *S. aureus* ATCC 25923 in the same condition. Antimicrobial activity of *n*-hexane fraction

Table 1. The *Staphyloccocus aureus* strains used in the experiments.

S. aureus strains	Class	mecA gene	Antibiotic Resistance pattern
ATCC25923	MSSA	-	-
ATCC33591	MRSA	+	AM, OX
DPS -1[a]	MRSA	+	AM, OX
DPS -2	MRSA	+	AM, OX
DPS -3	MRSA	+	AM, OX
DPS -4	MRSA	+	AM, OX
DPS -5	MRSA	+	AM, OX
DPS -6	MRSA	+	AM, OX
DPS -7	MRSA	+	AM, OX
DPS -8	MRSA	+	AM, OX
DPS -9	MRSA	+	AM, OX
DPS -10	MRSA	+	AM, OX
DPS -11	MRSA	+	AM, OX
DPS -12	MRSA	+	AM, OX
DPS -13	MRSA	+	AM, OX
DPS -14	MRSA	+	AM, OX

(+), positive; (-), negative;AM, ampicillin; OX, oxacillin;DPS-1[a] indicates *Staphyloccocus aureus* strains from the department of plastic surgery, Wonkwang University Hospital.

Table 2. Antimicrobial activity of *Kochia scoparia* ethanol extract, *n*-hexane, EtOAc, *n*-BuOH, and water fractions against *Staphyloccocus aureus* strains under dark.

S. aureus strain	Minimal Inhibitory Concentration(MIC)(µg/ml)						
	Ethanol extract	Fractions					
		n-hexane	EtOAc	*n*-BuOH	H_20	Ampicillin	Oxacillin
ATCC33591	1000	15.62	ND	ND	ND	1000	250
ATCC25923	250	15.62	ND	ND	ND	7.8	7.8
DPS 1[a]	1000	15.62	ND	ND	ND	31.25	500
DPS 2	500	7.8	ND	ND	ND	1000	500
DPS 3	500	15.62	ND	ND	ND	31.25	500
DPS 4	1000	15.62	ND	ND	ND	31.25	500
DPS 5	1000	15.62	ND	ND	ND	31.25	500
DPS 6	500	15.62	ND	ND	ND	31.25	250
DPS 7	500	15.62	ND	ND	ND	250	500
DPS 8	250	15.62	ND	ND	ND	250	500
DPS 9	500	31.25	ND	ND	ND	125	500
DPS 10	500	31.25	ND	ND	ND	250	500
DPS 11	1000	15.62	ND	ND	ND	250	500
DPS 12	500	31.25	ND	ND	ND	250	500
DPS 13	250	15.62	ND	ND	ND	31.25	1000
DPS 14	500	15.62	ND	ND	ND	250	500

DPS1[a] indicates *Staphylococcus* strains from the department of plastic surgery, Wonkwang University Hospital
ND no detected activity at this concentration.

was remarkable and had a MIC of from 7.8 to 31.25 µg/ml against *S. aureus* strains (Table 2).

The *n*-hexane fraction of *K. scoparia* showed antimicrobial activity inhibition zone from 15 to 18 mm against *S. aureus* strains (Table 3).

n-Hexane Fraction of *K. scoparia* lowered the MICs

Table 3. Antimicrobial activity of *n*-hexane fraction of Kochiae Fructus, Ampicillin, Oxacillin against *Staphyloccocus aureus* strains under dark.

S. aureus strain	Zone of Inhibition(mm)					
	n-hexane fraction (µg/ml)		Ampicillin (µg/ml)		Oxacillin (µg/ml)	
	500	250	500	250	500	250
ATCC33591	17	14	16	15	18	15
ATCC25923	16	14	43	41	37	35
DPS 1[a]	17	14	18	14	ND	ND
DPS 2	18	15	18	17	17	15
DPS 3	17	13	21	16	ND	ND
DPS 4	17	13	20	15	ND	ND
DPS 5	17	14	17	14	ND	ND
DPS 6	16	12	17	14	ND	ND
DPS 7	17	14	11	10	ND	ND
DPS 8	17	14	12	11	ND	ND
DPS 9	15	13	13	12	ND	ND
DPS 10	16	14	12	11	ND	ND
DPS 11	17	13	11	9	ND	ND
DPS 12	17	14	12	10	ND	ND
DPS 13	17	14	18	14	ND	ND
DPS 14	16	14	15	11	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. Result of the combined effect of *n*-hexane fraction of *Kochia scoparia* and AM against *Staphyloccocus aureus*.

	MICs(µg/ml)				
***S.aureus* strain**	**HFK[b] Alone**	**With AM**	**AM Alone**	**With HFK**	**FICI**
ATCC25923	15.62	3.9	7.8	1.95	0.5
ATCC33591	15.62	3.9	1000	250	0.5
DPS-1[a]	15.62	3.9	31.25	3.9	0.37
DPS-2	7.8	1.95	1000	62.5	0.31
DPS-3	15.62	7.8	31.25	3.9	0.62
DPS-4	15.62	7.8	31.25	3.9	0.62
DPS-5	15.62	7.8	31.25	1.95	0.56
DPS-6	15.62	7.8	31.25	1.95	0.56
DPS-7	15.62	3.9	250	62.5	0.5
DPS-8	15.62	3.9	250	62.5	0.5
DPS-9	31.25	7.8	125	62.5	0.75
DPS-10	31.25	7.8	250	31.25	0.37
DPS-11	15.62	7.8	250	31.25	0.62
DPS-12	31.25	7.8	250	31.25	0.37
DPS-13	15.62	3.9	31.25	7.8	0.5
DPS-14	15.62	3.9	250	15.62	0.31

DPS-1[a] indicates *Staphylococcus aureus* strains from the department of plastic surgery, Wonkwang University Hospital,HFK[b] *n*-hexan fraction of *Kochia scoparia* ; ND no detected activity at this concentration.

against the MRSA strain and MSSA but FICI values for HFK+AM and HFK+OX were 0.31 to 0.75 and 0.12 to 0.37 showing the increase of synergistic effect (Tables 4 and 5).

DISCUSSION

The most effective method is to develop antibiotics from the natural products without having any toxic or side

Table 5. Result of the combined effect of *n*-hexane fraction of *Kochia scoparia* and OM against *Staphyloccocus aureus*.

	MICs(µg/ml)				
***S. aureus* strain**	**HFK[b] Alone**	**With OM**	**OM Alone**	**With HFK**	**FICI**
ATCC25923	15.62	1.95	7.8	0.97	0.25
ATCC33591	15.62	1.95	250	62.5	0.37
DPS-1[a]	15.62	1.95	500	62.5	0.25
DPS-2	7.8	1.95	500	15.62	0.37
DPS-3	15.62	1.95	500	62.5	0.25
DPS-4	15.62	1.95	500	62.5	0.25
DPS-5	15.62	0.97	500	31.25	0.12
DPS-6	15.62	1.95	250	31.25	0.25
DPS-7	15.62	1.95	500	62.5	0.25
DPS-8	15.62	0.97	500	31.25	0.12
DPS-9	31.25	7.8	500	31.25	0.31
DPS-10	31.25	7.8	500	62.5	0.37
DPS-11	15.62	3.9	500	62.5	0.37
DPS-12	31.25	7.8	500	62.5	0.37
DPS-13	15.62	1.95	1000	125	0.25
DPS-14	15.62	1.95	500	62.5	0.25

DPS-1[a] indicates *Staphylococcus aureus* strains from the department of plastic surgery, Wonkwang University Hospital, HFK[b] *n*-hexan fraction of *Kochia scoparia.* ND no detected activity at this concentration.

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 mono-therapy may not be effective against all potential pathogens, and for preventing the emergence of resistant mutants. When combined together, these antibiotic effects were dramatically increased. The (methicillin-resistant) of 15 MRSA strains and *S. aureus* ATCC 25923 (methicillin-susceptible strain) to the tested antibiotics. Antimicrobial activity of *n*-hexane fraction was remarkable, and had a MICs ranging from 7.8 to 31.25 µg/ml and Checkerboard dilution test was performed to determine the action of HFK alone as well as its synergistic action with AM, or OX against the 16 strains. When tested against ATCC 33591, our data indicated that HFK alone only had moderate inhibitory effect on the growth of MRSA. However, in the presence of a non-growth inhibitory dose of HFK (7.8 to 31.25 µg/ml) or AM (1000 µg/ml), HFK together with AM was highly effective with a FICI of 0.31 to 0.75 µg/ml. Similar effects were also observed in MSSA strain. These results showed that HFK in combination with these antibiotics could effectively inhibit MRSA growth. It may be partly due to the fact that they had abundant Momordin Ic which contributed to their antimicrobial activity and should be further studied.

Conclusion

In conclusion, we found that *n*-hexane fraction of *K. scoparia* have an antibacterial effect on MRSA and MSSA, and showing the increase of synergistic effect. For future research, more in-depth study should be conducted to identify few things as follows. First, we need to identify which compounds would be responsible for the strong activity. Second, we have to investigate the mechanism of this effect in relation to those compounds.

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