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Antimicrobial Activities of *Eriobotrya japonica* Lindl. Leaf Extract against Methicillin-resistant *Staphylococcus aureus*

by

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메티실린계 내성 황색포도상구균에 대한 비파잎의 항균활성 기작

Advisor: Prof. Young-Mog Kim

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요 약

메치실린계 내성 황색포도상구균 (methicillin-resistant *Staphylococcus aureus* ; MRSA)은 메티실린에 내성을 지닐 뿐만 아니라 세팔로스포린, 암피실린, 나프실린 등과 같은 베타락탐 (β-lactam) 계열 항생제를 비롯한 많은 항생제에 내성을 지니고 있어 항생제 사용이 한정되어있다. 이에 따라 최근에는 항생제 내성 균주의 발생에 관한 우려가 없으면서 항생제를 대신 할 수 있는 천연물질, 즉 식물 또는 동물 유래 성분을 이용한 항균활성물질의 개발이 활발히 진행되고 있다. 본 연구는 사회적으로 문제가 되고 있는 항생제 내성균의 제어를 위한 연구의 일환으로 예로부터 민간과 한방에서 널리 이용되어 온 9개의 약용식물을 메탄올에 추출하여 항균활성을 조사하였고 그 중에서 MRSA에 대한 항균활성이 우수한 비파잎을 가지고 anti-MRSA 활성을 가지고 있는 fraction의 분리 및 항균 기작을 조사하였다. 그 결과 분획물 중 가장 뛰어난 항균활성을 나타내는 비파잎의 ethyl acetate 분획으로부터 8개의 fraction을 얻었고 ethyl acetate fraction 03 (EF03)에서 가장 높은 MIC 값 (32 μg/mL)의 anti-MRSA 활성을 확인할 수 있었다.

Ethyl acetate 분획물 중 EF03이 ß-lactam 계 항생제 내성에 관여하는

MRSA 내의 penicillin binding protein 2a (PBP2a) 단백질의 불활성 및 합성을 저해하는데 분자생물학적인 영향을 알아보기 위하여 RT-PCR과 western blot을 이용하여 PBP2a 단백질의 발현량과 MRSA의 ß-lactam 계 항생제 내성에 관여하는 유전자들의 전사량을 조사하였다. ß-lactam 계 항생제 내성에 관여하는 *mec* operon (*mecA*, *mecI*, *mecR1*) 중 *mecA*, *mecR1* 이 EF03에 의하여 농도 의존적으로 저해되는 것을 관찰할 수 있었고, *mec* operon 밖의 유전자인 *femA* 도 농도 의존적으로 감소되는 것을 확인하였다. *mecA* 유전자의 최종산물인 PBP2a 의 발현량 또한 비파잎 추출물의 ethyl acetate 분획물 중 EF03에 농도 의존적인 MRSA 항균작용을 가진다는 것을 확인하였다. 이와 같은 결과는 EF03 이 직접적으로 MRSA에서 ß-lactam 계 항생제의 세포 내 수송을 억제하는 PBP2a 단백질의 발현을 억제함으로써 ß-lactam 계 항생제의 MRSA 에 대한 항생제 감수성을 회복시킨다는 결론을 도출하였다. 본 연구에서 얻어진 결과는 향후 비파잎 추출물에 함유되어 있는 항균물질을 이용하여 MRSA 와 같은 항생제 내성균의 제어를 위한 alternative phytotherapeutic agent와 같은 치료제 개발에 연결될 것으로 기대된다.

Introduction

Drug resistance in pathogenic bacteria is a serious problem in the treatment of patients infected with such bacteria. It is currently very difficult to discover new antibiotics or to develop new antimicrobial drugs (Shimizu, 2001).

Methicillin-resistant *Staphylococcus 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, owing to the multidrug-resistance properties of MRSA, which is resistant to β-lactams as well as a host of other antibiotics. 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 and its analog teicoplanin are the most effective antibiotics for MRSA infection. However, their clinical use often results in unexpected side effects and the development of vancomycin-resistant *S. aureus* (VRSA) infection (Bailie and Neal, 1988; Berger-Bachi et al., 1992; Liu 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 glycans 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). The *mecA* gene is highly conserved among clinical MRSA isolates (>90% sequence identity between strains) and encodes PBP2a, a newly discovered PBP unlike any of the PBPs normally produced by *S. aureus* (<21% sequence identity) and for which no β-lactam-sensitive variants or direct homologs are known (Goffin and Ghusen, 1998).

This protein allows the resistance against all β-lactam antibiotics and obviates their clinical use during MRSA infections. As the results, MRSA has become resistant to almost all available antibiotics except vancomycin and teicoplanin. Vancomycin, the glycopeptide, is often deployed against MRSA (Lee, 2009). Recently, the susceptibility of MRSA to vancomycin has decreased; thus vancomycin-resistant *S. aureus* has been reported in several countries. Furthermore, a decrease in the susceptibility of MRSA to teicoplanin has been reported in several hospitals worldwide (Alim et al., 2009). For these reasons, a search for better drugs to combat this infection is urgently needed (Totsuka, 1999).

About a decade ago, much attention was focused on the exploration and utilization of plant extracts (phytochemicals) as alternatives to or synergistic enhancers of antibiotics used to treat MRSA infection (Iinuma et al., 1994; Lee et al., 2007; Peng et al., 2010). In these respects, natural resources including many compounds can be potential candidates. Also enormous studies about natural substances are being conducting in many countries (Park, 2012). Especially, plants contain a wide variety of chemicals that have potent antimicrobial activity. Long before mankind discovered the existence of microbes, the idea that certain plants had healing potential, indeed, that they contained what it was would currently characterize as antimicrobial principles, was well accepted. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies (Rios and Recio, 2005).

In this study, it was investigated antimicrobial activity of various medicinal plants against MRSA including *Artemisia princeps* Pamp., *Teucrium veronicoides* Maxim., *Taraxacum platycarpum* H. DAHLST., *Plantago asiatica* L., leaves of *Panax ginseng*, *Pinus densiflora* Sieb. et Zucc., *Houttuynia cordata* Thunb., *Eriobotrya japonica* Lindl. leaf, and *Hovenia dulcis* Thunb.

Based on the results on anti-MRSA activities of medicinal plants, the extract of *E. japonica* leaf was selected for further study. The loquat (Rosaceae) that is a small tree native to Japan and China that is widely cultivated for its succulent fruit. Its leaves have been used as a folk medicine for treatment of chronic bronchitis, coughs, phlegm, high fever, and ulcers in Japan and other Asian countries (Perry, 1980). *E. japonica* leaf has been reported to contain various activities against diabetes, inflammatory diseases, viral diseases, and skin diseases (Kawahara et al., 2002; Qa'dana et al., 2009; Kim et al., 2011). It was also reported an antimicrobial activity of *E. japonica* leaf against pathogenic bacteria (Bae et al., 2005). However, there is no obvious report on anti-MRSA activity of the medicinal plant. Therefore, the objective of this study, it was examined the antimicrobial activity of *E. japonica* leaf against MRSA and elucidated its antibacterial mechanism against MRSA.

Materials and Methods

1. Raw materials and extraction

The fresh leaves of *E. japonica* Lindl. (Rosaceae) were purchased from Seogwipo-city (Jeju, Korea) in March 2012. Fresh *E. japonica* leaf washed thoroughly with tap water and then dried for one week at room temperature. Dried leaf 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°C until required. The powder (1.5 kg) was exhaustively extracted three times with methanol (MeOH; 10 L) at 68°C for 3 h. The combined extracts evaporated using a rotary evaporator. MeOH extract (132.3 g) was suspended in 10% MeOH (1.0 L) and then partitioned between *n*-hexane (Hexane; 1.0 L × 3) and H_2O, CH_2Cl_2 (DCM; 1.0 L × 3) and H_2O, ethyl acetate (EtOAc; 1.0 L × 3) and H_2O, *n*-butanol (BuOH; 1.0 L × 3) and H_2O, in sequence. Each extract was concentrated using a rotary evaporator (Eyela, Tokyo, Japan) under vacuum at 45°C.

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°C 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 utilized to evaluate the antibacterial activity. In briefly, bacterial strains were cultured in TSB at 37°C 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 was spread on MHA plate and a paper disc (6 mm in diameter) containing 1 mg of each extract was then placed on the agar surface. After incubating for 24 h at 37°C, 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)

MIC means 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°C 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 Sephadex^{TM} LH-20 (particle size 18 - 111 mm, Amersham Bioscience Co., Uppsala, Sweden) instruments. Thin-layer chromatography (TLC) was performed using Kieselgel 60 F_{254} plates (0.25 mm thick ; EM Science, Gibbstown, NJ) and spots were detected by UV irradiation (254 and 365 nm).

6. RNA isolation and RT-PCR analysis

In order to elucidate an inhibitory effect of EtOAc-soluble fraction of *E. japonica* leaf on expression of drug resistance related genes, MRSA cells were treated with various concentrations of EtOAc-soluble fraction (Lee et al., 2007). After cell harvesting, total RNA was obtained by zirconia beads and RNAwiz kit (Ambion, Inc., Tex, USA) according to the manufacturer's protocols. RNA concentrations were estimated by spectrophotometer at 260 nm. 0.2 to 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 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°C for 20 min. One hundred units of Superscript II reverse transcriptase were added and incubated for 50 min at 37°C. The reaction was then suspended at 70°C for 15 min. One point five 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 0.1 uL Taq DNA polymerase. Twenty-eight 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: 51.9°C) F; 5′-ATGAGATTAGGCATCGTTCC-3′, R; 5′-TGGATGACAGTACCTGAGCC-3′; *mecI* (268 bp, PCR product, annealing temperature: 49.5°C) F; 5′-CTGCAGAATGGGAAGTTATG-3′, R; 5′-ACAAGTGAATTGAAACCGCC-3′; *mecR1* (235 bp, PCR product, annealing temperature: 53.9°C) F; 5′-AAGCACCGTTACTATCTGCACA-3′, R: 5′-GAGTAAATTTTGGTCGAATGCC-3′; *femA* (372 bp, PCR product, annealing temperature: 52.6°C) F; 5′-CAT GATGGCGAGATTACAGGCC-3′, R; 5′-CGCTAAAGGTACTAACACACGG-3′; GAPDH (514 bp, PCR product, annealing temperature: 51.0°C) F; 5′-ATGACCCCTTCATTGACC-3′, R; 5′-GAAGATGGTGATGGGATTTC-3′ (Lee et al., 2007; Lei et al., 2007).

7. Western blot analysis

In order to elucidate the inhibitory effect of EtOAc-soluble fraction of *E. japonica* leaf 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 the soluble fractions. After cell harvesting, 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., 2012). 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 medicinal plants extract

This study was performed to evaluate antimicrobial activity of methanol extract from medicinal plants and their fractions on MRSA. These effects were observed with organic solvents of the plant. Nine species of natural medicinal plants were used in this experiments (Table 1). Lyophilized each powder (1.0 kg) of medicinal plants was percolated in methanol (3 times × 1.0 L). The anti-MRSA activity of methanol soluble fraction was then evaluated by measuring inhibition zones and MICs (Table 2). Among them, *E. japonica* leaf extract showed the strongest anti-MRSA activity. Recently, Park (2012) reported an anti-MRSA activity form a brown algae, *Eisenia bicyclis*. The metabolic extract of seaweed exhibited also showed strong anti-MRSA activity in the range of 10 to 13 cm in inhibition zone in the presence of 1 mg extract. The *E. japonica* leaf methanolic extract also showed similar anti-MRSA activity compared to the activity of *E. bicyclis* (Eom, 2012).

2. Anti-MRSA activity of *E. japonica* leaf extract

The methanolic extract of *E. japonica* leaf 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 *E. japonica* leaf was percolated in methanol (3 times × 1.0 L), followed by fractionation with several organic solvents to yield Hexane soluble fraction (1.68 g), DCM soluble fraction (0.82 g), EtOAc soluble fraction (1.06 g), BuOH soluble fraction (11.4 g) and H_2O soluble fraction (15.0 g) (Fig. 1). The anti-MRSA activity of Hexane, DCM, EtOAc, BuOH and H_2O soluble fractions was appraised by measuring inhibition zones. Among them, the EtOAc soluble fraction showed the strongest anti-MRSA activity and followed by Hexane, DCM and BuOH soluble fraction in the order. No anti-MRSA activity was observed in H_2O soluble fraction (Table 3). These results were consistent with the reports of that EtOAc soluble fractions of *Flos Sophora japonica* Linne (Park et al., 2009), *Paeonia japonica* (Bae, 2011), and *E. bicyclis* (Park, 2012; Eom, 2012) exhibited the strongest anti-MRSA activity.

Table 1. List of nine medicinal plants used in this study

No.	Korean name of medicinal herbs	Scientific name	No.	Korean name of medicinal herbs	Scientific name
1	약쑥 (wormwood)	*Artemisia princeps* Pamp.	6	솔잎 (pine needles)	*Pinus densiflora* Sieb. et Zucc.
2	곽향 (betony)	*Teucrium veronicoides* Maxim.	7	비파잎 (loquat)	*Eriobotrya japonica* Lindl. leaf.
3	민들레 (dandelion)	*Taraxacum platycarpum* H. DAHLST.	8	어성초 (heartleaf)	*Houttuynia cordata* Thunb.
4	질경이 (plantain)	*Plantago asiatica* L.	9	헛개 (oriental raisin)	*Hovenia dulcis* Thunb.
5	인삼잎 (ginseng leaf)	*Panax ginseng* leaf			

Table 2. Antimicrobial activities of methanol (MeOH) extract from various extracts against methicillin-resistant *Staphylococcus aureus* KCCM 40511

MeOH extraction	Inhibition zone diameter (mm)[1)]		MeOH extraction	Inhibition zone diameter (mm)[1)]	
	1 mg/disc	5 mg/disc		1 mg/disc	5 mg/disc
Artemisia princeps Pamp.	10.5	16	*Pinus densiflora Sieb.* et Zucc .	10	14.5
Teucrium veronicoides Maxim.	8	14	*Eriobotrya japonica* Lindl. leaf.	10.5	16
Taraxacum platycarpum H. DAHLST.	7	10	*Houttuynia cordata* Thunb.	8.5	14.5
Plantago asiatica L.	7	11.5	*Hovenia dulcis* Thunb.	7	13
Panax ginseng leaf	9.5	13.5			

1) Disc diameter (6 mm) was included.

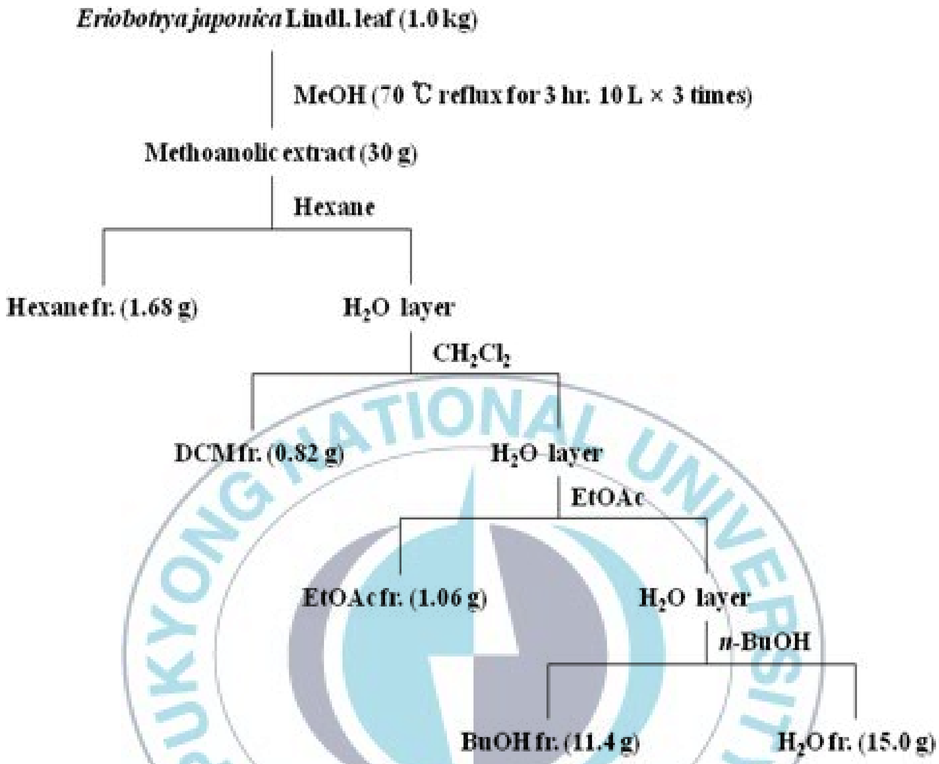

Fig. 1. Scheme of extraction and liquid-liquid solvent fractionation.

Table 3. Disk diffusion assay of the methanol extract and its fractions from *Eriobotrya japonica* Lindl. leaf 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]	9.5[c]	10.0	9.0	10.5	9.5	-[d]
	5 mg/disk	14.5	11.5	14.0	15.5	12.0	-
MRSA (KCCM 40510)	1 mg/disk	12.0	10.0	10.0	12.5	10.0	-
	5 mg/disk	16.0	18.5	19.0	20.0	15.0	-
MRSA (KCCM 40511)	1 mg/disk	10.5	9.0	9.5	11.0	10.0	-
	5 mg/disk	16.0	12.5	14.5	16.5	14.0	-

[a]Methanol extract and its fraction from *E. japonica* leaf 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 experiment
[d] - , no detected antibacterial activity.

Table 4. Minimum inhibitory concentrations (MIC) and minimum bacteriocidal concentration (MBC) of the methanol extract and its fractions from *Eriobotrya japonica* Lindl. leaf against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA)

	mecA[a]	MIC (μg/mL)					
		MeOH[b]ext.	Hexane fr.	DCM fr.	EtOAc fr.	BuOH fr.	H_2O fr.
MSSA (KCTC 1927)	-	512	512	256	64	512	>512
MRSA (KCCM 40510)	+	128	128	128	64	128	>512
MRSA (KCCM 40511)	+	128	128	64	32	128	>512

[a]+, *mecA* positive; -, *mecA* negative.

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

3. Determination of MIC of *E. japonica* leaf extract

The current study was focused on an antibacterial activity of *E. japonica* leaf extracts against MRSA. In order to quantitatively evaluate its antibacterial activity, it was investigated MIC values of the extracts against MRSA and MSSA (Table 4). The highest anti-MRSA activity was observed on 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 32 to 64 μg per mL against MSSA and MRSA strains (Table 4). Other soluble fractions (Hexane, DCM and BuOH) were in a range of 64 to 512 μg per mL for MICs. However, no antibacterial activity was observed in H_2O soluble extract (Table 4). These results strongly suggested that an anti-MRSA substance originated from the methanolic extract of *E. japonica* leaf will be abundant in the EtOAc soluble fraction.

It was perversely reported that the EtOAc extract of this plant showed the highest antibacterial activity against Gram positive bacteria, *Bacillus cereus* and *S. aureus* (Bae et al., 2005). However, to our knowledge, there is no obvious report on anti-MRSA activity of this plant. Thus, this is the first report on anti-MRSA activity of *E. japonica* leaf. The anti-MRSA activity was equal to that of the EtOAc fraction of *E. bicyclis* (Eom et al., 2011). However, the anti-MRSA activities of other soluble fractions was inferior to those of the fractions of *E. bicyclis* (Eom et al., 2011).

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). Among them, ampicillin, penicillin, and oxacillin against 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).

Among *S. aureus* strains tested in the current study, two standard MRSA strains were *mecA* positive and one standard MSSA strain was found to be *mecA*-negative (Table 4). It has been known that *mecA* is a key gene involved in β-lactam antibiotic resistance (Eom, 2012). All of the MRSA strains were highly resistant to the β-lactams, including ampicillin, penicillin and oxacillin, and evidenced MICs equal to or greater than 64 μg per mL (Eom et al., 2011). The MICs of ampicillin, penicillin, and oxacillin against standard MSSA were less than 1 μg per mL, respectively, as has been established in other studies (Eom et al., 2012). The MICs of EtOAc fraction raining in 32 to 64 μg per mL were equal to or less than those of the β-lactams tested against the MRSA strains (Table 4) (Eom et al. 2011).

4. Isolation of an active compound against MRSA from EtOAc soluble fraction

In order to study the activity in more detail, more, the methanolic extract was further fractionized using liquid-liquid solvent fractionation (Fig.1). Among soluble fractions, the EtOAc fraction showed the strongest anti-MRSA activity in tested model system. The aim of the present study was to elucidate antimicrobial mechanism of *E. japonica* leaf extract against MRSA. However, only limited information is available concerning their anti-MRSA activity. Therefore, the EtOAc fraction was subject on a column chromatography to obtain an active fraction exhibiting strong anti-MRSA activity.

The EtOAc-soluble extract (17.41 g) was chromatographed on a column (4.0 cm i.d. × 50 cm) packed with Sephadex™ LH-20. Eight sub-fractions (EF01-EF08) were obtained by MeOH elution as a mobile solvent. The yield of each sub-fractions was EF01 (5.35 g), EF02 (1.43 g), EF03 (6.41 g), EF04 (1.29 g), EF05 (0.50 g), EF06 (1.81 g), EF07 (0.25 g), and EF08 (0.34 g), respectively.

5. Minimum inhibitory concentrations (MICs) of EtOAc sub-fractions derived from EtOAc soluble fraction

In order to quantitatively evaluate anti-MRSA activity of each sub-fractions against MSSA and MRSA strains, MIC assay was performed. The MIC values of the sub-fractions were obtained by a two-fold serial dilution method (Table 5). The MICs of EtOAc sub-fractions raining in 32 to 128 μg per mL were equal to or less than those of the β-lactams tested against the MRSA strains (Eom, 2012). Among EtOAc sub-fractions, EF03 showed the highest anti-MRSA activity indicating that the sub-fraction contains an active substance against MRSA and MSSA. These results also suggest that this fraction may merit performing additional studies including mechanism assays and structure modifications.

Table 5. Minimum inhibitory concentrations (MICs) of sub-fractions derived from EtOAc soluble fraction against methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA)

Strains	MIC (μg/ml)*						
	EF01	EF02	EF03	EF04	EF05	EF06	EF07
MRSA (KCCM40510)	128	128	64	128	128	64	128
MRSA (KCCM40511)	128	128	32	128	128	64	128
MSSA (KCTC 1927)	128	128	64	128	128	128	128

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

6. Inhibitory activity of EtOAc sub-fraction of *E. japonica* leaf on the expression of genes and PBP2a related in drug resistance

The resistance of the β-lactam group of antibiotics, including ampicillin, penicillin and oxacillin is primarily mediated by the penicillin-binding protein 2a (PBP2a) encoded by the *mecA* gene (Foster, 2004; Lee et al., 2008). The mechanism underlying methicillin resistance appears to be quite complex and has yet to be thoroughly elucidated; however, it is believed to involve the overproduction of β-lactamase (Sabath, 1982) and the expression of PBP2a, which has a low affinity for β-lactam antibiotics, as well as a change in PBP type (Hiramatsu et al., 2001; Guignard et al., 2005).

The gene that encodes PBP2a, *mecA*, 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). In a previous study, factors other than the expression of the *mecA* gene were detected (Lee et al., 2007).

These factors appear to control the induction of PBP2a production in MRSA, and are collectively termed as *mecR*, which has been shown to be present in some variants of *S. aureus*. It suppresses the synthesis of PBP2a present in a certain genetic background, and mediates influences on the expression of methicillin resistance. This *mecR* has been identified as two different genes, *mecI* and *mecR1*, both of which are located within the upstream region of the *mecA* gene (Shukla et al., 2004). These genes were previously cloned and sequenced (Zhang et al., 2001), and it was also explained that *mecI* encodes for the *mecI* suppression protein. Upon contact with β-lactam, *mecR1* is activated, and its signal binds to the promoter region of *mecA* and is transduced to *mecI*, thereby suppressing transcription. In addition, reported that the *femA* gene also performs a crucial role in this process (Berger-Bachi et al., 1989).

Other study also reported that a chromosomally determined factor for methicillin resistance is *femA* gene (Vannuffel et al., 1995). The action mechanism of the *femA* gene remains to be fully elucidated at present; nevertheless, it is believed that it plays not part in PBP 2a synthesis. Rather, it appears to be involved in the synthesis of cell wall components of *S. aureus*, and also mediates drug sensitivity, and thus in involved in methicillin resistance (Meng et al., 2009). The *femA* gene is 48 kDa proteins (Kobayshi et al., 1994). 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 was employed in my comparison of resistant gene expression patterns. The *S. aureus* micrococcal *nucA* is a small (~18 kDa), stable, extracellular enzyme, which is secreted as a target protein in *S. aureus* (Dutton et al., 2000; Myscofski et al., 2001). Also, GAPDH gene, which is a housekeeping gene in all eubacteria, was used as a control (Lee et al., 2007).

The results of synergistic effect between phlorofucofuroeckol-A (PFF) and β-lactams suggested that β-lactams can restore the inhibitory activity to cell wall synthesis of MRSA cell in the combination with anti-MRSA substance (Eom, 2012). Also, it was reported PFF inhibits expression of *mecA* and PBP2a in MRSA cells.

Considering these results, it was supposed that anti-MRSA activity of EtOAc fraction of *E. japonica* leaf might be related directly or indirectly to expression of *mecA* and PBP2a. In order to verify this hypothesis, an inhibitory effect of sub-fraction EF03 on the expression of genes (*mecA*, *mecI*, *mecR1* and *femA*) and the production of PBP2a related in β-lactams resistance was investigated.

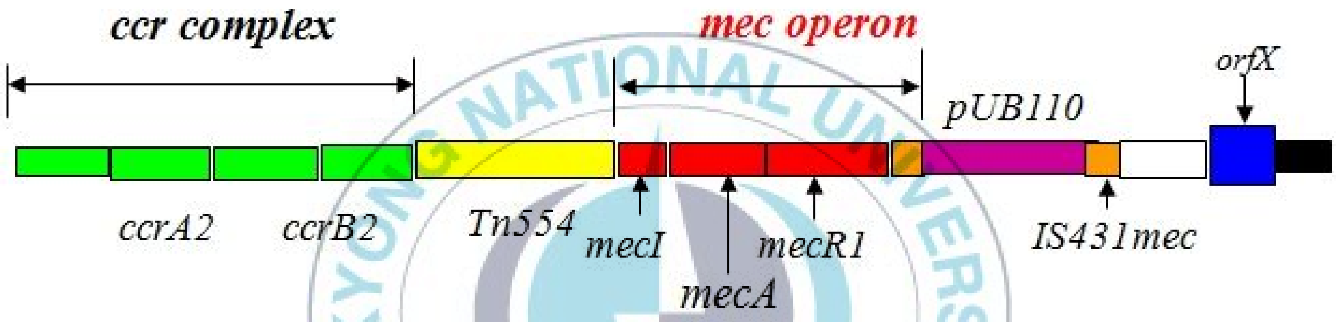

Fig. 2. *mec* operon of methicillin-resistant *Staphylococcus aureus* (MRSA).

All mRNA of *mecA*, *mecI*, *mecR1*, and *femA*, *nucA*, and GAPDH genes in MRSA KCCM 40511 strain were clearly detected by a reverse transcriptase-polymerase chain reaction (RT-PCR). However, *mecR1* gene was not unable to confirm in MRSA KCCM 40510 strain (data not shown). It has been previously reported that some clinical isolates of MRSA contain deletions of *mecI* and partial deletion of *mecR1* (Shukla et al., 2004). From this report, it was supposed two possibilities; the gene(s) is deleted full or partial in SCC*mec* of KCCM 40510 strain, and / or the primer(s) used for RT-PCR in this study was not proper due to its mutation caused by the treatment of EF03 sub-fraction (Eom, 2012).

As shown in Fig. 3, the mRNA expression of *mecA*, *femA*, and *mecR1* gene were inhibited in a dose-dependent manner by EF03 sub-fraction. However, the expression of *mecI*, *nucA* and GAPDH was not affected by the treatment of EF03 at the concentration of 16 μg per mL. Thus, the results obtained from Fig. 3 indicated that EF03 fraction of *E. japonica* leaf can selectively inhibit the mRNA expression of *mec* operon genes and eventually led to the reduction of resistance on MRSA. In general, mRNA expression of target gene would not directly correlate with its protein production (Eom, 2012). Therefore, it needs to investigate the production of PBP2a encoded by *mecA*.

The results of Western blotting assay indicated that the EF03 sub-fraction attenuated the production level of PBP2a in a dose-dependent manner (Fig. 4). Considering the results obtained from mRNA expression and Western blotting assay, it was concluded that the EF03 sub-fraction 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, 2012). In the study, the EF03 sub-fraction from *E. japonica* leaf is expected to be recognized as a natural source for the development of an alternative therapeutic method against MRSA. Also, with more detailed research into the isolation, identification, action mechanisms, and toxicity of active compounds, *E. japonica* leaf extract is expected to be recognized as natural sources for the development of new functional foods, and will also comprise a component of the fight against MRSA.

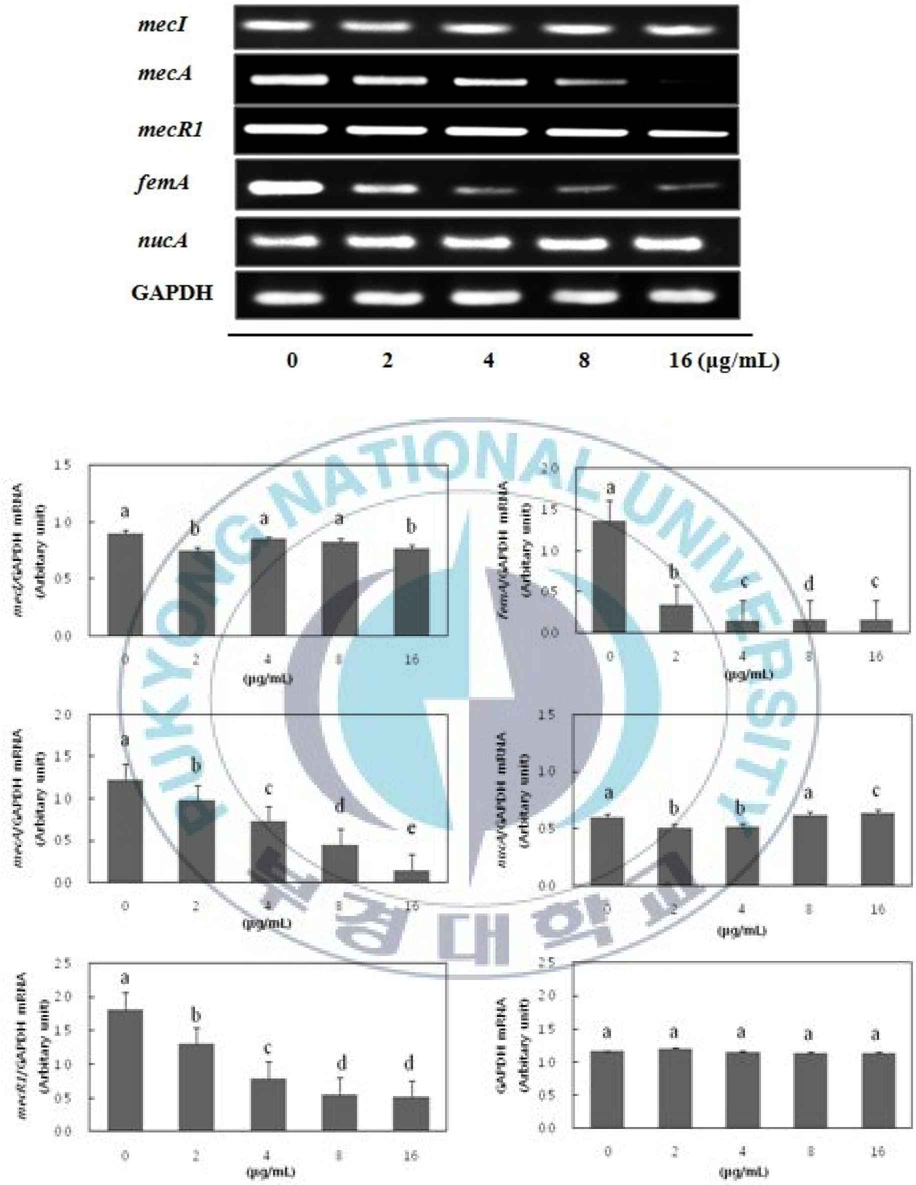

Fig. 3. Effect of ethyl acetate sub-fraction (EF03) of *Eriobotrya japonica* Lindl. leaf on the mRNA expression of *mecA*, *mecI*, *mecR1*, *femA*, and *nucA* genes. Methicillin-resistant *Staphylococcus aureus* (MRSA) KCCM 40511 strain was treated with the indicated concentrations of EF03 fraction.

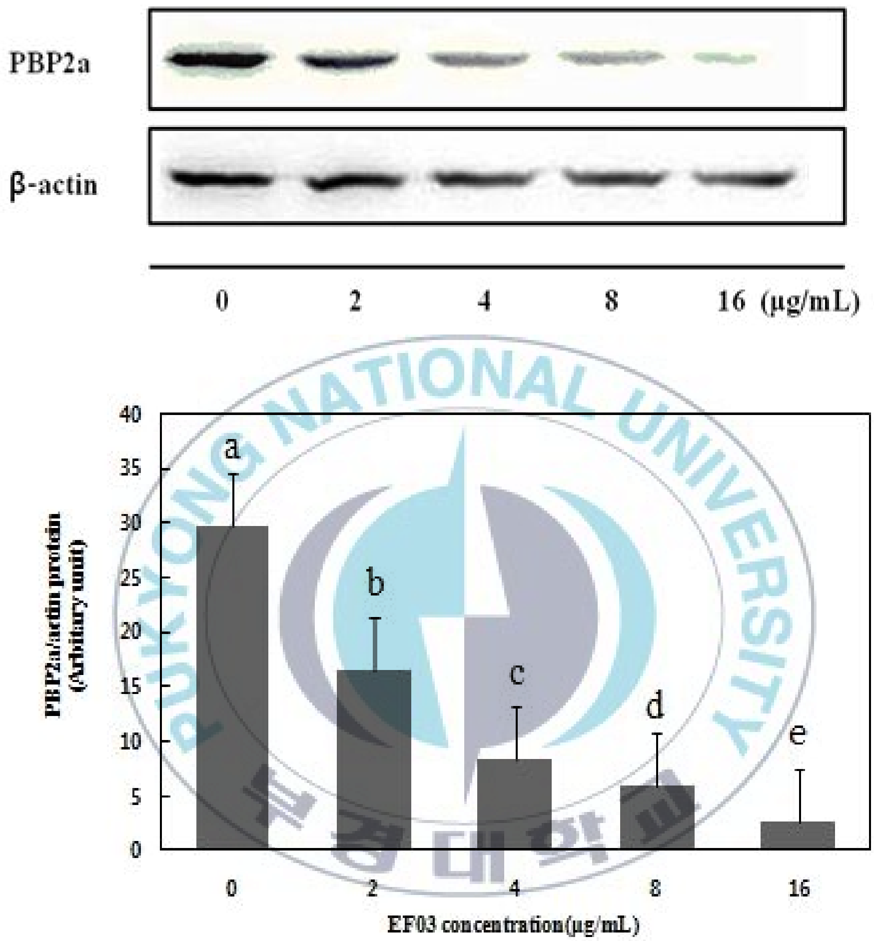

Fig. 4. Effect of ethyl acetate sub-fraction (EF03) of *Eriobotrya japonica* Lindl. leaf on the expression of PBP2a protein against methicillin-resistant *Staphylococcus aureus* (MRSA) strain. MRSA KCCM 40511 strain was treated with the indicated concentrations of EF03 fraction.

Summary

In an effort to discover an alternative antibiotic against MRSA, natural resources provide a rich source of chemical diversity that can be used to develop new, potential and useful therapeutic agents. Among natural resoureces, *E. japonica* leaf exhibits activities against diabetes, inflammatory diseases, viral diseases, and skin diseases. However, study of the antimicrobial acitivity of MRSA from *E. japonica* leaf has not reported yet. Therefore, the objectives of this study are to investigate an anti-MRSA activity of *E. japonica* leaf and to elucidate the its anti-MRSA mechanism.

In this study, the highest anti-MRSA activity was observed from EtOAc sub-fraction 03 (EF03) of *E. japonica* leaf methanolic extract. The MIC values of EF03 are ranging in 32 to 64 μg per mL against MSSA and MRSA. Furthermore, the mRNA expression of the *mec* operon genes was selectively inhibited in a dose-dependent manner by EF03 fraction. The EF03 fractions inhibited the expression of the resistant genes *mecA*, *mecR1*, and *femA* in a dose-dependent manner. Moreover, the results of Western blotting assays indicated that the EF03 fractions inhibited the expression levels of the resistant protein, PBP2a in a dose-dependent manner. As far as could be ascertained in my literature survey, the active compounds implicated herein have proven effective in other studies. However, the majority of the reports published has addressed the antimicrobial activity of these compounds

only against MRSA, and did not address the capacity of the extract and fractions to regulate resistance properties.

In conclusion, it was determined herein that the EtOAc soluble fraction of *E. japonica* leaf evidenced profound antimicrobial activity, and inhibited resistant gene expression against MSSA and MRSA. Therefore, this study expects to contribute to the development of an alternative phytotherapeutic agent against MRSA, and in applications of the treatment of MRSA infection. However, in needed more detailed research into the identification, action mechanisms, and toxicity of active compounds of the anti-MRSA activity.

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본 논문이 완성되기 까지 부족한 저를 격려해주시고 학문과 인생의 길에서 뒤처지지 않게 이끌어주셨던 김영목 교수님, 진심으로 감사드립니다. 항상 교수님의 가르침을 잊지 않고 살아가겠습니다. 또한 부족한 논문이지만 자상하게 심사해주시고 유익한 지도 편달을 해주셨던 김선봉 교수님, 양지영 교수님, 석사 수업시간에 많은 가르침을 주셨던 조영제 교수님, 전병수 교수님, 안동현 교수님, 이양봉 교수님께도 감사하단 말씀을 전하고자 합니다.

본 논문의 완성은 주위에 계신 많은 분들의 도움이 있었기에 가능한 일이었다고 생각합니다. 특히 학문적인 시야를 넓혀주셨던 수과원에 이희정 연구관님, 박큰바위 연구사님, 조미라 연구사님, 유전자 실험을 할 수 있게 자리를 마련해주셨던 김현우 교수님, 단시간에 많은 내용을 습득 할 수 있도록 꼼꼼하게 지도해 주셨던 전정민 박사님, 보광선배, 부족한 저에게 실험적으로 많은 도움을 주고 가르쳐주셨던 성환선배, 물어보면 귀찮아하지 않고 항상 친절하게 가르쳐주었던 한성선배, 석사 하는 동안 부족한 저를 많이 챙겨주셨던 재홍선배, 대웅선배, 현철선배 2년이란 시간을 함께 지내며 동고동락했던 지일언니, 저에게 부산의 따뜻함을 알게 해준 연주언니, 민아언니, 내 친구 최은혜, 현지, 진심으로 감사의 마음을 전합니다. 실험실에서 실험적으로나 그 밖에 여러 부분을 도와주었던 실험실 방장 홍엽이, 가끔은 이해할 수 없는 말로 웃겨주었던 혜림이, 베이비 페이스지만 마음은 어른스러운 송이, 어른스럽지만 웃길 땐

웃길 줄 아는 대규, 학부생 막내 장원이, 석사 막내 은혜, 카리스마와 부드러움을 갖춘 실장님 연중선배, 정 많이 들었지만 영국으로 공부 간다는 개미 기언이, 같이 있었던 시간은 짧았지만 정들었던 지윤이, 지훈선배, 덕훈이, 호준선배, 승용선배, 선영언니, 형훈선배, 신국이, 부산에 와서 유일하게 마음 터놓고 이야기 할 수 있는 친구 동륜이 정말 감사합니다. 광주에서 석사를 하며 석사의 고충을 누구보다 잘 이해해주는 친구 소피 혜정이, 나의 영원한 지기 미골이 성숙이, 고등학교 때부터 지금까지 옆에서 응원해 주던 건빵 은진이, 특수교육과의 미모 3인방 재연이, 엄양 현숙이, 지영이, 대학교 때부터 힘이 되어준 친구 친절한지현씨, 사기꾼 동아리의 귀염둥이 오빠 재성씨, 효댕오빠, 승기오빠, 경훈오빠, 개구쟁이 동생들 범이, 세준이, 능력 있는 내 친구 지련이, 빈자리를 채워주던 수현이, 모두 감사드립니다. 작은 공간에 다 열거하지 못한 그 밖의 소중한 인연을 맺은 분들 모두에게 고마움을 전합니다. 그리고 부족한 누나지만 항상 자랑스러워 해주고 아껴주고 챙겨주었던 우리 집의 복덩이 준희 누나가 너무너무 사랑한다. 하나 밖에 없는 딸이지만 효도도 제대로 못하고 아직 어리게만 보이는 큰 딸을 항상 믿어주고 든든한 지원군이 되어 어렵고 힘든 상황을 버틸 수 있게 힘이 되어준 사랑하는 우리 엄마 아빠 항상 존경하고 자랑스럽습니다.

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