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Thesis for the Degree of Master of Engineering

Elucidation of Antibacterial Mechanism of *Poncirus trifoliata* Extract against Methicillin-resistant *Staphylococcus aureus*

by

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Department of Food Science & Technology

The Graduate School

Pukyong National University

February 2014

Elucidation of Antibacterial Mechanism of *Poncirus trifoliata* Extract against Methicillin-resistant *Staphylococcus aureus*

메티실린계 내성 황색포도상구균에 대한 탱자 추출물의 항균활성 기작

Advisor: Prof. Young-Mog Kim

by

Yeoun-Joong Jung

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Engineering

in Department of Food Science & Technology, The Graduate School, Pukyong National University

February 2014

Elucidation of Antibacterial Mechanism of *Poncirus trifoliata* Extract against Methicillin-resistant *Staphylococcus aureus*

A dissertation

by

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PUKYONG NATIONAL UNIVERSITY 부경대학교

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요 약

최근 항생제 내성균의 증가는 세계적으로도 심각하게 문제 되고 있다. 그 중 메티실린계에 내성을 가지는 메티실린계 내성 황색포도상구균 (methicillin-resistant *Staphylococcus aureus*; MRSA) 은 메티실린에 내성을 지닐 뿐만 아니라 페니실린, 엠피실린 같은 모든 베타락탐 (β-lactam) 계열 항생제뿐만 아니라 다른 많은 항생제에 내성을 가지는 다중 내성균이다. 이에 따라 항생제를 대신 할 수 있는 천연 항생제 개발이 시급한 가운데 천연 물질에서 합성한 천연 항균제가 각광 받고 있다. 본 연구에서는 예로부터 약으로 많이 이용되어왔던 탱자를 이용하여 MRSA에 대한 항균 활성 물질을 분리하고 그 기작을 조사하였다. 그 결과,

탱자의 분획물 중 가장 뛰어한 anti-MRSA 활성을 띄는 ethyl acetate 분획물을 이용하여 실험을 진행하였다. Ethyl acetate 분획으로부터 gel column chromatography를 이용하여 분리한 결과 11개의 fraction을 얻을 수 있었고 fraction 08(EF08)에서 가장 높은 활성(256 μg mL^{-1})을 얻을 수 있었다. 이 결과로 미루어 보아 ß-lactam 계 항생제 내성에 관여하는 MRSA 내의 penicillin binding protein 2a (PBP2a) 단백질의 불활성 및 합성을 저해할 것이라고 판단되어 EF08을 이용하여 분자생물학적인 기법으로 규명하기 위하여 RT-PCR 및 western blot을 실시하였다. ß-lactam 계 항생제 내성에 관여하는 *mec* operon (*mecA, mecI, mecR1*) 중 MRSA의 핵심 유전자인 *mecA*가 EF08에 의하여 농도의존적으로 저해되는 것을 관찰할 수 있었다. 또한 western blot의 결과를 통해 *mecA* 유전자의 최종산물인 PBP2a 의 발현량 또한 ethyl acetate 분획물 중 EF08에 농도 의존적인 MRSA 항균작용을 가진다는 것을 확인하였다. 본 연구에서 얻어진 결과는 향후 탱자의 ethyl acetate 추출물이 MRSA에 대한 천연 항균제로 사용되어 질수 있다는 가능성을 보여주고 있을 뿐 만 MRSA 와 같은 항생제 내성균의 제어를 위한 alternative phytotherapeutic agent와 같은 치료제 개발으로도 이용 될수 있을 것으로 기대된다.

INTRODUCTION

The evolving resistance of many bacterial strains makes the exploration of new antimicrobial therapies paramount especially for *Staphylococcus aureus* prevalent in both intense and skin infection in humans (Sana et al., 2013).

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 (Choi et al., 2009). MRSA infections are quite difficult to cure, due to the multidrug-resistance properties of MRSA, which is recently a critical problem because it exhibits multidrug resistant not only to β-lactams, but also to aminoglycosides, fluoroquinolones, chloramphenicol, and macrolides (Isnansetyo and Kamei, 2003; Lee, 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). Vancomycin has been the drug of choice for the treatment of MRSA-related infections. With the increasing use of vancomycin, vancomycin-intermediate and -resistant *S. aureus* (VISA and VRSA) have been reported in a number of countries (Seqreti et al., 2008). β-Lactam antibiotics, such as penicillin and methicillin, are substrate analogs

penicillin-binding proteins (PBPs), which catalyze the formation of peptide cross-links (transpeptidation) between glycan chain of the cell wall. Covalent inhibition of PBPs by β-lactam results in a weakened cell wall and eventual cell lysis and death (Ghuysen, 1997). The resistance mechanism against methicillin is mediated via the *mec* operon, part of the staphylococcal cassette chromosome *mec* (SCC*mec*) (Shiota et al., 2000; Lee, 2009). MRSA exhibits resistance to β-lactam antibiotics because of acquisition of the *mecA* gene, which encodes PBP2a which has a lower affinity for binding β-lactams (penicillins, cephalosporins and carbapenems) (Shiota et al., 2004; Lee, 2009).

This protein allows the resistance against all β-lactam antibiotics and obviates their clinical use during MRSA infections. Recently, the susceptibility of MRSA to vancomycin has decreased; thus vancomycin-resistant *S. aureus* has been reported in several countries (Tenover et al., 2001). Furthermore, a decrease in the susceptibility of MRSA to teicoplanin has been reported in several hospitals worldwide (Alim et al., 2009). Thus, the development of new drugs or alternative therapies is clearly a matter of urgency (Hanaki, 1997).

Especially, plants contain a wide variety of chemicals that have potent antimicrobial activity. The use of plant compounds to treat infections is an age-old practice in a large part of the world, especially in developing

countries, where there is dependence on traditional medicine for a variety of diseases (Katsuyama et al., 2005).

Poncirus trifoliata used in this study has been used against allergic diseases for generations, and still occupies an important place in traditional Oriental medicine (Lee et al., 1997). However, study of the anti-MRSA activity of *P. trifoliata* has not reported yet. Therefore, the objective of this study, it was examined the antimicrobial activity of *P. trifoliata* against MRSA and elucidated its antibacterial mechanism against MRSA.

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Materials and Methods

1. Raw materials and extraction

The fresh *P. trifoliata* (Rutaceae) was purchased from a local market located at Youngchun-si (Kyungbuk, Korea) in late Febrary 2013. Fresh *P. trifoliata* was washed meticulously with tap water and then dried for two weeks at room temperature. The dried powder was ground and then finely powdered with a food mixer (HMF-1000A; Hanil Electronics, Seoul, Korea). The dried powder was stored in a freezer at -20℃ until needed. The powdered *P. trifoliata* (1.0 kg) was thoroughly extracted three times with methanol (MeOH; 10 L) at 70℃ for 3 h. The combined extracts were concentrated by rotary evaporation. MeOH extract (147.3 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), and n-butanol (BuOH; 1.0 L × 3), in sequence. Each extract was concentrated using a rotary evaporation (Eyela, Tokyo, Japan) under vacuum at 45℃.

2. Microorganisms and culture

Standard bacteria strains were obtained from the Korean Collection of Type Cultures (KCTC; Daejeon, Korea) and the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea). The bacterial strains utilized for evaluation of antibacterial activity in this study were a methicillin-susceptible *S. aureus* (MSSA; KCTC 1927), and two MRSA strains (MRSA; KCCM 40510 and KCCM 40511). All strains were grown aerobically at 37℃ in Mueller-Hinton broth (MHB; Difco, Detroit, USA) or tryptic soy broth (TSB; Difco) for a minimum inhibitory concentration (MIC) assay and in Mueller-Hinton agar (MHA; Difco) for a disc diffusion assay.

3. Disk diffusion assays

The disk diffusion assay described by the National Committee for Clinical Laboratory Standards (NCCLS, 2004) was used to evaluate the antibacterial activity. In brief, bacterial strains were cultured in TSB at 37℃ until the cell concentrations reached at about 0.5 of optical density at 600 nm. One mL of bacterial culture containing approximately 10^4 CFU mL^{-1} was spread on MHA plate and a paper disc (6 mm in diameter) containing 1-5 mg of each extract was then placed on the agar surface. After incubating for 24 h at

37℃, the diameter of inhibition zone was measured. The experiment was done three times and the mean values were presented.

4. Measurement of minimum inhibitory concentration (MIC)

The MIC can be defined as the lowest concentration of antimicrobials that will inhibit the visible growth of microorganisms after overnight incubation (Grierson and Afolayan, 1999). The MIC of the extracts and vancomycin was determined by the 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℃ for 20-24 h and was performed in triplicates (Grierson and Afolayan, 1999).

5. Isolation and purification of anti-MRSA substance

Column chromatography was performed using LiChroprep RP-18 (Merck, Darmstadt, Germany) and Silica gel 60 (0.063-0.200 mm). Thin-layer chromatography (TLC) was performed using Kieselgel 60 F254 plates (0.25 mm thick; EM Science, Gibbstown, NJ) and spots were detected by UV irradiation (254 and 365 nm)

6. Synergy test by the combination with commercial antibiotics

The interaction between EtOAc-soluble fraction of *P. trifoliata* and β-lactams including ampicillin, penicillin, and oxacillin against MRSA were tested by the checkerboard method (Norden et al., 1979). The synergy effect between EtOAc-soluble fraction of *P. trifoliata* and the antibiotics was evaluated as a fractional inhibitory concentration (FIC) index (Norden et al., 1979). The FIC was calculated as the MIC of an antibiotic or EtOAc-soluble fraction of *P. trifoliata* in combination divided by the MIC of the antibiotic or EtOAc-soluble fraction of *P. trifoliata* alone as follows. The FIC was then summed to derive the FIC index, which indicated synergy when index values were :

synergistic ≤ 0.5, partially synergistic > 0.5 to ≤ 1.0,

additive $=1.0$, indifferent > 1 to ≤ 2.0, antagonistic > 2.0

$FIC_A = MIC_A$ in combination / MIC_A , $FIC_B = MIC_B$ in combination / MIC_B,

FIC Index = $FIC_A + FIC_B$

7. RNA isolation and RT-PCR analysis

MRSA cells were treated with various concentrations of EtOAc-soluble fraction of *P. trifoliata* (Lee et al., 2007) 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℃ for 5 min, then cooled in ice at 30 sec and preincubated for 2 min at 37℃ after the 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℃ for 20 min. One hundred units of Superscript II reverse transcriptase were added and incubated for 50 min at 37℃. The reaction was then suspended at 70℃ for 15 min. Two percent of the RT products were added to a 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 uL Taq DNA polymerase. Thirty-four PCR cycles were then conducted as follows: denaturation at 95℃, extension at 72℃. Primer sequences were as follows: *mecA* (554 bp, PCR product, annealing temperature: 54.4℃) F; 5'-ATGAGATTAGGCATCGTTCC-3', R; 5'-TGGATGACAGTACCTGAGCC-3'; *mecI*

(268 bp, PCR product, annealing temperature: 52.7℃) F; 5'-CTGCAGAATGGGAAGTTATG-3', R; 5'-ACAAGTGAATTGAAACCGCC-3'; *mecR1* (235 bp, PCR product, annealing temperature: 53.4℃) F; 5'-AAGCACCGTTACTATCTGCACA-3', R: 5'-GAGTAAATTTTGGTCGAATGCC-3'; *femA* (372 bp, PCR product, annealing temperature: 52.6℃) F; 5'-CAT GATGGCGAGATTACAGGCC-3', R; 5'-CGCTAAAGGTACTAACACACGG-3'; *nucA* (270 bp, PCR product, annealing temperature: 52.7℃) F; 5'-GCGATTGATGGTGATACGGTT-3', R; 5'-AGCCAAGCCTTGACGAACTAAAGC-3' (Lee et al., 2007; Lei et al., 2007).

8. Western blot analysis

For the purpose of elucidating the inhibitory effect of EtOAc-soluble fraction of *P. trifoliata* on expression of drug resistance related protein, PBP2a, MRSA cells were treated with various concentrations of EtOAc-soluble fraction (Lee et al., 2007). MRSA were treated with various concentrations of *P. trifoliata* extracts. 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,

the supernatant was obtained as the cell lysate. Protein concentrations were measured with Bradford protein assay (Ku et al., 2013). Then, an equal amount of 2X 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 the tubes containing the cell lysate and boil tubes for 3 min. Aliquots of cellular proteins (10 μg/lane) were then electrophoresed on 10% sodium dodecyl sulfate –polyacrylamide gel electrophoresis (SDS-PAGE).

Results and Discussion

1. Anti-MRSA activity of *P. trifoliata* extract

The methanolic extract of *P. trifoliata* exhibited an antibacterial activity against MRSA strains, suggesting that the extract contains an antibacterial substance against MRSA. Also, the extract exhibited almost similar antibacterial activity against MSSA. In order to identify an antimicrobial substance against MRSA and MSSA, the further fractionation on extract was performed with organic solvents (Fig. 1). Lyophilized powder (1.0 kg) of *P. trifoliata* was percolated in methanol (3 times × 1,0 L), followed by fractionation with several organic solvents to yield Hexane soluble fraction (3.23 g), DCM soluble fraction (5.62 g), EtOAc soluble fraction (4.29 g), BuOH soluble fraction (18.3 g) and water soluble fraction (19.1 g) (Fig. 1). The anti-MRSA activity of hexane, DCM, EtOAc, BuOH and water soluble fractions was appraised by measuring inhibition zones. Among them, the EtOAc soluble fraction showed the strongest anti-MRSA activity and followed by BuOH soluble fraction. No anti-MRSA activity was observed in hexane, DCM and water soluble fraction (Table 1). These results were

consistent with the reports of (Lee et al., 2008), Bae (2011) and Park (2012) that EtOAc soluble fraction of *Eisenia bicyclis* and *Paeonia japonica* exhibited the strongest anti-MRSA activity.

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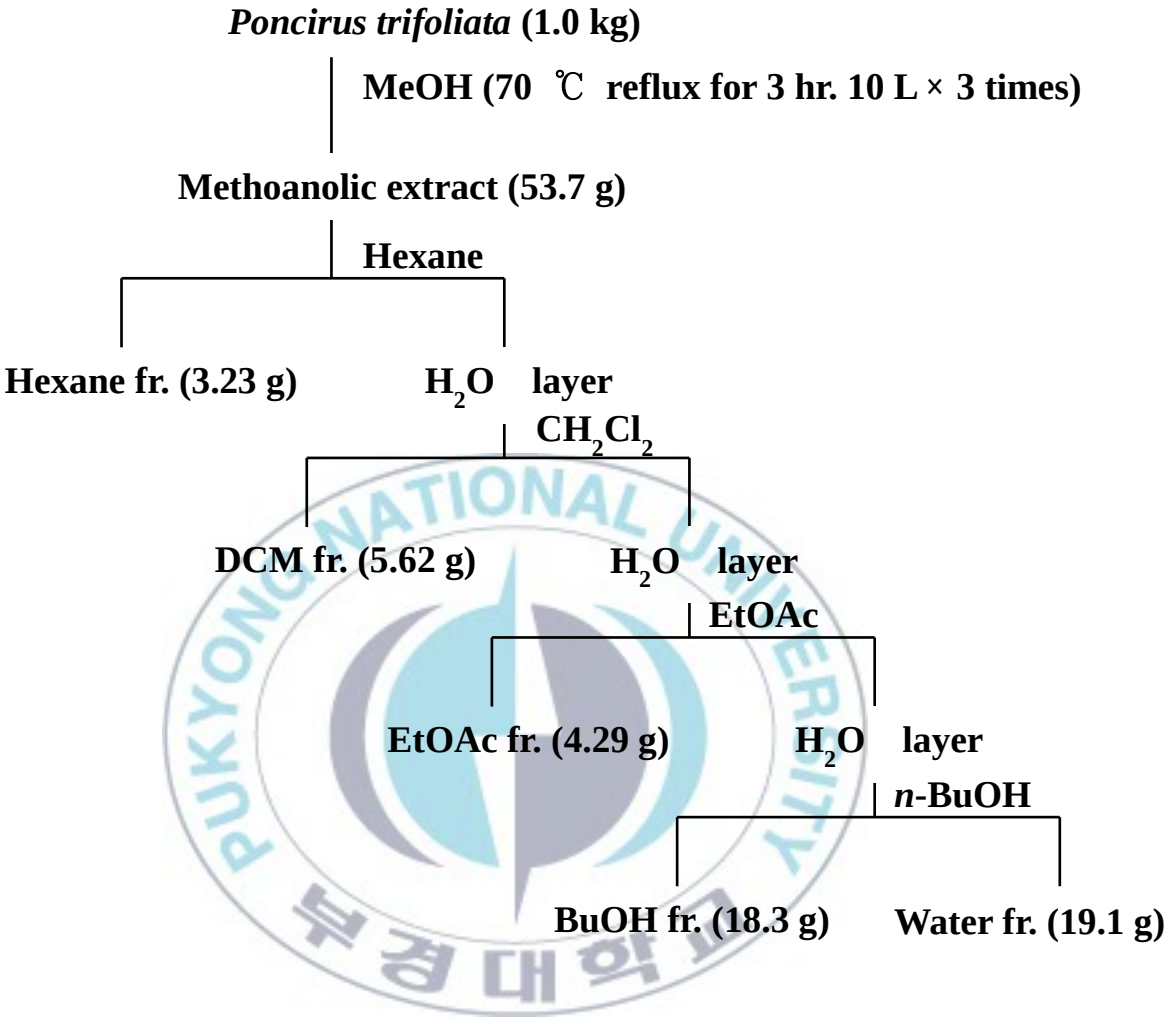

Fig. 1. Scheme of extraction and liquid-liquid solvent fractionation

Table 1. Disk diffusion assay of the methanol extract and *Poncirus trifoliata* extracts against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA)

Gram-positive bacteria	Concn.	Zone of inhibition (mm)[a]					
		MeOH[b] ext.	Hexane fr.	DCM fr.	EtOAc fr.	BuOH fr.	H_2O fr.
MSSA (KCTC 1927)	1 mg/disk[b]	6.0[c]	-	-	7.5	6.5	-[d]
	5 mg/disk	9.0	-	-	10.0	8.5	-
MRSA (KCCM 40510)	1 mg/disk	8.5	-	-	9.0	8.0	-
	5 mg/disk	10.5	-	-	12.0	10.5	-
MRSA (KCCM 40511)	1 mg/disk	8.0	-	-	8.5	7.0	-
	5 mg/disk	10.0	-	-	11.0	10.0	-

[a]Methanol extract and its fraction from *P. trifoliata* was loaded onto a disk (6 mm in diameter).

[b]MeOH ext., methanolic extract; DCM fr., dichloromethane fraction.; EtOAc fr., ethyl acetate fraction; BuOH fr., butanol fraction; H_2O fr., water fraction

[c]Data are the averages of duplicate experiments.

[d]–, no detected antibacterial activity.

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Table 2. Minimum inhibitory concentrations (MIC) of the methanol extract and *Poncirus trifoliata* extracts against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA)

Gram-positive bacteria	MIC (μg mL^{-1})					
	MeOH* ext.	Hexane fr.	DCM fr.	EtOAc fr.	BuOH fr.	H_2O fr.
Staphylococcus aureus (KCTC 1927)	>512	>512	>512	512	512	>512
MRSA (KCCM 40510)	512	>512	>512	256	512	>512
MRSA (KCCM 40511)	512	>512	>512	256	512	>512

*MeOH ext., methanolic extract; DCM fr., dichloromethane fraction.; EtOAc fr., ethyl acetate fraction; BuOH fr., butanol fraction; H_2O fr., water fraction

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2. Determination of MIC of *P. trifoliata* extract

The current study focused on an antibacterial activity of *P. trifoliata* extracts against MRSA. In order to quantitatively evaluate its antibacterial activity, it was investigated MIC values of the extract against MSSA and MRSA (Table 2). The highest anti-MRSA activity was observed on the ethyl acetate (EtOAc) soluble fraction. These results were also consistent with the results obtained by the disk diffusion assay. The MICs of EtOAc soluble extract were determined in a range of 256 to 512 μg per mL against MSSA and MRSA strains. BuOH soluble fraction was 512 μg per mL for MICs. However, no antibacterial activity was observed in H_2O-soluble extract (Table 2). These results thoroughly suggested that an anti-MRSA substance originated from the *P. trifoliata* methanolic extract will be abundant in the EtOAc soluble fraction.

It has been previously reported that the EtOAc extract of this plant also showed the highest antibacterial activity against a cavity-causing Gram positive bacterium, *Bacillus subtilis* Eom (2008). Thus, it was investigated the *P. trifoliata* extract will exhibit an antibacterial activity against several kinds of pathogenic or spoilage bacteria. The MICs of the EtOAc fraction against these bacteria were in a range of 256 to 512 μg per mL, respectively. These results were consistent with the report of Eom (2008), suggesting that

there might be a different mechanism to inhibit cell growth as well as the inhibition of bacterial cell wall synthesis.

3. Isolation of fraction from *P. trifoliata*

It has been reported on the biological activities of the EtOAc extract on potential inhibition against tumor cell line (Lee et al., 2003) and anti-inflammatory activity to human body (Zhou et al., 2007). In this study, the EtOAc fraction exhibited the most potent antimicrobial activity. However, only limited information is available concerning their antimicrobial activities.

The aim of the present study was to investigate the antimicrobial activity of methanolic extract and its solvent soluble fractions derived from *P. trifoliata*. In order to elucidate the anti-MRSA action mechanism, the EtOAc fraction was subjected to a chromatogram to obtain single or more purified compounds.

The EtOAc-soluble extract (15.72 g) was chromatographed on a Silica gel 60 (0.063-0.200 mm) column using DCM and MeOH as solvent to yield 11 subfractions. A portion (15.72 g) of the EtOAc extract was chromatographed on a Silica gel 60 (0.063-0.200 mm) column with DCM, MeOH and fractioned into eleven subfractions (EF01-EF011). Subfractions were EF01

(0.15 g), EF02 (0.13 g), EF03 (1.27 g), EF04 (0.57 g), EF05 (1.12 g), EF06 (0.45 g), EF07 (4.12 g), EF08 (2.71 g), EF09 (0.14 g), EF10 (3.1 g), and EF11 (0.55 g).

4. Minimum inhibitory concentrations (MICs) of EtOAc fraction derived from *P. trifoliata*

The present study focused on quantitatively evaluate of antibacterial activity against MSSA and MRSA strains. The MIC values of EtOAc subfractions were obtained by a two-fold serial dilution method. Among them, EF08 was the highest anti-MRSA activity with MIC values of 256 μg mL^{-1} (Table 3). This result was equal to or less than those of the β-lactams tested against the MRSA strains (Eom, 2012).

These results are in accordance with the disc diffusion assay obtained in this study. These results strongly suggested that the EF08 subfraction might possess potential antimicrobial activity against MRSA and merit performing additional studies including action mechanism.

Table 3. Minimum inhibitory concentrations (MICs) of EtOAc subfractions against methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA)

Gram-positive bacteria	MIC (μg mL^{-1})*										
	EF01	EF02	EF03	EF04	EF05	EF06	EF07	EF08	EF09	EF10	EF11
MSSA(KCTC 1927)	>512	>512	>512	>512	512	>512	512	256	>512	>512	>512
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.

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5. Synergy effects between EtOAc subfraction 08 and β-lactams against MSSA and MRSA

The β-lactam groups of antibiotics are derived from a β-lactam structure and inhibit several enzymes associated with the final step of peptidoglycan synthesis (Foster, 2004). The great number of chemical modifications based on this structure permits an astonishing array of antibacterial and pharmacological properties within this valuable family of antibiotics. Among them, ampicillin, penicillin, and oxacillin preferentially bind to PBP in the cell wall and inactivate their transpeptidase and carboxypeptidase activities; these activities are responsible for catalyzing the final transpeptidation step of bacterial cell wall biosynthesis (Foster, 2004). The MRSA strains that are *mecA* positive exhibited a resistance to the β-lactams, including ampicillin, penicillin and oxacillin, and showed the MICs of 128 μg mL^{-1} or higher than those (Table 4).

The ever-increasing development of pathogenic microbial resistance to traditional antibiotics has already reached alarming levels (Eom et al., 2012). As mentioned above, MRSA is the most serious gram-positive bacterium because it has become resistant to almost all available antibiotics. Therefore, there is currently a pressing need to develop new drugs or alternative

therapies (Hiramatsu et al., 1997; Hanaki et al., 1998). It has been demonstrated that one of the more effective strategies in this regard is the restoration of antibiotic activity in combination with antibacterial materials derived from natural products and traditional medicines against drug-resistance bacteria (Zaho et al., 2001; Shioda et al., 2004; Jeong et al., 2006). Thus, the synergy effects of the EtOAc subfraction 08 of *P. trifoliata* on MRSA was assessed with FIC test when administered in combination with commercial β-lactams.

As is shown in Table 4, the MICs of β-lactams against two standard MRSA strains (KCCM 40510 and 40511) were reduced dramatically, when administered in combination with 128 μg mL^{-1} of the subfraction 08, thereby indicating that the subfraction reversed the high-level β-lactams resistance of MRSA. The synergy was evaluated in terms of a FIC index between the sub fraction 08 and β-lactams, as described in the Materials and Methods section.

The FIC indices of ampicillin were 0.75 in combination with a low concentration of the subfraction 08 (128 μg mL^{-1}) against two standard MRSA, thereby indicating the partially additive synergy effect of the sub fraction 08-ampicillin combination. The FIC indices of penicillin were also 0.75 in combination with 128 μg mL^{-1} of the subfraction 08 against two standard MRSA strains. Also The FIC indices of oxacillin were 0.53 in combination with 128 μg mL^{-1} of the subfraction against all tested MRSA

strains, thereby indicating that the subfraction and two β-lactams, such as penicillin and oxacillin, inhibit the growth of MRSA in the manner of partially synergistic. Synergistic effects of an anti-MRSA substance-β-lactams combination were also reported in the combination phlorotannins from brown alga and β-lactams (Lee et al., 2009; Eom, 2012). Among of phlorotannins, dieckol also evidenced the synergistic effect against MRSA in combination with ampicillin and penicillin. However, no synergistic effect was observed with a dieckol- oxacillin combination (Lee et al., 2009). These results suggested that there will be a difference antibacterial mechanism against MRSA between the EtOAc subfraction 08 of *P. trifoliata* and the dieckol. Also, the synergistic effect between catechins and β-lactams was reported by Hu et al. (2001).

The results of the checkerboard assay revealed that the EtOAc subfraction 08 of *P. trifoliata* (128 μg mL^{-1}) can remarkably reduce the MICs of the β-lactams against MRSA. This finding shows that the presence of EtOAc subfraction 08 could restore the antibacterial activity of old-fashion β-lactams against MRSA. Thus, the EtOAc subfraction 08 of *P. trifoliata* might have potential for use as an adjunct in the treatment of antibiotic-resistant bacteria.

Table 4. Minimum inhibitory concentrations (MICs) and fractional inhibitory concentration (FIC) indices of EtOAc fraction 08 of *Poncirus trifoliata* in combination with β-lactams against methicillin-resistant *Staphylococcus aureus* (MRSA)

Strain	Ampicillin					Penicillin					Oxacillin				
	MIC (㎍/㎖)			FIC index		MIC (㎍/㎖)			FIC index		MIC (㎍/㎖)			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 40511)	512	128	32	0.75	1.06	256	64	16	0.75	1.06	512	16	8	0.53	1.02

A, without EtOAc fraction 08 of *P. trifoliata*; B to C and b to c, EtOAc fraction 08 of *P. trifoliata* at 128 and 256 μg mL^{-1}, respectively. [a)] The FIC index indicated as followed. ≤ 0.5, synergistic; > 0.5 to ≤ 1, partially synergistic; =1.0, additive; > 1 to = 2, indifferent; > 2, antagonistic.

6. Inhibitory activity of *P. trifoliata* extract on the expression of genes and the production of PBP2a related in drug resistance

The resistance of the β-lactam group of antibiotics, including ampicillin, penicillin and oxacillin is primarily mediated by the PBP2a encoded by the *mecA* gene (Foster, 2004; Lee et al., 2008). Unlike β-lactam-sensitive PBP that is normally produced by MSSA, PBP2a provides transpeptidase activity to permit cell wall synthesis at β-lactam concentration due to its low affinity for β-lactam (Scheffers and Pinho, 2005; Park et al., 2012).

The results of synergy effect between the EtOAc subfraction and β-lactams suggested that β-lactams can restore the antibiotic activity against MRSA in the combination with the EtOAc subfraction. Thus, the PBP2a protein, which is a fundamental protein of β-lactam antibiotic resistance, would be unexpressed or inactivated by the EtOAc subfraction.

Based on this presumption, it was supposed that the anti-MRSA activity of EtOAc subfraction 08 of *P. trifoliata* might be related directly or indirectly to PBP2a. An inhibitory effect of the subfraction on the expression of genes (*mecA, mecI, mecR1* and *femA*) and the production of PBP2a related in β-lactams resistance was investigated for verifying this hypothesis (Eom et al., 2012).

The *mecA* gene that encodes PBP2a was acquired from an unknown donor bacterium, together with 30 to 50 kb of additional DNA, and inserted at a specific chromosomal site (Tanya et al., 2001). The *mecA* gene is located on a mobile element, staphylococcal cassette chromosome *mec* (SCC*mec*), which is horizontally transferable among staphylococcal species. This instability of *mecA* in some genetic backgrounds may account in part for the relatively restricted clonal clustering of the mobile SCC*mec* element (Katayama et al., 2005) (Fig. 2).

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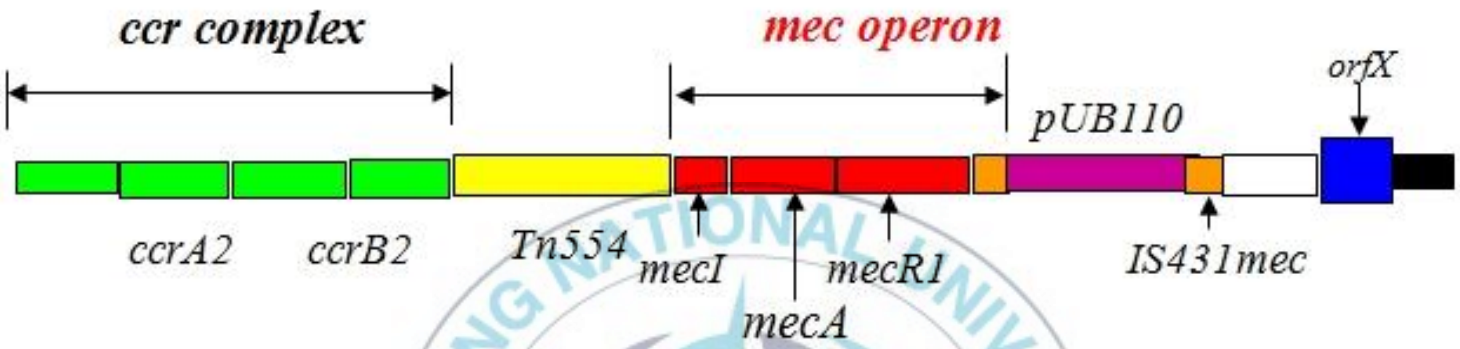

Fig. 2. *mec* operon of methicillin-resistant *Staphylococcus aureus* (MRSA).

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The transcription of *mecA* can be regulated by two regulatory genes (*mecI* and *mecR1*), which is located upstream or downstream of *mecA*. The *mecR1* gene encodes a membrane-bound signal transduction protein while *mecI* encodes a transcriptional regulator (Meng et al., 2009). Other chromosomally determined factor for methicillin resistance is *femA* gene (Vannuffel et al., 1995). The *femA* gene, which is known to be related to cell wall composition, encodes a factor which is essential for methicillin resistance and exists universally in all *S. aureus* isolate (Mehrotra et al., 2000). The expression levels of *femA* in high-level resistant MRSA (non-β-lactamase-producing) were found to be higher than in low-level resistant MRSA and MSSA. A regulatory gene of *femA* is probably present in MRSA and *femA* was shown to be essential for the expression of high-level methicillin resistance (Li et al., 2008). The *nucA* gene is essential gene in all *S. aureus* (Lee et al., 2007).

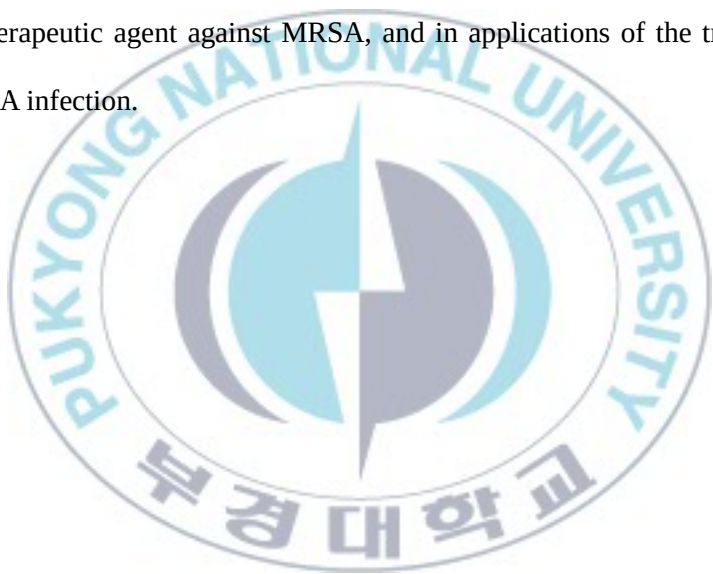
The expression of genes related in the antibiotic resistance in MRSA cells was monitored by RT-PCR (reverse transcriptase-polymerase chain reaction). All mRNA of *mecA*, *mecI*, *mecR1*, and *femA* genes in MRSA KCCM 40511 strain were clearly detected (Fig. 3). As shown in Fig. 3, the mRNA expression of *mecA* and *mecR1* gene was inhibited in a dose-dependent manner by the subfraction 08 but not *nucA*. However, the inhibitory effect on

the mRNA expression of *mecI* and *femA* gene was observed at the concentration of over 64 μg mL^{-1}, suggesting that both genes were not directly related the *mecA* gene expression.

The *mecR1* encodes for the activator protein to induce the PBP2a production (Lee et al., 2007). By exposure to β-lactam, *mecR1* is activated and the resulting protein, *mecRI*, binds to the promoter region of *mecA*, and mediated the production of PBP2a. In this manner, it was hypothesized that the subfraction 08 inhibited the mRNA expression of the key antibiotic resistant gene, *mecA*, by the transcriptional inhibition of activator gene, *mecRI*. Based on this hypothesis, it was supposed that the PBP2a production would be attenuated or inhibited in a dose-dependent manner. Thus, the results indicated that the subfraction 08 can inhibit the mRNA expression of the *mecA* gene and eventually led to the reduction or inhibition of the resistance protein, PBP2a, in MRSA cells.

Generally, it has been believed that mRNA expression of target gene would not directly correlate with its protein production. Therefore, the production of PBP2a encoded by *mecA* was investigated. The results of Western blotting assay indicated that the subfraction 08 of *P. trifolilata* attenuated the production level of PBP2a in a dose-dependent manner (Fig. 4). The results obtained from mRNA expression and Western blotting assay,

it was concluded that the subfraction 08 inhibited the expression of the resistant protein, PBP2a, through transcriptional inhibition. The majority of published reports did not address the capacity of the plant and herbal extracts to regulate drug-resistant properties in molecular levels (Eom et al., 2012). These results show that the EtOAc fraction from *P. trifoliata* is expected to be recognized as natural sources for the development of an alternative phytotherapeutic agent against MRSA, and in applications of the treatment of MRSA infection.



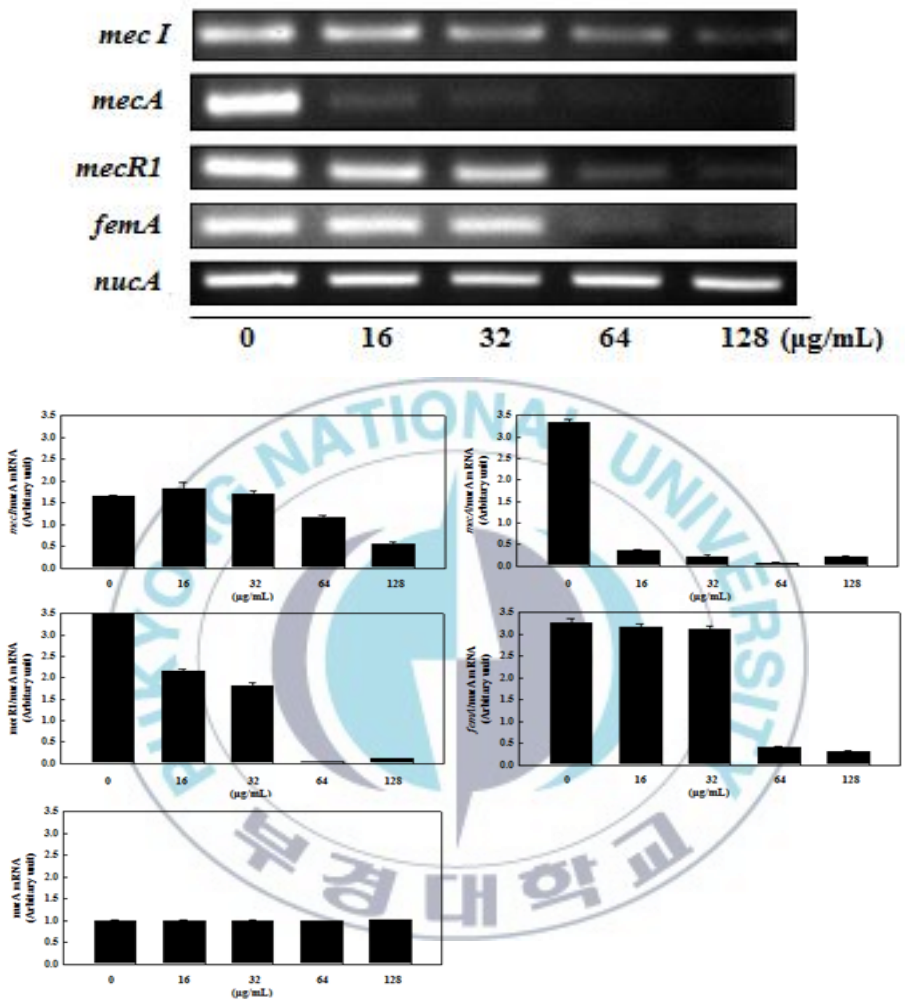


Fig. 3. Effect of ethyl acetate subfraction 08 of *Poncirus trifoliata* on the mRNA expression of *mecI*, *mecA*, *mecR1*, *femA*, and *nucA* genes. Methicillin-resistant *Staphylococcus aureus* (MRSA) KCCM 40511 strain was treated with the indicated concentrations of the subfraction.

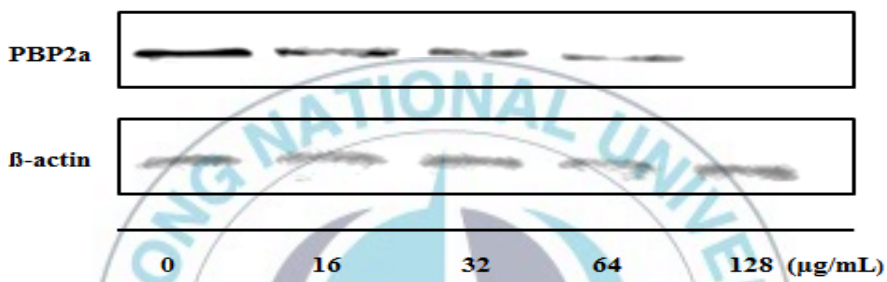


Fig. 4. Effect of ethyl acetate subfraction 08 of *Poncirus trifoliata* on the expression of PBP2a protein against MRSA strain. The MRSA KCCM 40511 strain was treated with the indicated concentrations of the subfraction of *Poncirus trifoliata.*

Summary

MRSA is notorious as multidrug- resistant Gram-positive bacteria. MRSA has been acquired vancomycin resistant (vancomycin-intermediate and -resistant *S. aureus*) with increasing use of vancomycin to treat MRSA-related infections. Thus, the development of new drugs or alternative therapies is clearly a matter of urgency. In an effort to discover an alternative antibiotic against MRSA, many plants have been interested to discover an alternative therapeutic agent against MRSA. Among plants, *P. trifoliata* used to elucidate its anti-MRSA mechanism.

The EtOAc subfraction 08 of *P. trifoliata* exhibited the highest anti-MRSA activity with MIC values of 256 μg mL^{-1}. This result shows that the EtOAc extract could restore the antibacterial activity of β-lactams against MRSA. Thus, the PBP2a protein, which is a central protein of β-lactam antibiotic resistance, would be unexpressed or inactivated by EF08 fraction. In addition, the EtOAc extract could suppress in a dose-dependent manner on mRNA expression of genes (*mecA*, *mecI*, *mecR1* and *femA*) related in β-lactam antibiotics resistance and on expression of penicillin binding protein (PBP) 2a encoded by *mecA*. As a result, the EF08 fraction dramatically inhibited the expression of the resistant genes *mecA* and *mecR1* in a dose-

dependent manner at a concentration of 128 μg mL^{-1}. The results of the present study show that the ethyl acetate fractions of *P. trifoliata* evidenced profound antimicrobial activity, and inhibited resistant gene expression against *S. aureus* and MRSA. However, further study is needed to identify the rest of active compound and to investigate the characteristics of the anti-MRSA activity

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