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Short Communication

A Study on Antimicrobial Activity of *Lysimachia clethroides* Duby Root Extracts against Methicillin-resistant *Staphylococcus aureus*

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Abstract - Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium responsible for a number of infections in humans that are difficult to treat, and as a result, is a substantial contributor to morbidity and mortality. In the present study, in search of natural products capable of inhibiting this multidrug-resistant bacterium, we investigated the antimicrobial activity of *Lysimachia clethroides* Duby *root*. The antibacterial activities of EtOH extract of *Lysimachia clethroides* Duby root and its *n*-hexane, EtOAc, *n*-BuOH and water fractions were evaluated against 15 strains of methicillin-resistant *staphylococcus aureus* (MRSA) and 1 standard methicillin-susceptible *S. aureus* (MSSA) strain by using the minimal inhibitory concentrations (MICs) assay, colorimetric assay using MTT test, checkerboard dilution test. Antimicrobial activity of *n*-hexane fraction of *Lysimachia clethroides* Duby root was remarkable. Against the 16 strains, the minimum inhibitory concentrations (MICs) were in the range of 31.25−62.5 ㎍/㎖ and FICI values for *n*-hexane fraction of *Lysimachia clethroides* Duby root+AM and *n*-hexane fraction of *Lysimachia clethroides* Duby root+OX were checkerboard method performed using the MRSA, MSSA and one clinical isolate strains via MICI 0.12-1 and 0.25-0.75, 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.

Key words − Antimicrobial, *Lysimachia clethroides* Duby, Methicillin-resistant *staphylococcus aureus* (MRSA), Synergism

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; Joung *et al*., 2012). 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 *Lysimachia clethroides* Duby. The primary purpose of this study was to investigate the *in vitro* effect against MRSA. *Lysimachia clethroides* Duby, one of the species of genus *Lysimachia*, is a traditional folk Chinese medicine, distributed widely in many provinces of China. This plant has been used widely for treatment of throat ache, edema, and menoschesis (Bae, 1998).

It has also been shown to have antimicrobial activity on food-borne microorganisms (Han *et al*., 2001). Chemical study showed Astragalin, Isoquercitrin, Kaempferol-3-rutinoside, kaempferol-3-0-(2,6-di-o-rhamnopyranosylglucopyranoside), Kaempferol-3-0-(rhamnopyranosylglucopyranoside) (Yasukawa *et al*., 1986). Flavonoids and saponins (Zou *et al*., 2004; Ren *et al*., 2001) were present in this plant and the flavonoids were proved to be the main biological constituents, with the activities of anti-tumor, anti-bacterial and anti-platelet aggregation (Xu *et al*., 2003). However, little is known

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about its antimicrobial effects on MRSA. Thus, we present the current study demonstrating the antimicrobial activity of *Lysimachia clethroides Duby* against MRSA and methicillin-sensitive (MSSA) strains, as well as its synergistic effect.

Materials and Methods

Plant material and sample preparation

Lysimachia clethroides Duby roots were collected from Sunchon, southern Republic of Korea, in June, 2017. A voucher specimen was deposited in the Laboratory of Oriental Pharmacology (N.1369). *Lysimachia clethroides* Duby root was air-dried, and boiled in ethanol (2L for 3h). The ethanol extract of *Lysimachia clethroides Duby* root (5.67% 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 ℃.

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

S. aureus strains	Class	mecA gene	Antibiotic resistance pattern
ATCC25923	MSSA	-[z]	-
ATCC33591	MRSA	+[z]	AM[y], OX[y]
DPS -1x	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

[z](+), positive; (-), negative.
[y]AM, ampicillin; OX, oxacillin.
[x]DPS-1 indicates *Staphylococcus aureus* strains from the Department of Plastic Surgery, Wonkwang University Hospital.

Test Microorganisms

Fourteen 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 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.

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 1.5×10^8CFU/㎖). 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, *Lysimachia clethroides* Duby 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 fourteen patients via MIC determination, according to the CLSI guidelines (Mazumdar et al., 2005). The MIC was defined as the lowest

Table 2. Antimicrobial activity of *Lysimachia clethroides* Duby root ethanol extract, *n*-hexane, EtOAc, *n*-BuOH and water fractions against *S. aureus* strains under dark

Minimal Inhibitory Concentration(MIC) (㎍/㎖)							
S. aureus strain	Ethanol extract	Fractions					
		n-hexane	EtOAc	*n*-BuOH	H_20	Ampicillin	Oxacillin
ATCC33591	250	31.25	250	ND[y]	ND	1000	250
ATCC25923	250	31.25	250	ND	ND	7.8	7.8
DPS -1[z]	250	31.25	250	ND	ND	31.25	500
DPS -2	250	15.62	125	ND	ND	1000	500
DPS -3	250	31.25	250	ND	ND	31.25	500
DPS -4	250	31.25	250	ND	ND	31.25	500
DPS -5	125	31.25	250	ND	ND	31.25	500
DPS -6	250	62.5	250	ND	ND	31.25	250
DPS -7	250	62.5	250	ND	ND	250	500
DPS -8	250	62.5	250	ND	ND	250	500
DPS -9	250	31.25	250	ND	ND	125	500
DPS -10	250	31.25	250	ND	ND	250	500
DPS -11	250	31.25	250	ND	ND	250	500
DPS -12	250	31.25	250	ND	ND	250	500
DPS -13	250	31.25	250	ND	ND	31.25	1000
DPS -14	250	31.25	250	ND	ND	250	500

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

Table 3. Result of the combined effect of *n*-hexane fraction of *Lysimachia clethroides* Duby root and AM against *S. aureus*

MICs (㎍/㎖)					
S. aureus strain	[b]HFL Alone	With AM	AM Alone	With HFL	[c]FICI
ATCC 25923	31.25	1.95	7.8	0.48	0.12
ATCC 33591	31.25	7.8	1000	250	0.5
DPS-1[a]	31.25	7.8	31.25	3.9	0.37
DPS-2	15.62	7.8	1000	62.5	0.56
DPS-3	31.25	7.8	31.25	7.8	0.5
DPS-4	31.25	7.8	31.25	7.8	0.5
DPS-5	31.25	7.8	31.25	7.8	0.5
DPS-6	62.5	7.8	31.25	15.62	0.62
DPS-7	62.5	15.62	250	31.25	0.37
DPS-8	62.5	15.62	250	31.25	0.37
DPS-9	31.25	15.62	125	7.8	0.56
DPS-10	31.25	7.8	250	62.5	0.5
DPS-11	31.25	7.8	250	62.5	0.5
DPS-12	31.25	15.62	250	62.5	0.75
DPS-13	31.25	15.62	31.25	15.25	1
DPS-14	31.25	7.8	250	62.5	0.5

[c]FICI; fractional inhibitory concentration index.
[b]HFL; n-hexane fraction of Lysimachia clethroides Duby root.
[a]DPS; indicates *Staphylococcus aureus* strains from the Department of Plastic Surgery, Wonkwang University Hospital.

Table 4. Result of the combined effect of *n*-hexane fraction of *Lysimachia clethroides* Duby root and OX against *S. aureus*

	MICs(㎍/㎖)				
S. aureus strain	[b]HFL Alone	With OX	OX Alone	With HFL	[c]FICI
ATCC 25923	31.25	3.9	7.8	0.97	0.25
ATCC 33591	31.25	7.8	250	62.5	0.5
DPS-1[a]	31.25	1.95	500	250	0.56
DPS-2	15.62	3.9	500	125	0.5
DPS-3	31.25	15.62	500	125	0.75
DPS-4	31.25	15.62	500	125	0.75
DPS-5	31.25	7.8	500	125	0.5
DPS-6	62.5	15.62	250	62.5	0.5
DPS-7	62.5	15.62	500	62.5	0.37
DPS-8	62.5	15.62	500	62.5	0.37
DPS-9	31.25	7.8	500	125	0.5
DPS-10	31.25	7.8	500	125	0.5
DPS-11	31.25	7.8	500	125	0.5
DPS-12	31.25	15.62	500	62.5	0.62
DPS-13	31.25	7.8	1000	125	0.37
DPS-14	31.25	7.8	500	31.25	0.31

[c]FICI; fractional inhibitory concentration index
[b]HFL; n-hexan fraction of Lysimachia clethroides Duby root
[a]DPS; indicates *Staphylococcus aureus* strains from the department of plastic surgery Wonkwang University Hospital.

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 5 ㎎/㎖ MTT (Sigma) was prepared in phosphate-buffered saline and kept at -70℃. A final concentration of 1 ㎎/㎖ 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

Ethanol extract had a MIC of 250 ㎍/㎖ against *S. aureus* ATCC 33591 under dark, and had a MIC of 250 ㎍/㎖ against *S. aureus* ATCC 25923 in the same condition. Antimicrobial activity of *n*-hexane fraction was remarkable, and had a MIC of from 15.62 ㎍/㎖ to 62.5 ㎍/㎖ against *S. aureus* strains (Table 1 and Table 2). *n*-hexane fraction of *Lysimachia clethroides* Duby root (HFL) lowered the MICs against the MRSA strain and MSSA but FICI values for HFL+AM and HFL+OX were 0.12-1 and 0.25-0.75, showing the increase of synergistic effect (Table 3 and 4).

Discussion

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 infections 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 for preventing the emergence of resistant mutants (Drago *et al*., 2007; Joung *et al.,* 2016). When combined together, these antibiotic effects were dramatically increased. Different drug combinations are reported to treat infections caused by pathogens (Miranda-Novales *et al*., 2006; Drago *et al*., 2007; Liu *et al*., 2000). 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 31.25 ㎍/㎖ to 62.5 ㎍/㎖ and checkerboard dilution test was performed to determine the action of HFL alone as well as its synergistic action with AM, or OX against the 16 strains. When tested against ATCC 33591, our data indicated that HFL alone only had moderate inhibitory effect on the growth of MRSA. However, in the presence of a nongrowth inhibitory dose of HFL (31.25 ㎍/㎖) or AM (1000 ㎍/㎖), HFL together with AM was highly effective with a FICI of 0.5. Similar effects were also observed in MSSA strain. These results showed that HFL in combination with these antibiotics could effectively inhibit MRSA growth. It may be partly due to the fact that they had abundant flavonoids which contributed to their antimicrobial activity and should be further studied. In conclusion, we found that *Lysimachia clethroides* Duby root extracts and *n*-hexane fraction have an antibacterial effect on MRSA and MSSA, and showing the increase of synergistic effect.

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