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Studies on antimicrobial activity of *Poncirus trifoliata* ethyl extract fraction against methicillin-resistant *Staphylococcus aureus* and to elucidate its antibacterial mechanism

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Abstract

Traditional medicinal plants contain a wide variety of chemicals that have potent antibacterial activity. To find an alternative agent of overcoming the problems of methicillin-resistant *Staphylococcus aureus* (MRSA), the antibacterial mechanism of *Ponciruss trifoliata* against MRSA was investigated. Ethyl acetate (EtOAc)-soluble extract of *P. trifoliata* methanolic extract was evaluated for antibacterial activity using minimum inhibitory concentration (MIC). An EtOAc sub-fraction 08 (EA08) from silica-gel open column chromatography exhibited strong anti-MRSA activity. Apart from the study to isolate single compound from EA08, a synergistic antibacterial effect between the sub-fraction and β-lactam antibiotics against MRSA was determined. In order to elucidate the antibacterial restoring mechanism of EA08 on MRSA, mRNA expression of *mecA* gene and production penicillin-binding protein 2a (PBP2a) encoded by *mec*A gene were monitored. EA 08 showed the strongest antibacterial activity with MIC value of 256 μg ml^{-1}. MIC of oxacillin against MRSA was dramatically reduced from 512 to 16 μg ml^{-1} in combination with 256 μg ml^{-1} of EA08. The fractional inhibitory concentration index of oxacillin was measured at 0.53 in combination with EA08 against MRSA, suggesting that EA08-oxacillin combinations exert synergetic effect against MRSA. The analysis of RT-PCR and Western blotting profiles revealed that EA08 inhibited mRNA expression of *mecA* gene and production PBP2a, which is a key determinant for β-lactam antibiotic resistance, in a dose-dependent manner. These results indicated that EA08 eventually led to the reduction or inhibition of PBP2a production through translational inhibition in MRSA.

Key words

Antibacterial activity, *MecA* gene, *Poncirus trifoliata*, *Staphylococcus aureus*, Synergy effect

Introduction

Evolution of resistant bacteria makes the exploration of new antimicrobial agents especially, for *Staphylococcus aureus* infections including skin and soft-tissue, respiratory, bone, joint, and endovascular infections in humans (Bramley *et al*., 1989). In the presence of antibiotic-resistant bacteria, methicillin-resistant *S. aureus* (MRSA) is a principal cause of nosocomial infectious diseases, and has become a serious problem in hospitals. MRSA infections are quite difficult to

treat, due to the multidrug-resistance properties of MRSA, which is recently a critical problem because it exhibits multidrug resistance not only to β-lactam antiobitcs, but also to aminoglycosides, fluoroquinolones, chloramphenicol and macrolides (Choi *et al*., 2009; Nikaido, 2009).

Due to the emergence of increasing drug resistance, most notably methicillin resistance in staphylococci, much attention has been focused on the search for new antimicrobial agents (Bramley *et al*., 1989; Hiramatsu *et al*., 1997). As alternatives to methicillin, vancomycin has been the drug of choice for the treatment of MRSA-related infections. However, as the use of vancomycin increased with the spread of MRSA, vancomycin-intermediate and -resistant *S. aureus* (VISA and VRSA) have been reported in several of countries (Tarai *et al*., 2013).

The penicillin-binding domains of penicillin-binding proteins (PBPs) are transpeptidases or carboxypeptidases involved in peptidoglycan metabolism (Zapun *et al*., 2008). Covalent inhibition of PBPs by β-lactam antibiotics results in a weakened cell wall and eventual cell lysis and death (Lovering *et al*., 2012). The resistance mechanism against methicillin is mediated via *mecA* gene, a part of the *mec* gene complex in staphylococcal cassette chromosome *mec* (SCC*mec*) (Shore *et al*., 2011). MRSA exhibits resistance to β-lactam antibiotics because of acquisition of the *mecA* gene encoding penicillin-binding protein 2a (PBP2a), which confers a lower affinity for binding β-lactam antibiotics (penicillins, cephalosporins and carbapenems) (Katayama *et al*., 2000; Shiota *et al*., 2000). This protein allows resistance against all β-lactam antibiotics and obviates their clinical use during MRSA infections. Recently, the susceptibility of MRSA to teicoplanin has been reported in several hospitals worldwide (Loomba *et al*., 2010). Thus, development of new drugs or alternative therapies is clearly a matter of urgency (Sacks and Behrman, 2010).

To find an alternative agent, traditional medicinal plants for treatment of MRSA have been investigated. Traditional medicinal plants contain a wide variety of chemicals that have potent antimicrobial activity. Among traditional medicinal plants, we examined the possibility that *Poncirus trifoliata* may be effective in MRSA treatments. *P. trifoliata* has been used against allergic diseases for generations, and still occupies an important place in traditional oriental medicine in China and Korea (Kim *et al*., 1999). However, research of anti-MRSA activity of *P. trifoliata* has not been reported yet. Therefore, the antimicrobial activity of *P. trifoliata* against MRSA and its antibacterial mechanism against MRSA were elucidated in the present study.

Materials and Methods

Raw materials and extraction : Fresh *P. trifoliata* (Rutaceae) was purchased from local market located at Youngchun-si (Kyungbuk, Korea) in late February 2013. Fresh *P. trifoliata* was washed meticulously with tap water and then dried for two weeks at room temperature. Dried powder was ground and then finely powdered with a food mixer (HMF-1000A; Hanil Electronics, Seoul, Korea). Dried powder was stored in a freezer at -20°C until used. The powdered *P. trifoliata* (1.0 kg) was thoroughly extracted three times with methanol (MeOH; 10 L) at 67°C for 3 hrs. The combined extracts were concentrated by rotary evaporation. MeOH extract (147.30 g) was suspended in 10% MeOH (1.0 L) and then was fractionated with *n*-hexane (Hexane; 1.0 L × 3), dichloromethane (DCM; 1.0 L × 3), ethyl acetate (EtOAc; 1.0 L × 3), *n*-butanol (BuOH; 1.0 L × 3), and water (H_2O) in sequence. Each extract was concentrated using a rotary evaporation (Eyela, Tokyo, Japan) under vacuum at 45°C to yield Hexane-soluble extract (3.23 g), DCM-soluble extract (5.62 g), EtOAc-soluble extract (4.29 g), BuOH-soluble extract (18.30 g) and H_2O-soluble extract (19.10 g).

Microorganisms and culture : Standard bacterial strains were obtained from the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea). All strains were grown aerobically at 37°C in Mueller-Hinton broth (MHB; Difco, Detroit, USA) for a minimum inhibitory concentration (MIC) assay.

Measurement of minimum inhibitory concentration (MIC) : MIC can be defined as the lowest concentration of antimicrobials that will inhibit the visible growth of microorganisms after overnight incubation. MIC of extracts and vancomycin was determined by two-fold serial dilution method in MHB (NCCLS, 2003). MIC was defined as the lowest concentration of crude extract that inhibited the visual growth after incubation at 37°C for 20-24 hr and was performed in triplicates.

Isolation and purification of anti-MRSA substance : Column chromatography was performed using Silica gel 60 (Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) was performed using Kieselgel 60 F_{254} plates (0.25 mm thick; Merck). Spots were detected by UV irradiation (254 and 365 nm) and by spraying with 10% H_2SO_4 reagent. EtOAc-soluble extract was found to be active in an antibacterial activity against MRSA with MIC value of 256 and 512 μg ml^{-1}. To identify anti-MRSA component from *P. trifoliata*, a portion (22.0 g) of EtOAc extract was chromatographed on a Silica gel 60 column (4.0 cm i.d. × 50 cm) with DCM $MeOH^{-1}$ (10:1, 7:1, 5:1, 1:1) to yield eleven sub-fractions (EA01-EA11).

Synergy test by the combination with commercial antibiotics : Interaction between *P. trifoliata* extract and β-lactam antibiotics including ampicillin, penicillin, and oxacillin against MRSA was tested by checkerboard method (Bennett *et al*., 1979). Synergistic effect between EA08 of *P.*

trifoliata and the antibiotics was evaluated as a fractional inhibitory concentration (FIC) index. FIC was calculated as the MIC of an antibiotic or EA08 of *P. trifoliata* in combination divided by MIC of β-lactam antibiotics or EA08 of *P. trifoliata* alone as follows. $FIC_A = MIC_A$ in combination MIC_A^{-1}, $FIC_B = MIC_B$ in combination MIC_B^{-1}

$$FIC\ Index = FIC_A + FIC_B$$

Interaction was defined as synergistic if FIC index was <0.5, additive if FIC index was between >0.5 and 1.0, and indifferent if the FIC index was between >1.0 and <2.0 (Takatsu *et al*., 1987) respectively.

RNA isolation and RT-PCR analysis :MRSA cells were treated with various concentrations with EA08 of *P. trifoliata* to elucidate an inhibitory effect on expression of drug resistance related genes. Total RNA was obtained by zirconia beads and RNAwiz kit (Ambion, Inc., Tex, USA) according to the manufacturer's protocols after cell harvesting. RNA concentrations were estimated by spectrophotometer at 260 nm. 0.2-1.4 μg of total RNA plus 1.4 μg of random primer was denatured at 65°C for 5 min, then cooled in ice at 30 sec and preincubated for 2 min at 37°C after addition of 10 mM dithioothreitol (DTT), 2.5 mM each of dNTPs and reaction buffer. Any remaining RNA was removed via the addition of 2 units RNase H at 37°C for 20 min. One hundred units of Superscript II reverse transcriptase were added and incubated for 50 min at 37°C. Reaction was then suspended at 70°C for 15 min. Two percent of the RT products were added to PCR reaction which included PCR buffer (pH 8.4, 20 mM Tris, 50 mM KCl), 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 2 pM primers and 0.2 μl Taq DNA polymerase. Thirty-four PCR cycles were then conducted as follows: denaturation at 95°C, extension at 72°C. Primer sequences were as follows: *mecA* (554 bp, PCR product, annealing temperature: 54.4°C) F: 5'-ATGAGATTAGGCATCGTTCC -3', R: '-TGGATGACAGTACCTGAGCC-3' (Kim *et al*., 1997); *nucA* (270 bp, PCR product, annealing temperature: 52.7°C) F: 5'-GCGATTGATGGTGATACGGTT-3', R: 5'-AGCCAAGCCTTGACGA ACTAAAGC-3' (Gupta *et al*., 1998).

Western blot analysis : For the purpose of elucidating the inhibitory effect of EA08 on expression of drug resistance related protein, PBP2a, MRSA cells were treated with various concentrations of EA08. The bacterial lysates were prepared in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM ethylene glycol tetra acetic acid (EGTA), 2 mM ethylene diamine tetra acetic acid (EDTA) and 0.25 M sucrose. The pellets were resuspended by sonication in lysis buffer 2 times for 20 sec. Following 10 min of centrifugation at 13,000 × g, supernatant was obtained as cell lysate. Protein concentrations were measured with Bradford protein assay (Bradford, 1976). Then, an equal amount of 2 × SDS-PAGE sample buffer (pH 7.5, 20 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 1% SDS, 150 mM NaCl) was added to tubes containing cell lysate and boil tubes for 3 min. Aliquots of cellular proteins (10 μg $lane^{-1}$) were then electrophoresed on 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Hartman and Tomasz, 1986).

Statistical analysis : Analyses were performed in triplicate, and data were averaged over three measurements. Standard deviation was also calculated. Multiple comparisons were evaluated by two-way analysis of variance using SPSS ver. 12.0 statistical software (SPSS Inc., Chicago, USA). Significant differences between means were determined using Duncan's multiple range test. A $P<0.05$ was considered significant.

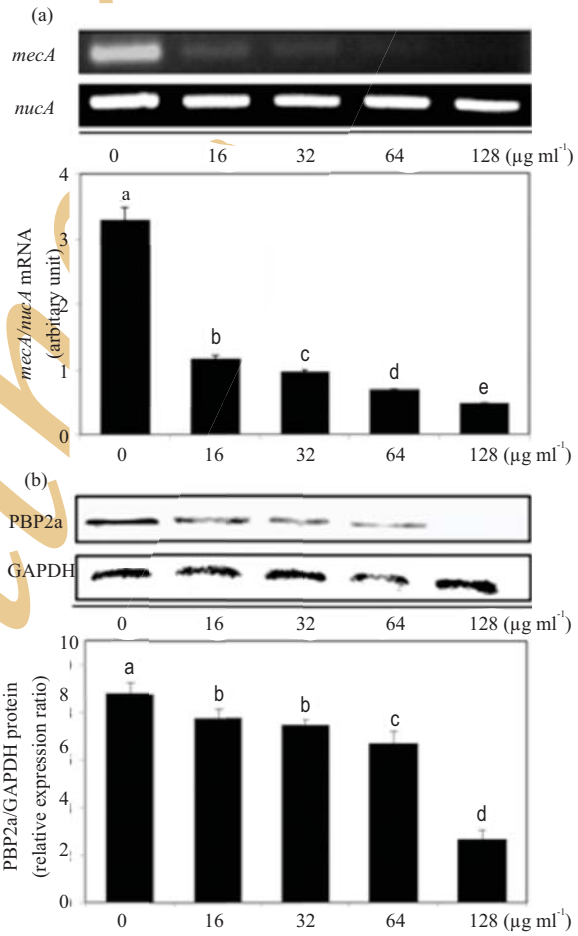

Fig. 1: Effect of ethyl acetate sub-fraction 08 (EA08) of *Poncirus trifoliata* on the mRNA expression of *mecA* (a) and on the production of penicillin-binding protein 2a (PBP2a) (b) in methicillin-resistant *Staphylococcus aureus* (MRSA) KCCM 40511. The MRSA KCCM 40511 strain was treated with the concentrations of EA08 indicated

Table 1.: Minimum inhibitory concentrations (MIC) of the *Poncirus trifoliata* methanol extract and its solvent-soluble extracts against methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA	MIC (μg ml^{-1})*					
	MeOH**	Hexane	DCM	EtOAc	BuOH	H_2O
MRSA (KCCM 40510)	512	>512	>512	256	512	>512
MRSA (KCCM 40511)	512	>512	>512	256	512	>512

*MIC of each solvent extract was determined by the two-fold serial dilution method in Mueller Hinton broth; **MeOH, methanolic extract; Hexane, n-hexane-soluble extract; DCM, dichloromethane-soluble extract; EtOAc, ethyl acetate-soluble extract; BuOH, n-butanol-soluble extract; H_2O, water-soluble extract

Table 2 : Minimum inhibitory concentrations (MICs) of EtOAc sub-fractions against methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA	MIC (μg ml^{-1})*										
	EA01	EA02	EA03	EA04	EA05	EA06	EA07	EA08	EA09	EA10	EA11
MRSA (KCCM 40510)	>512	>512	>512	>512	256	>512	512	256	>512	512	>512
MRSA (KCCM 40511)	>512	>512	>512	>512	256	>512	512	256	>512	512	>512

*MIC of each solvent extract was determined by the two-fold serial dilution method in Mueller Hinton broth;

Table 3.: Minimum inhibitory concentrations (MICs) and fractional inhibitory concentration (FIC) indices of ethyl acetate sub-fraction 08 (EA08) of *Poncirus trifoliata* in combination with β-lactam antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA	Ampicillin					Penicillin					Oxacillin				
	MIC (μg ml^{-1})			FIC index[a]		MIC (μg ml^{-1})			FIC index		MIC (μg ml^{-1})			FIC index	
	A	B	C	b	c	A	B	C	b	c	A	B	C	b	c
MRSA (KCCM 40510)	512	128	64	0.75	1.13	128	32	16	0.75	1.13	512	16	8	0.53	1.02
MRSA (KCCM 0511)	512	128	32	0.75	1.06	256	64	16	0.75	1.06	512	16	8	0.53	1.02

A, without EA08 of *P. trifoliata*; B to C and b to c, EA08 of *P. trifoliata* at 128 and 256 μg ml^{-1}, respectively; [a]The FIC index indicated synergistic effect: <0.5, synergy; >0.5 to 1.0, additive; >1.0 and <2.0, indifferent

Results and Discussion

The methanolic extract of *P. trifoliata* exhibited antibacterial activity against MRSA strains, suggesting that *P. trifoliata* contains antibacterial substance(s) against MRSA. To quantitatively evaluate its antibacterial activity, MIC values of extracts, against MRSA were investigated (Table 1). Highest anti-MRSA activity was observed with the EtOAc-soluble extract were determined in a range of 256-512 μg ml^{-1} against MRSA strains. The MIC value of BuOH-soluble extract was 512 μg ml^{-1}. However, no antibacterial activity was observed in H_2O-soluble extract (Table 1). These results suggested that an anti-MRSA substance originating from *P. trifoliata* methanolic extract was abundant in EtOAc-soluble extract of *P. trifoliata*, as previously reported to have potent antibacterial activity against food borne pathogenic bacteria (Rahman *et al*., 2009). These results were consistent with those of our study, which found that *P. trifoliata* might possess potential anti-MRSA activities. However, there has been no scientific report on anti-MRSA activity of *P. trifoliata*.

Following the results above, the EtOAc-soluble extract of *P. trifoliata* showed strongest antibacterial activity against MRSA. Because only limited information was available concerning the antimicrobial activity against MRSA, EtOAc-soluble extract was subjected to silica gel 60 chromatography to separate compounds. A portion (5.72 g)

of the EtOAc-soluble extract was subjected to silica gel 60 (0.063-0.200 mm) column chromatography with DCM·MeOH^{-1} gradient (10:1, 7:1, 5:1, 1:1), and fractioned into 11 sub-fractions (EA01-EA011). Sub-fractions quantities obtained were EA01 (0.15 g), EA02 (0.13 g), EA03 (1.27 g), EA04 (0.57 g), EA05 (1.12 g), EA06 (0.45 g), EA07 (4.12 g), EA08 (2.71 g), EA09 (0.14 g), EA10 (3.1 g), and EA11 (0.55 g). Among the sub-fractions, EA08 showed highest anti-MRSA activity with a MIC of 256 μg ml^{-1} (Table 2), suggesting thatEA08 contained anti-MRSA compound(s). Further study to isolate an active component is needed to determine the most active MRSA component of *P. trifoliata*, since EA08 does not contain a single compound. Separate from the study to isolate single compound(s) exhibiting anti-MRSA activity from EA08, a synergistic antibacterial effect between the most active fractions and β-lactam antibiotics against MRSA was determined the present research.

As mentioned above, MRSA is a problem because it has become resistant to almost all available antibiotics. Thus, there is a pressing need to develop new drugs or alternative therapies (Hiramatsu *et al*., 1997). It has been demonstrated that one of the more effective strategies in this regard is restoration of antibiotic activity in combination with antibacterial materials derived from natural products and traditional medicines against drug-resistant bacteria (Zhang *et al*., 2001). Thus, the synergistic effects of EA08 on MRSA was assessed with FIC test when administered in combination with commercial β-lactam antibiotics that inhibit several enzymes associated with the final steps of peptidoglycan synthesis (Foster, 2004). MRSA strains have shown resistance to β-lactam antibiotics, including ampicillin, penicillin, and oxacillin with MICs of 128 μg ml^{-1} or higher (Table 3). The MICs of β-lactam antibiotics against two MRSA strains (KCCM 40510 and 40511) were reduced markedly when coadministered with EA08. The MIC of oxacillin against MRSA was reduced markedly, from 512 to 16 μg ml^{-1} in combination with 256 μg ml^{-1} of EA08. The MICs of ampicillin and penicillin against MRSA were also reduced (Table 3). These results indicate that β-lactam antibiotics in combination with EA08 restored the antibacterial activity against MRSA exhibiting high-level β-lactam antibiotics resistance.

The synergistic effects between EA08 and β-lactam antibiotics were evaluated in terms of FIC index, as described in the Materials and Methods. The FIC indices of ampicillin were 0.75 in combination with a low concentration of EA08 (128 μg ml^{-1}) against MRSA strains, indicating a additive effect of the EA08-ampicillin combination. The FIC indices of penicillin were also 0.75 in combination with 128 μg ml^{-1} of EA08 against the MRSA strains. Additionally, the FIC indices of oxacillin were 0.53 in combination with 128 μg ml^{-1} of EA08 against MRSA strains, indicating strong additive or weak synergistic effects of EA08-oxacillin combinations. According Zhao *et al*. (2001), the FIC indices of the β-lactam antibiotics (penicillin and oxacillin) against 25 isolates of MRSA were from 0.126 to 0.625 in combination with 6.25, 12.5 or 25 μg of epigallocatechin gallate ml^{-1}. Although EA08 was less effective at reducing the MIC values in the presence of oxacillin, the combination of EA08-oxacillin provides an example of therapeutic potential of such combinations. The results of FIC assays revealed that the EA08 from *P. trifoliata* (128 μg ml^{-1}) could reduce the MIC values of the β-lactam antibiotics markedly against MRSA. This finding shows strong additive or weak synergistic interactions between EA08 and β-lactam antibiotics could restore the antibacterial activity of 'old' β-lactam antibiotics against MRSA. Thus, EA08 may have potential for use as an adjunct in treatment of antibiotic-resistant bacteria.

Resistance to β-lactam group of antibiotics, including ampicillin, penicillin and oxacillin, is mediated primarily by PBP2a, encoded by *mecA* gene (Lee *et al*., 2007; Foster, 2004). The PBP2a of MRSA enables transpeptidase activity in the presence of β-lactam antibiotics to permit cell wall synthesis, resulting in low affinity for β-lactam antibiotics (Scheffers and Pinho, 2005). Based on the results of synergitic the effect between EA08 and β-lactam antibiotics, the *mecA* gene or the PBP2a protein, which is a fundamental element of β-lactam antibiotic resistance, would be unexpressed or inactivated by EA08. To examine this, any inhibitory effect of EA08 on mRNA expression of the *mecA* gene and production of PBP2a in relation to β-lactam antibiotic resistance was investigated. The mRNA expression level of *mecA* gene and production of PBP2a protein in MRSA cells were monitored by RT-PCR and Western blotting, as described in the Materials and Methods. As shown in Fig. 1, the mRNA expression of *mecA* and PBP2a production were inhibited by EA08 treatment in a dose-dependent manner. These results indicated that EA08 can inhibit the mRNA expression of *mecA* gene and eventually lead to the reduction in or inhibition of β-lactam resistance protein, PBP2a, through transcriptional inhibition in MRSA cells. Thus, β-lactam antibiotics, in combination with EA08, will be able to bind to penicillin-binding domains of PBPs in MRSA cells, resulting in the restoration of antibiotic activity.

The results presented here provide the possibility of an alternative phytotherapeutic approach using natural sources for treatment of MRSA infection, and a clue to determining the antibacterial restoring mechanism in combination with natural compounds and less effective antibiotics.

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