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Antibacterial activity of *Eriobotrya japonica* leaf extracts against methicillin-resistant *Staphylococcus aureus*

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Abstract

Aim : To investigate antimethicillin-resistant *Staphylococcus aureus* (MRSA) activity of *E. japonica* leaf and to elucidate its anti-MRSA mechanism.

Methodology : Methanolic extract of dried *E. japonica* leaf was partitioned in *n*-hexane, dichloromethane, ethyl acetate (EtOAc) and *n*-butyl alcohol. Antibacterial activity of extracts was determined by disk diffusion assay and minimum inhibitory concentration. Total RNA isolation was performed by treating MRSA culture with EtOAc sub-fractions. All MRSA isolates were tested for the presence of genes by reverse transcription polymerase chain reaction, followed by Western Blot.

Results : The highest MRSA activity was observed from EtOAc sub-fraction 03 of methanolic extract. The minimum inhibitory concentration values of EF03 ranged from 32 to 64 µg ml⁻¹ against methicillin-susceptible and -resistant *S. aureus*. EF03 fractions inhibited the expression of resistant genes in a dose-dependent manner. Moreover, the results of Western Blot assay indicated that the EF03 fractions inhibited the expression levels of resistant protein, PBP2a in a dose-dependent manner.

Interpretation : EtOAc soluble fraction of *E. japonica* leaf evidenced profound antimicrobial activity, and inhibited expression of resistant genes against MRSA.

Key words: Antibacterial activity, *Eriobotrya japonica*, MRSA activity, Synergistic effect

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Introduction

The widespread of antimicrobial-resistant (AMR) bacteria is undoubtedly recognized as one of the world's major health crisis in the past two decades (Kamenshchikova *et al.*, 2018). As indicated in the Review on Antimicrobial Resistance (2016) by the UK government, up to 700,000 deaths and illnesses caused by AMR have been recorded annually. Regardless of geographical locations and ages, placing devastating burdens on health-care systems, economy and social community worldwide. Despite extensive explorations of new antimicrobials or antibiotics over the last 50 years, the situation yet remains uncontrollable as the extreme complications (associated with antibiotic resistance) in genetics and ecology derived during exposure to antibiotics enable AMR bacteria to constantly emerge antimicrobial resistant genes or genetic mutation and transfer to another species or next generation to survive the antibiotics pressure and further transfer the antimicrobial resistant genes to other bacteria or to the next generations. Previously established AMR bacteria from individual hospitals and health-care facilities, have widely invaded the community rapidly posing catastrophic threat to the public health (Abreu *et al.*, 2017; Kamenshchikova *et al.*, 2018). One of such threatening AMR bacteria are known as methicillin-resistant *Staphylococcus aureus* (MRSA).

Gram-positive *Staphylococcus aureus* is a common human opportunistic pathogen present in one-third of healthy population and numerous diseases ranging from skin and soft tissue infections, invasive infections (bacteremia and pneumonia) to toxic-shock syndrome and food poisoning (Tong *et al.*, 2015). Back in 1950s after the bacteria had developed resistance against penicillin by self-production of penicillin-inactivating enzyme (penicillinase), the bacteria has continuously developed resistance to confront a number of antibiotics, including the semisynthetic penicillin namely MRSA. Since a healthcare-associated strain was clinically introduced to UK in 1959, MRSA has rapidly evolved to community-associated and livestock-associated forms and has spread globally. Resistance was found to root from bacterial capability to suppress the antibiotic function using altered transpeptidase named penicillin-binding protein 2a (PBP2a) encoded by *mecA* or *mecC* via homologous transformation (Boswihi and Udo, 2018; Udo, 2013).

A significant number of approaches have been developed with the aim of conferring the widespread occurrence of MRSA, including discovering new β -lactam antibiotics (ceftaroline and ceftobiprole), shifting to non- β lactam antibiotics (vancomycin, daptomycin and linezolid), and promoting infection control programs (screening, hand hygiene and decolonisation therapy) (Alm *et al.*, 2014; Roca *et al.*, 2015; Weigel *et al.*, 2003). Despite achieving local effectiveness, a vast majority of current approaches are predicted to fail against the threat in the long run due to unpredictably complicated remodeling of MRSA pathogenicity and epidemiology. Due to incapability of

commercial antibiotics for MRSA, the shift to natural sources of antimicrobials appears to be interesting. With recent advances in extraction and separation technology, research has discovered remarkable antimicrobial potential of plant extracts, which are highly concentrated with various secondary metabolites (phytochemicals), typically flavonoids, alkaloids, phenolics, terpenes and tannins (Shin *et al.*, 2018). In the case of MRSA, in particular, extracts of *Camellia sinensis* (Cho *et al.*, 2008; Aboulmagd *et al.*, 2011), *Berberis micophylla* (Manosalva *et al.*, 2016) and *Bauhinia kockiana* (Chew *et al.*, 2018) have shown significant anti-MRSA activities either as a single natural antimicrobial or as an antibiotic enhancer (Savoia, 2012). However, in order to prevent MRSA resistance development over time, it is recommended to constantly diversify the antimicrobial phytochemicals as well as their extract sources.

E. japonica (Rosaceae) commonly known as loquat, is a subtropical fruit tree originating from south-eastern China and is now cultivated in various countries worldwide, including Japan, India and Turkey. Currently, there are two *E. japonica* cultivars, red-colored and white-colored fruits with distinct nutritional content and values (Liu *et al.*, 2016). Leaves of *E. japonica* are generally utilized in liquid extract form. Discovered hundreds of years ago, *E. japonica* leaf extract has been recognized for its medicinal values as an effective treatment for chronic bronchitis, coughs, fever and skin inflammation (Baljinder *et al.*, 2010). Besides anti-inflammatory property, recent pharmacological studies have further discovered anticancer, antidiabetic, antibacterial activities and liver function improvement of the leaf extract due to the presence of flavonoids, polyphenols and terpenoids (Ito *et al.*, 2000; Liu *et al.*, 2016; Gordon *et al.*, 2010). However, the inhibitory activity of this extract against MRSA has yet remained unclear. Therefore, the objective of the present study was to examine the anti-MRSA activity of *E. japonica* leaf extract, its derived fractions and sub-fractions using bacterial susceptibility testing (disc diffusion and minimum inhibitory concentration determination). Further, the expression as well as PBP2a production was also determined.

Materials and Methods

Plant materials: Fresh leaves of *E. japonica* were directly purchased from a farm, which has been registered as producing organic *E. japonica* by the Ministry of Agriculture, Food and Rural Affairs, Korea. The plant samples were washed thoroughly with tap water and allowed to dry at room temperature for one week. Dried leaves were ground to powder and stored in freezer at -20°C before analysis.

Preparation of plant extracts: One kg of dried leaf powder was extracted three-time repeatedly with 10 l of methanol at 70°C for 3 hrs. The obtained extract was evaporated using a rotary evaporator and resuspended in 1.0 l of 10% methanol. The extract was then partitioned in sequence with 3 × 1.0 l of following solvents: *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and *n*-butyl alcohol (*n*-BuOH). All resulting fractions (*n*-

hexane, DCM, EtOAc, *n*-BuOH and H₂O) were combined and concentrated using a rotary evaporator for subsequent antibacterial activity tests.

Bacterial strains and culture conditions: Two MRSA standard bacterial strains KCCM 40510 and KCCM 40511 and one methicillin-susceptible *S. aureus* (MSSA) standard bacterial strain KCTC 1927 were obtained from Korean Collection of Type Cultures (KCTC) (Daejeon, Korea) and were inoculated and enriched aerobically at 37°C in Tryptic Soy Broth (TSB; Difco, Detroit, USA). Muller Hinton agar (MHA; Difco, Detroit, USA) and Muller Hinton broth (MHB; Difco, Detroit, USA) were used as media for antibacterial susceptibility testing.

Antibacterial susceptibility testing: The *in-vitro* antibacterial activity of *E. japonica* leaf extract was determined by disk diffusion assay and broth microdilution methods described in the Guidelines of Clinical Laboratory Standards Institute (CLSI, 2009). For disk diffusion assay, 0.1 ml of standardized bacterial density of 10⁶ colony forming (0.5 on the McFarland scale, CFU ml⁻¹) were spread on MHA plates using a sterilized cotton swab. Sterile paper disks (6 mm in diameter; Oxoid) loaded with 10 mg and 50 mg of crude methanolic extract and its fractions (*n*-hexane, DCM, EtOAc, *n*-BuOH) were placed onto the agar surface. Blank disks loaded with 10% DMSO were used as negative control. The plates were then incubated at 37°C for 24 hr. After incubation, diameter of clear inhibition zone (mm) of tested bacteria was measured to evaluate the antibacterial activity. All tests were performed in triplicate.

For broth microdilution, 20 µl suspension of *S. aureus* MRSA and *S. aureus* MSSA strains with cell density adjusted approximately to 10⁶ CFU ml⁻¹ were added to 80 µl of Muller Hinton broth contained in a sterile 96-well microtiter plate. The methanolic crude leaf extract and its soluble fractions (*n*-hexane, DCM, EtOAc and *n*-BuOH) at 10 mg ml⁻¹ and H₂O were serially two-fold diluted to obtain the following concentrations (µg ml⁻¹): 512; 256; 128; 64 and 32. Negative control wells contained bacterial suspension and DMSO while positive control wells contained extract and fractions and DMSO. Minimum inhibitory concentration test (MIC) is defined as the lowest concentration (µg ml⁻¹) of an antimicrobial agent that inhibits visible growth of microorganisms after 24 hr of incubation at 37°C in broth. Bacterial growth in microdilution wells were detected directly by unaided eye. The assay was performed in triplicate for each solvent extract against all bacterial strains. MIC values were determined as a mean value of each assay.

Isolation and purification of anti-MRSA substance and MIC measurement: As EtOAc soluble fraction of methanolic *E. japonica* leaf extract was revealed by previous susceptibility tests to possess the strongest anti-MRSA activity. This fraction was subjected to isolation and purification for its bioactive substance using LiChroprep RP-18 (Merck silica gel, Darmstadt, Germany) and Sephadex LH-20 column (4.0 x 50 cm) chromatographic instrument (Amersham Bioscience Co., Uppsala, Sweden).

Methanol was used as mobile phase with ratio to water of 1:1, 1:2, 1:3; 1:4 and 1:5 and the spots were visualized under visible and UV light at 254 and 365 nm, respectively. The MIC value of individual sub-fraction was measured by broth micro-dilution, following the methodology mentioned earlier (Gupta *et al.*, 2018).

RNA isolation, cDNA synthesis and RT-PCR analysis: Isolation of total RNA was performed firstly by treating MRSA KCCM 40511 cell culture with EtOAc sub-fractions at 2, 4, 8 and 16 µg ml⁻¹. Culture without EtOAc treatment served as negative control. Total RNA was then extracted and purified using Zirconia Beads and RNeasy kit (Ambion Inc., Austin, USA) following manufacturer's instructions. Concentration of total RNA was quantified by measuring absorbance at 260 nm (A₂₆₀) on a Biochrom Libra S22 spectrophotometer (Biochrom Ltd., UK) and checked for contamination. A portion of 0.2-1.4 µg of isolated RNA was reverse transcribed to complementary DNA (cDNA) via reaction with random primers (1.4 µg) catalyzed by 100 units of SuperScript II reverse transcriptase (Life Technologies Inc., Gaithersburg, USA) as described in manufacturer's protocol. The remaining isolated RNA was removed by RNase H at 37°C for 20 min. The reaction was terminated by heating the reaction mixture at 70°C for 15 min.

All MRSA isolates were tested for the presence of *mecA*, *mecI*, *mecR1* and *femA* genes by RT-PCR using 1.5% of resulted cDNA from each bacterial strain and gene-specific pairs of forward and reverse primers as previously reported (Weller, 1999). The primer sequences are listed in Table 1. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control for normalization of mRNA levels of the target genes. The cycling conditions for total 28 PCR cycles were maintained as follow: denaturation at 94°C for 30 sec, annealing at specific temperatures of each primer for 30 sec and extension at 72°C for 30 sec. Gel electrophoresis of amplified PCR product was performed on 1.5 % agarose gel in Tris-borate-EDTA buffer. The resulting bands were stained with ethidium bromide for 20 min and visualized under Transluminator BioView UV light. All PCR reactions were replicated three times.

Western Blot analysis: After *S. aureus* culture was inoculated into fresh TSB (1:100 dilution) and incubated for 24 hr at 37°C, culture was subjected to centrifugation (10,000 x g; 4°C; 1 min). The resultant *S. aureus* cell pellets were suspended in a lysis buffer (20 mM Tris-HCl, 2 mM ethylene diamine tetra acetic acid (EDTA), 2 mM ethylene glycol tetra acetic acid (EGTA) and 0.25 M sucrose, pH 7.5) and sonicated at 95 µA for two times, 20 sec time⁻¹ with an ultrasonicator (Ultrasonic Ltd., Hants, UK) prior to centrifugation at 13,000 x g in 10 min. The protein concentration of the supernatant was estimated by Bradford method (Bradford, 1976). Protein aliquots (10 µl per lane⁻¹) were electrophoresed in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and boiled for 3 min. The aliquots were transferred from the gel to a Immobilon-P membranes (Millipore, USA) using a wet transfer method. The membrane was probed with anti-PBP2a (Abcam, UK), washed and re-probed with anti-mouse immunoglobulin G

(IgG) at room temperature. MRSA 40511 strains were incubated with EF03 concentrations of 0, 2, 4, 8 and 16 $\mu\text{g ml}^{-1}$ and the difference in PBP2a quantity was indicated by band intensity. Polyclonal. Polyclonal anti-actin antibody was used to normalize the loading difference (Lee et al., 2007).

All analyses were performed in triplicate. Averaged values and standard deviations were calculated and used. Two-way analysis of variance was applied for multiple comparisons using SPSS ver. 12.0 statistical software (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was applied to determine significant differences between means.

Results and Discussion

Liquid-liquid solvent fractionation of crude *E. japonica* methanolic leaf extracts (30 g) yielded six different soluble fractions, including *n*-hexane (1.68 g), DCM (0.82 g), EtOAc (1.06 g), BuOH (11.4 g) and H₂O (15.0 g). The antibacterial activities of resulting fractions were determined by measuring the diameter of their inhibition zones and data (Table 2). Overall, on increasing the concentration from 1 mg ml⁻¹ to 5 mg ml⁻¹ significantly increased the size of inhibition zone ranging from 9.5-12.5 mm to 14.5-20 mm ($P < 0.05$). This indicated enhanced extract activity against all MRSA and MSSA strains

targeted in this study. Among the soluble extracts obtained from solvent fractionation of *E. japonica* leaf extracts, EtOAc soluble extracts showed the highest anti-MRSA activity with maximum inhibition diameter up to 20 mm, followed by DCM, hexane and two alcoholic-soluble extracts. No MRSA and MSSA inhibitory activity was observed in water-soluble extract. Initial quantitative assessment by disk diffusion was subsequently followed by MIC determination obtained from serial two-fold broth dilution as described by the National Committee for Clinical Laboratory Standards (NCCLS, 1997) in order to further evaluate quantitatively the antimicrobial activity of *E. japonica* leaf extracts. As shown in Table 3, MIC values varied with each sample, of which the highest ranged from 128 to 512 $\mu\text{g ml}^{-1}$ for *n*-hexane, MeOH and BuOH fractions, followed by 64 to 256 $\mu\text{g ml}^{-1}$ for DCM fraction and the lowest ranged from 32 to 64 $\mu\text{g ml}^{-1}$ for EtOAc fraction.

Overall, considering the expanded inhibition zone and the lowest MIC value (32 $\mu\text{g ml}^{-1}$ to 64 $\mu\text{g ml}^{-1}$) obtained from disk diffusion and two-fold serial microdilution, it can be concluded that EtOAc fraction derived from *E. japonica* leaf extract showed the most effective anti-MRSA activity. Similar results were obtained from previous studies, where EtOAc fraction yielded from ethanolic extract of cinnamon bark (Julianti et al., 2017) and methanolic extract of Menthapulegium leaf

Table 1: Primer sequence of genes used for RT-PCR analysis

Gene	Size (bp)	Annealing temperature (°C)	Sequence	
			Forward primer	Reverse primer
<i>mecA</i>	554	51.9	ATGAGATTAGGCATCGTTCC	TGGATGACAGTACCTGAGCC
<i>mecI</i>	268	49.5	CTGCAGAAATGGGAAGTTATG	ACAAGTGAATTGAAACCGCC
<i>mecR1</i>	235	53.9	AAGCACCGTTACTATCTGCACA	GAGTAAATTTTGGTCGAATGCC
<i>femA</i>	372	52.6	CAT GATGGCGAGATTACAGGCC	CGCTAAAGGTACTAACACACGG
<i>nucA</i>	130	52.7	GCGATTGATGGTGATACGGTT	AGCCAAGCCTTGACGAACCTAAAGC
GAPDH	514	51.0	ATGACCCCTTCATTGACC	GAAGATGGTGATGGGATTTC

Table 2: Disk diffusion assay of methanol extract and its fractions from *Eriobotrya japonica* leaf against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA)

Gram-positive bacteria	Concn.	Zone of inhibition (mm) ^a					
		MeO Hext. ^b	<i>n</i> -Hexane fr.	DCM fr.	EtOAc fr.	BuOH fr.	H ₂ O fr.
MSSA (KCTC 1927)	1 mg disk ⁻¹	9.5 ^c	10.0	9.0	10.5	9.5	- ^d
	5 mg disk ⁻¹	14.5	11.5	14.0	15.5	12.0	-
MRSA (KCCM 40510)	1 mg disk ⁻¹	12.0	10.0	10.0	12.5	10.0	-
	5 mg disk ⁻¹	16.0	18.5	19.0	20.0	15.0	-
MRSA (KCCM 40511)	1 mg disk ⁻¹	10.5	9.0	9.5	11.0	10.0	-
	5 mg disk ⁻¹	16.0	12.5	14.5	16.5	14.0	-

^aMethanol extract and its fraction from *E. japonica* leaf was loaded onto a disk (6 mm in diameter); ^bMeOH ext., methanolic extract; DCM fr., dichloromethane fraction.; EtOAc fr., ethyl acetate fraction; BuOH fr., butanol fraction; H₂O fr., water fraction; ^cData are the averages of duplicate experiment; ^dno detected antibacterial activity

(Ceyhan-Guvensen and Keskin, 2016) also showed the highest bactericidal activity against MRSA. In addition, Eom *et al.* (2013) reported that an EtOAc extract containing abundant phenolic compounds exhibited strong antibacterial activities against MSSA and MRSA. Thus, these results suggested that EtOAc extract of *E. japonica* possess high content of phenolic compounds.

Column chromatographic purification of *E. japonica* leaf EtOAc soluble fraction (17.41 g) yielded eight sub-fractions, designated as 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). The MICs of each sub-fraction against *S. aureus* strains were also determined using two-fold serial broth microdilution method and the results are presented in Table 4. The MIC values of EtOAc sub-fractions did not very highly, however, most sub-fractions showed MIC value of 128 $\mu\text{g ml}^{-1}$. Only sub-fraction EF03 yielded significantly low MIC of 64 $\mu\text{g ml}^{-1}$ with MRSA KCCM 40510 and MSSA KCTC 1927 strains and 32 $\mu\text{g ml}^{-1}$ with MRSA KCCM 40511. This finding sets a platform for further phytochemical analysis to identify the profile of bioactive compounds present in EF03 sub-fraction from methanolic *E. japonica* leaf extract that pose antimicrobial activity towards MRSA and MSSA.

Intrinsic resistance to β -lactam group of antibiotics such as ampicillin, penicillin and oxacillin is primarily mediated by PBP2a encoded by *mecA* gene. Particularly, the targeted *S. aureus* strains in the present study ranged from susceptible (MSSA-KCTC 1927) to resistant (MRSA-KCCM 40510 and

KCCM 40511) to methicillin corresponding to the presence of *mecA*. Acquired horizontally from an unknown bacterium donor, *mecA* is located on a 21 - 67 kb novel mobile DNA element termed *Staphylococcus* chromosome cassette *mec* (SCCmec) and its upstream transcriptional regulatory system named *mecR1-mecI*. *mecR1* encodes for an extracellular signal sensor-transducer which trigger transcription of *mecA*, while *mecI* encodes for a repressor of this transcription. Upon sensing signals from β -lactam antibiotics, *mecR1*-encoded sensors undergo conformational change that activates an intracellular metalloprotease domain to cleave the *mecI*-encoded repressor from *mecR1-mecI* operator, enabling *mecA* transcription, and thus resulting in PBP2a production that confers antibiotics resistance (Cha *et al.*, 2007). In addition to down-regulated *mecA* transcription by *mecI-mecR1* system, *S. aureus* resistance was found to be optimized by a group of over 20 housekeeping genes named *fem* (factors essential for methicillin resistance), which are localized in all wild-type *S. aureus* genome and are primarily involved in peptidoglycan cell wall synthesis. In particular, *femA*, along with *femB* and *femH*, is considered most crucial as it synthesizes pentaglycine side chain-a unique peptide structure of *S. aureus* peptidoglycan cell wall that actively supports PBP2a resistant to a wide variety of antibiotics (Bachi and Rohrer, 2002; Li *et al.*, 2008). The present study also includes the gene encoded for an extracellular 18kDa-monomeric enzyme called *Staphylococcus* nuclease A (*nucA*) which is known to commonly express in Gram-positive bacteria such as *Staphylococcus gordonii*, *Bacillus subtilis* and *Lactococcus lactics* (Dutton *et al.*, 2000; Kovacevic *et al.*, 1985; Poquet, 1998). The enzyme is not

Table 3: Minimum inhibitory concentration (MIC) of methanol extract and its fractions from *Eriobotrya japonica* leaf against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA)

	<i>mecA</i> ^a	MIC ($\mu\text{g ml}^{-1}$)					
		MeOH ^b ext.	n-Hexane fr.	DCM fr.	EtOAc fr.	BuOH fr.	H ₂ O 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; ^bMeOH ext.: methanolic extract; DCM fr.: dichloromethane fraction; EtOAc fr.: ethyl acetate fraction; BuOH fr.: butanol fraction; H₂O fr.: water fraction

Table 4: Minimum inhibitory concentrations (MICs) of sub-fractions derived from EtOAc soluble fraction against methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA)

Strains	MIC ($\mu\text{g ml}^{-1}$)*						
	EF01	EF02	EF03	EF04	EF05	EF06	EF07
MSSA(KCTC 1927)	128	128	64	128	128	128	128
MRSA (KCCM40510)	128	128	64	128	128	64	128
MRSA (KCCM40511)	128	128	32	128	128	64	128

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

Fig. 1: Effect of ethyl acetate (EtOAc) sub-fraction (EF03) of *Eriobotrya japonica* leaf on mRNA expression of *mecA*, *mecl*, *mecR1*, *femA*, and *nucA* genes. Methicillin-resistant *Staphylococcus aureus* (MRSA) KCCM 40511 strain was treated with the indicated concentrations of EF03 fraction.

Fig. 2: Effect of ethyl acetate (EtOAc) sub-fraction (EF03) of *Eriobotrya japonica* leaf on the expression of PBP2a protein against methicillin-resistant *Staphylococcus aureus* (MRSA) strain. The MRSA KCCM 40511 strain was treated with the indicated concentrations of EF03 fraction.

involved in methicillin resistance; instead, it is important for the bacterial surface protein expression system (SPEX), along with other antigens, growth factors or cytokines (Dutton *et al.*, 2000).

Overall, due to their essential roles in MSSA and MRSA, it is therefore important to investigate the expression levels of *mecA*, *mecI*, *mecR1*, *femA* and *nucA* using RT-PCR as well as to study the suppression effect of EF03 sub-fraction on expression of these methicillin resistance determinants. Results from RT-PCR analysis revealed that two MRSA strains were *mecA* positive, by which both MRSA strains yielded about 550bp band. Other methicillin resistance genes were found in MRSA KCCM 40511, though *mecR1* gene-encoded product were unexpectedly undetectable in MRSA KCCM 40510. Similar results were reported by Lee *et al.* (2007), where *mecR1* of MRSA KCCM 40510 also remained undetectable. The possible explanation is that the gene has either been completely deleted in SCCmec of KCCM 40510 strain or due to impaired function of primer used in RT-PCR due to mutations resulting from the treatment of EF03 sub-fraction (data not shown).

As shown in Fig.1, mRNA expression of *mecA*, *femA* and *mecR1* gene were inhibited in a dose-dependent manner by EF03 sub-fraction; whereas expression of *mecI*, *nucA* and GAPDH appeared to be unaffected. Thus, it can be concluded that EF03 sub-fraction of *E. japonica* leaf methanolic extract is capable of selectively inhibiting the mRNA expression of *mec* operon genes and eventually resulting in reduced MRSA resistance. Interestingly, such selective inhibition towards specific *mec* genes have also occurred when natural antimicrobials were applied for MRSA KCCM 40511 strain, as it was previously reported that *Eiseniabicyclis*-sourced phlorofucofuroeckol-A inhibited only *mecA*, *mecI* and *mecR1* (Eom *et al.*, 2014); or *n*-hexane fraction of *Salvia miltiorrhiza* Bunge inhibited only *mecA*, *mecR1* and *femA* (Lee *et al.*, 2007). These noticeable findings may be helpful for further research on MRSA KCCM 40511 pathways of interaction between these natural anti-MRSA agents and MRSA genome.

The strain MRSA KCCM 40511 was selected to detect PBP2a in the PBP profile using Western Blot analysis, as its resistance-essential genes (*mecA*, *mecI*, *mecR1*, *femA* and *nucA*) were fully expressed. Fig.2 shows a visible decrease of band intensity as the sub-fraction concentrations increased from 0 to 16 $\mu\text{g ml}^{-1}$ ($P < 0.05$), which indicated that EF03 sub-fraction, particularly at 16 $\mu\text{g ml}^{-1}$ attenuated the production PBP2a level in a dose-dependent manner. Considering the results obtained from mRNA expression and Western Blot assay, it was concluded that EF03 sub-fraction from *E. japonica* leaf extract inhibited the expression of resistant protein PBP2a through transcriptional inhibition. The results of this study suggest that the extract of *E. japonica* exhibited strong inhibitory effect on gene expression associated with β -lactam antibiotic resistance in agreement with the previous studies (Lee *et al.*, 2007; Eom *et al.*, 2014). Further, the extract possess antibacterial activity through inhibition of PBP2a production against MRSA will provide a clue to develop an alternative therapy for fighting antibiotic resistance bacteria.

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