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Full Length Research Paper

Antibacterial and synergistic effects from aerial part of *Kalimeris yomena* Kitamura extract against methicillin-resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is recognized as a serious clinical problem worldwide. Consequently, a recent investigation was conducted on the antibacterial activity and aerial part of *Kalimeris yomena* Kitamura. The antibacterial activities of ethanol (EtOH) extract of *K. yomena* and its *n*-hexane, EtOAc, *n*-BuOH and H_2O fractions were evaluated against 15 strains of MRSA and 1 standard methicillin-susceptible *S. aureus* (MSSA) strain. The *n*-hexane fraction showed a positive antibacterial activity against all the tested strains. The minimum inhibitory concentrations (MICs) of *n*-hexane fraction were in the range of 31.3 to 250 µg/ml. The disc diffusion test diameter of the inhibition zone was in the range of 12 to 17 mm. Thereafter, the combination of *n*-hexane fraction of *K. yomena (*HFK) + ampicillin (AM) or oxacillin (OX), fractional inhibitory concentrations index (FICI) values for HFK + AM ranged between 0.25 and 0.5, and HFK + Ox ranged between 0.5 and 0.56, which show increased synergistic effects. Determining time-kill curves also confirmed the improved synergism of HFK and AM combination against 4, 8, 12 and 24 h cultured MRSA. This result encouraged us to think that *n*-hexane fraction of aerial part of *K. yomena* can be used as a natural antibacterial agent.

Key words: *Kalimeris yomena* Kitamura*,* aster yomena, antibacterial, ethanol (EtOH) extract, combination methicillin-resistant *Staphylococcus aureus* (MRSA).

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a problem since the 1960s as its infection is associated with higher mortality and increase cost in the hospitals (Klevens et al., 2007). It becomes more and more evident that bacteria, when faced with a new developed drug, respond with clever mechanisms of resistance (Tenover, 2006). Today, with this emergence of antibiotic resistant pathogens like MRSA, a new approach to natural products must be taken. These natural products are increasingly in demand due to their non-side effect benefit (Ghosh et al., 2008). Therefore, our ongoing efforts to find bioactive natural products have led us to study the antibacterial activity of *Kalimeris yomena*. *K. yomena (*Asteraceae) is a perennial herb that is found in Korea, Japan, China and Siberia. As a kind of folk remedy, it has been used for the treatment of cough, asthma, insect bite (Jung et al., 2005; Lee, 1996). In addition, though not scientifically tested until now, its cancer prevention qualities (Jung et al., 2005),

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Table 1. *Staphylococcus aureus* strains used in the experiments.

S. aureus strain	Class	mecA gene	β-Lactamase activity	Antibiotic resistance pattern
ATCC 33591	MRSA	+	+	AM, OX
ATCC 25923	MSSA	-	-	-
Clinical isolates MRSA				
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.

antioxidant qualities (Bae and Kim, 2009) has been proven. The essential oils were obtained from *K. yomena and* were analyzed for its chemical composition by gas chromatography-mass spectrometry; there were 68 constituents in the essential oil: 48 hydrocarbons, 8 alcohols, 3 ketones, 2 oxides, 2 acetates, 1 aldehyde, 1 acid, and 1 anhydride. The Major constituents were germacrene D (11.56%), camphor (5.23%), caryophyllene oxide (3.38%), caryophyllene (3.18%) and germarcrene B (3.09%). Through solid-phase micro-extraction (SPME), 13 constituents were identified: 12 hydrocarbons and 1 ketone. Major constituents of the SPME extracted sample were germarcrene D (45.75%), γ-gurjunene (9.39%), γ-selinene (8.33%), camphor (4.81%) and β-caryophyllene (3.05%). (Yeon et al., 2011) However, the antibacterial activity of *K. yomena* against MRSA has not been studied. The present study investigated antibacterial activity of *k. yomena* and the synergistic effects of the mixture of standard antibiotics [Ampicillin (AM), Oxacillin (OX)], performed minimum inhibitory concentrations (MICs), the disc diffusion method, and a time-kill assay to evaluate the susceptibility of *K*. *yomena* against 16 MRSA strains.

MATERIALS AND METHODS

Plant material and sample preparation

Aerial parts of *K. yomena* were collected from suncheon, southern republic of Korea, in September, 2010. 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.7718). Thereafter, Aerial part of *K. yomena* was air-dried to 200 g, which were then boiled in 2 L of ethanol (EtOH) for 3 h. The EtOH extract aerial part of *K. yomena* (10.68% w/w) was partitioned with organic solvents of different polarities to yield *n*-hexane, EtOAc, *n*-BuOH and H_2O fractions, in sequence. The samples were stored at 4°C.

Equipment

An incubator (vision co, seoul, Korea).

Test microorganisms

Among the 16 *S. aureus* strains used in this study (Table 1), Fourteen (14) clinical isolates (MRSA) were obtained from 14 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).

ATCC 25923 (American Type Culture Collection, Manassas, VA, USA) and ATCC 33591 were commercially purchased. 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 MHB and then incubated at 37°C for 24 h.

Antibiotics

AM and OX (Sigma Chemical Co. St.Louis, M0, USA).

Minimum inhibitory concentration (MIC)

The 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 was done on a 24 h broth cultures, and the suspensions were adjusted to a 0.5 McFarland standard turbidity (approximately 1.5×10^8 CFU/ml). Final inoculums were adjusted to the 1.5×10^5 CFU/ml. These serially diluted cultures were then incubated at 37°C for 18 h. MIC was defined at the lowest concentration of AM, OX, aerial part *K. yomena* extract, Fractions (*n*-hexane, EtOAc, *n*-BuOH, H_2O).

Disc diffusion method

The disc diffusion method was as prescribed by the Clinical and Laboratory standards Institute standards, 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 1.5×10^8 CFU/ml). The MHA was poured into petri dishes and inoculated with 100 µl of the suspension sterile paper discs (diameter 6mm: Tokyo Roshi Kaihsa, Japan) were punched in the agar and filled with 500 and 250 µg. The dissolution of the organic extracts was facilitated with the addition of 50% (v/v) dimethyl sulfoxide (DMSO) (50% DMSO was not active against all strains). (DMSO, Sigma, USA) AM and OX was used as positive controls, and the disks treated with 50% DMSO were used as the negative control. The plates were placed in an incubator (Vision Co, Seoul, Korea) at 37°C for 24 h, after which the diameter of the zone of inhibition around each of the discs was measured and recorded. Each experiment was performed in triplicate.

Checkerboard dilution test

The synergistic combinations were investigated in the preliminary checkerboard method performed using the MRSA, methicillin-susceptible *S. aureus* (MSSA) and one clinical isolate strains via MIC determination, according to the CLSI guidelines (CLSI, 2006). The MIC was defined as the lowest concentration of drug alone or in a combination that inhibited the visible growth. The *in vitro* interaction was quantified by determining the fractional inhibitory concentration (FIC). The FIC index (FICI) 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 (Mazumdar et al., 2005; Guadalupe et al., 2006). All experiments were independently repeated three times.

The time-kill curve assay

The time-kill curve assay was performed according to the method described (Nascimento et al., 2007) in order to study the combined effects of time and antimicrobial agent concentration on the bacterial growth. For this assays, a standard inoculums of approximately 10^6 CFU/ml of an overnight culture was used. *n*-hexane fraction of *K. yomena* (HFK) (0.5 MIC) and AM (0.5 MIC) were used. Combinations of HFK + AM were also evaluated. A test plate containing only MHB was inoculated and served as control. Counts of viable strains were carried out at different intervals up to 24 h at 37°C. The rate and extent of killing was determined by plotting viable colony counts (CFU/ml) against time in MHA. According to the method described (Nascimento et al., 2007), all experiments were independently repeated three times.

Colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (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°C. A final concentration of 1 mg/ml of MTT was used in the assay. After 24 h of incubation a 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

Antimicrobial activity of *K. yomena* EtOH extract and *n*-hexane, EtOAc, *n*-BuOH and H_2O fractions for exhibited antimicrobial activities against 15 MRSA and 1 MSSA strains.

Among them, the *n*-hexane fraction was remarkable which was even higher than that of the EtOH extract shown on Table 2. Other fractions (except EtOAc) did not show antimicrobial activity against MRSA.

The *n*-hexane fraction showed a significant antibacterial activity against all the tested strains. The MICs of *n*-hexane against MRSA strains ranged from 31.3 to 250 µg/ml and excellent antibacterial effect than AM and OX against some clinical isolates (DPS-2, 8, 10) MRSA (Table 3).

Antibacterial activity *n*-hexane fraction used as the control group showed no significant difference AM, and seven other strains (DPS-7, 8, 9, 10, 11, 12, 14) rather showed better antibacterial activity. OX did not indicate any antibacterial activity within clinical isolates strains used in the control group, however, *n*-haxane fraction showed more antibacterial activity (Table 4).

For the determination of in vitro combinations test we selected a clinical isolate MRSA, a standard MRSA strain, and a standard MSSA strain. This test was performed to determine the action of HFK alone as well as its synergistic action with AM or OX against the 3 strains. HFK lowered the MICs of AM and OX against the MRSA strains. For the standard MSSA strain, the HFK lowered the MICs of both AM and OX. The combined activity of HFK and two antibacterial agents (AM, OX) against 2 MRSA strains resulted in FICI ranged from 0.25 to 0.5 and from 0.5 to 0.56, respectively (Tables 5 and 6). So the combination effect of HFK with AM and OX was found to be synergistic or partially synergistic. We found that HFK reduced MICs of AM and OX.

Time-kill tests were performed to study the synergis ticeffects of HFK and AM with time. Figures 1 and 2 show the test results of the time-kill assay against a standard MRSA strain, and the DPS-3 strain. On the tested MHA plates, remarkably lower numbers of colo nies were detected as compared to the MHA plates

Table 2. Antimicrobial activity aerial part of *K. yomena* EtOH extract and fractions against *S. aureus* strain ATCC33591.

S. aureus strain	Minimal inhibitory concentration (MIC)(µg/ml)						
	Extract	Fractions				Antibiotic resistance pattern	
	EtOH	*n*-hexane	EtOAc	*n*-BuOH	H_2O	Ampicillin	Oxacillin
ATCC 33591	500	250	2000	ND	ND	125	125
ATCC 25923	250	62.5	1000	ND	ND	0.24	0.49
DPS-1[a]	500	125	2000	ND	ND	62.5	500
DPS-2	250	31.3	1000	ND	ND	250	125
DPS-3	500	125	2000	ND	ND	62.5	500
DPS-4	500	125	2000	ND	ND	62.5	500
DPS-5	250	250	2000	ND	ND	15.63	500

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

Table 3. Antimicrobial activity aerial part of *K. yomena* n-hexane fraction against *S. aureus* strain ATCC33591, ATCC25923, and 14 MRSA.

S. aureus strain	Minimal inhibitory concentration (MIC) (µg/ml)		
	n-hexane fraction	Ampicillin	Oxacillin
ATCC 33591 (MRSA)	250	125	125
ATCC 25923 (MSSA)	62.5	0.24	0.49
Clinical isolates MRSA			
DPS-1[a]	125	62.5	500
DPS-2	31.3	250	125
DPS-3	125	62.5	500
DPS-4	125	62.5	500
DPS-5	250	15.6	500
DPS-6	62.5	31.3	500
DPS-7	125	125	500
DPS-8	62.5	125	500
DPS-9	125	125	500
DPS-10	62.5	125	500
DPS-11	125	125	500
DPS-12	125	125	500
DPS-13	250	62.5	500
DPS-14	125	125	500

[a], DPS indicates *S. aureus* strains from the Department of Plastic Surgery, Wonkwang University Hospital.

treated with each HFK and AM. The effect of HFK wi th AM was synergistic in FICIs. Each of these combi nation regimens were then tested together in a time kill at 4 times the respective isolate MIC. In this sam e time kill, HFK significantly enhanced AM activity. (Fi gures 1 and 2)

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 most effective method is to develop antibiotics from the natural products without having any toxic or side effects and develop substances showing synergistic effect by the combined application with conventional antibiotics. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. (Ahmad et al., 1998; Berahou et al., 2007).

Conclusion

We conclude that the aerial part of *K. yomena* extract has an antibacterial effect on MRSA and MSSA. Especially within *n*-hexane fraction for the case of MRSA was found

Table 4. Antimicrobial activity aerial part of *K. yomena* n-hexane fraction against *S. aureus* strain ATCC33591, ATCC25923, and 14 MRSA.

S. aureus strain	Zone of Inhibition (mm) (µg/ml)					
	***n*-hexane fraction**		**Ampicillin**		**Oxacillin**	
	500 µg	**250 µg**	**500 µg**	**250 µg**	**500 µg**	**250 µg**
ATCC 33591	16	15	17	14	18	14
ATCC 25923	16	13	46	44	41	39
Clinical isolates						
DPS-1[a]	15	13	18	14	ND	ND
DPS-2	17	15	19	18	20	19
DPS-3	15	14	19	18	ND	ND
DPS-4	16	13	19	14	ND	ND
DPS-5	16	14	19	14	ND	ND
DPS-6	15	13	17	14	ND	ND
DPS-7	15	14	13	11	ND	ND
DPS-8	15	14	13	12	ND	ND
DPS-9	14	13	14	12	ND	ND
DPS-10	15	14	12	9	ND	ND
DPS-11	14	12	13	11	ND	ND
DPS-12	14	12	14	11	ND	ND
DPS-13	15	12	18	13	ND	ND
DPS-14	15	14	14	11	ND	ND

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

Table 5. The interpreted FICI response for HFK + AM combinations against a standard MRSA strain, a standard MSSA strain and an MRSA isolate.

	MIC of HFK + AM (µg/mL)					
Strian	**HFK**		**Ampicillin**			
S. aureus strain	Alone	with AM	Alone	with HX	FICI[b]	Outcome
ATCC 33591	250	31.3	125	15.6	0.25	Synerge
ATCC 25923	62.5	0.06	0.24	0.015	0.13	Synerge
Clinical isolates						
DPS-3[a]	125	31.3	62.5	15.6	0.5	Partial synergy

[a], DPS indicates *S. aureus* strains from the Department of Plastic Surgery, Wonkwang University Hospital. [b]FICI, fractional inhibitory concentration index; HFK, *n*-hexane fraction aerial part of *K. yomena* Kitamura.

Table 6. The interpreted FICI response for HFK + OX combinations against a standard MRSA strain, a standard MSSA strain and an MRSA isolate.

	MIC of HFK + OX (µg/ml)					
Strain	**HFK**		**Oxacillin**			
S. aureus strain	Alone	with OX	Alone	with HX	FICI[b]	Outcome
ATCC 33591	250	62.5	125	15.6	0.5	Partial synergy
ATCC 25923	62.5	3.9	0.48	0.03	0.06	Synerge
Clinical isolates						
DPS-3[a]	125	125	500	31.7	0.56	Partial synergy

[a], DPS indicates *S. aureus* strains from the Department of Plastic Surgery, Wonkwang University Hospital.

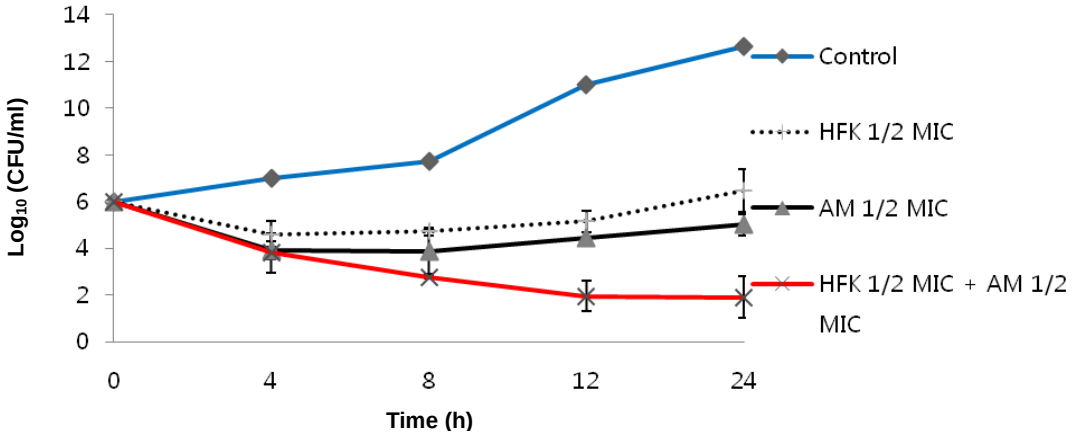

Figure 1. The time-kill curves of HFK and AM against the standard MRSA (ATCC 33591) strain.

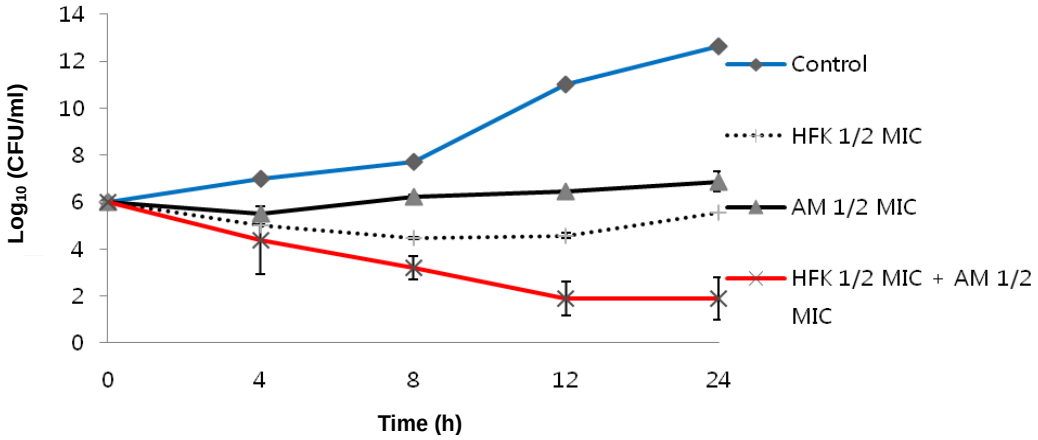

Figure 2. The time-kill curves of HFK and AM against the Clinical Isolate MRSA (DPS-3) strain.

to have good antimicrobial activity. Additional research in the future with detailed analysis to determine exact composition and development of a new antibiotic by deriving natural products such as *K. yomena* should be positively considered.

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