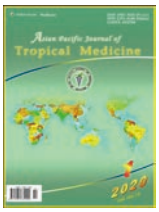

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Orostachys japonicus ethyl acetate fraction suppresses MRSA biofilm formation

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ABSTRACT

Objective: To investigate the effect of *Orostachys* (*O.*) *japonicus*, a perennial herbaceous plant of the Family Crassulaceae, on biofilm formed by methicillin-resistant *Staphylococcus aureus* (MRSA).

Methods: Powdered *O. japonicus* was extracted by 95% methanol, concentrated, and then, systematically fractionated with *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc), *n*-butanol, and H_2O according to polarity. Among them, the flavonoid-rich EtOAc fraction demonstrated the highest antibacterial activity and was used in this study. Using the biofilm inhibition assay, cell-surface attachment assay, confocal laser scanning microscopy, latex agglutination assay, and real time qRT-PCR, we examined whether the EtOAc fraction inhibited the formation of MRSA biofilm.

Results: The EtOAc fraction exhibited distinct activity against biofilm formation and cell-surface attachment of MRSA up to 1 mg/mL through down-regulating the expression of *mecA* gene and the production and agglutination of penicillin-binding protein 2a as solidly observed in biofilm inhibition assay, cell-suface attachment assay, confocal laser scanning microscopy, latex agglutination assay, and real time qRT-PCR analysis.

Conclusions: These results suggest that *O. japonicus* could be utilized as a potential resource for the development of new anti-biofilm formation of MRSA and antibacterial agents in the future.

KEYWORDS: *Orostachys japonicus*; MRSA; Biofilm formation

1. Introduction

Staphylococcus (S.) aureus is a spherical, Gram-positive bacterium that is commonly found in the nose, respiratory tract, and skin[1,2]. Methicillin-resistant *S. aureus* (MRSA) is a strain that has developed multiple resistance to beta-lactam antibiotics, including penicillins (methicillin, dicloxacillin, nafcillin, oxacillin, and so on) and cephalosporins, through horizontal gene transfer and natural selection[3-6]. MRSA is particularly troublesome in prisons, nursing homes, and hospitals, where patients with open wounds, weakened immune systems, and invasive devices are at higher risk of nosocomial infection than the general public. The illness caused by this pathogen started as a hospital-acquired infection, but has developed limited endemic situation and is now sometimes community- and livestock-acquired[7].

Many reports have shown that bacterial cells growing within biofilms are resistant to lots of antibiotics. Biofilms play an primary role in keeping bacterial cells from any variations of their microenvironment as well as any possible antimicrobial agents[7-9]. The ability of MRSA to form biofilms is a principal cause of pathogen infection, and the abilities of cells within the biofilm to produce toxins and to avoid the host immune responses make the treatment of the illness more complex[10]. There are basically two main phases in production of biofilms. The first phase is cell-surface attachment, also known as the premiere attachment step, where the bacterial cells adhere to a surface to form colonies. The second stage involves cell-cell interaction and is an accumulative step in which the microorganisms create a multilayered structure that leads to bacterial biofilm development[11,12]. Biofilm formation by MRSA has been reported to be mediated by penicillin-binding protein 2a (PBP2a), a modified protein avoiding the antimicrobial activity of

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beta-lactam antibiotics as a result of its low binding affinity to these drugs[13]. A previous study had reported that PBP2a elevates cell-cell interactions in biofilm formation[14].

Orostachys (*O.*) *japonicus*, called Wa-song in Korea, is a perennial herbaceous plant. It is used as a folk remedy for hemorrhage, inflammation, intoxication, fever, and cancer[15-20]. Previous studies have reported the presence of flavonoids (*e.g.*, quercetin and kaempferol), sterols (*e.g.*, campesterol and beta-sitosterol), triterpenoids (*e.g.*, friedelin, beta-amyrin, glutinone, and glutinol), and other phytochemicals (*e.g.*, flavonoid glycosides and sterol glucosides) in this plant[17,18,21,22]. *O. japonicus* has been proven to block oxidative stress by enhancing the expression of heme oxygenase-1 and nuclear factor (erythroid-derived 2)-like 2, as well as regulating the mitogen-activating protein kinase signaling pathway[23]. It was reported that *O. japonicus* could also inhibit the inflammatory response *via* suppression of nuclear factor-kappaB activation and mitogen-activating protein kinase and phosphoinositide 3-kinase/protein kinase B signaling transduction[15,24]. Moreover, the plant has exhibited anticancer activity against various cancer cells (*e.g.*, gastric cancer, hepatic cancer and colon cancer, etc.) through induction of apoptosis and/or cell cycle arrest[25-30]. In addition, *O. japonicus* has been reported to have antibacterial and antifungal activities[28].

In recent years, interest in antibiotic-resistant bacteria like MRSA has been increasing worldwide. In Korea, especially the issue of antibiotic resistance is very serious because antibiotics have been used indiscriminately compared with the use in other developed countries. Hence, there has been increasing interest in natural plant-derived antibacterial materials that can effectively handle infectious diseases without affecting resistance of antibiotics. In this study, we used various diagnostic methods to examine whether the extracted ethyl acetate (EtOAc) fraction of this plant can inhibit MRSA biofilm formation.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The MRSA strain confirmed by disk diffusion test using cefoxitin and used in this study was isolated from clinical specimens at a university hospital in Seoul The bacterium was cultured on either Nutrient Agar (NA, Difco Laboratories, Franklin Lakes, NJ, USA) containing Bacto peptone (5 mg/mL) and Bacto beef extract (3 mg/mL), or Tryptic Soy Broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing papaic digest of soybean (3 mg/mL), pancreatic digest of casein (17 mg/mL), sodium chloride (5 mg/mL), dextrose (2.5 mg/mL), and dipotassium phosphate (2.5 mg/mL), at 37 ℃ under the atmosphere of the incubator.

2.2. Plant material and extraction

The *O. japonicus* whole plants (Dried Wa-song, 100 g ×2) provided by Jirisanwasong Co. (Sancheong, Korea) were dried, sliced, and powdered. The resultant sample (200 g) was extracted by 95% methanol (MeOH) for 6 h. After concentrating the MeOH extract by rotary evaporation at 50 ℃, the concentrate was dissolved in water and then fractionated with the following order of organic solvents: *n*-hexane, dichloromethane (DCM), EtOAc, *n*-butanol, and H_2O. The EtOAc fraction was applied to thin-layer chromatography to investigate whether any of its chromatogram spots correspond to the known *O. japonicus* components, such as kaempferol and quercetin (data not shown).

2.3. MTT based MIC test

MIC test were performed according to the method described previously[31]. A 96 well tissue culture plate was taken, to which was added the 100 μL of bacterial culture (0.5 McFarland) in each well, then 100 μL of different concentrations of EtOAc fraction (0.25, 0.5, 1, 2, 4, 8, 16, 32 mg/mL) were put into each well, the plate was incubated at 37 ℃ for 6 h. And then 20 μL of solution of MTT (Sigma-aldrich®, USA) (5 mg/mL) in phosphate buffer was added in each well and the plate was reincubated for 1 h. The formation of blue color denotes the presence of viable bacteria and MICs were determined by visual breakpoints at each concentration.

2.4. Confocal laser scanning microscopy

MRSA biofilms were established on glass-bottomed confocal dishes (SPL Life Sciences, Pocheon, Korea). Following exposure to 0.5 or 1.0 mg/mL of the *O. japonicus* EtOAc fraction for 24 h, the biofilms were visibly stained with the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, Carlsbad, CA, USA) according to the instructions of manufacturer. The biofilms were stained with the LIVE/DEAD BacLight Bacterial Viability Kit and were inspected using confocal laser scanning microscopy (LSM 510 META; Carl Zeiss, Germany). This kit utilizes a mixture of SYTO 9 (a green-fluorescent stain) and propidium iodide (a red-fluorescent stain), both of which differ in their spectral characteristics and ability to penetrate healthy bacterial cells. When used solely, the SYTO 9 stain normally labels all bacterial cells in a population. And then, the final sample suspension was examined under a confocal laser scanning microscope (magnification power was 400×).

2.5. Biofilm inhibition assay

The biofilm inhibition assay was performed according to the method described previously[32]. In brief, the EtOAc fraction was

added to the 96-well plate at the concentrations of 4.0, 2.0, 1.0, and 0.5 mg/mL. An aliquot of an MRSA suspension (200 μL/well, final inoculum size of 1×10^5 CFU/mL) was then applied to each well and incubated for 24 h at 37 ℃. After incubation, they were washed with physiological buffered saline solution, and the production of biofilm was quantified by crystal violet staining method[33]. The experiment was conducted in triplicate on three separate occasions.

2.6. Microtiter attachment assay

The microtiter attachment assay was carried out according to the method described previously[34]. It was conducted in the same manner as in biofilm inhibition assay except changing only the inoculum size of MRSA (1×10^7 CFU/mL) and the incubation time (1 h). The experiment was performed in triplicate on three separate occasions.

2.7. HPLC analysis of kaempferol, quercetin and EtOAc fraction

Methanol solutions of kaempferol, quercetin, and the EtOAc fraction were analyzed by high-performance liquid chromatography (HPLC) according to the method described previously[35]. An Agilent 1100 Series liquid chromatography system (Agilent, Palo Alto, CA, USA), equipped with a quaternary gradient pump, vacuum degasser, diode array detector, and autosampler, was used and was controlled by Agilent ChemStation software. A C18 column (250.0 mm×4.6 mm *id*, 5 μm; YMC Co., Ltd., Kyoto, Japan) was used at a column temperature of 25 ℃. The mobile-phase gradient applied was methanol/water/formic acid (50: 47: 3; v/v/v) at a flow rate of 1.0 mL/min. The UV detector was operated at 340 nm and the sample injection volume was 5 or 10 μL.

2.8. PBP2a latex agglutination test on MRSA biofilm

The PBP2a latex agglutination test was carried out according to the method described previously[5]. MRSA cells were cultured in 50 mm-diameter Petri dishes containing 10 mL of TSB with 1 mg/mL EtOAc fraction, for 24 h at 37 ℃. After incubation, the broth was removed and 0.5 mL of physiological buffered saline was added to the dishes, using a sterile 5 μL inoculating loop, enough of the biofilm layer was scraped off (rendering approximately 1.5×10^9 CFU/mL). The obtained bacterial biofilm was processed according to the manufacturer's instructions for the MRSA Screening Kit (Denka Seiken, Tokyo, Japan) to detect PBP2a. Semi-quantitative estimation of PBP2a production in the biofilms was done according to the protocol described previously[36]. In the test, *S. aureus* KCTC 1621 strain distributed from Korean Collection for Type Cultures (KCTC) was used as methicillin- sensitive *S. aureus* (MSSA), and dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, USA) was used. The intensity of agglutination was scored between – and ++, whereas the control latex showing no reactivity without PBP2a was scored as –.

2.9. RNA extraction, cDNA synthesis, and real time qRT-PCR

The MRSA strain was grown in the presence of 2.0, 1.0, or 0.5 mg/mL of the EtOAc fraction until the late exponential phase (24 h). Total RNA and DNA were then extracted using the ExiProgen Fully Automated Nucleic Acid Extraction System and ExiPrep Viral DNA/RNA Kit (Bioneer Co., Daejeon, Korea). RNA was obtained by removing the DNA from the extracted sample using the Riboclear Plus Kit (GeneAll Biotechnology Co., Seoul, Korea). The RNA concentration was assayed by measuring the absorbance of the sample at 260 and 280 nm, after which 300 ng was used for cDNA synthesis using the M-MLV Reverse Transcriptase Kit (Bioneer Co.). The synthesized cDNA was then used to quantify the expression of target genes by the quantitative reverse-transcription polymerase chain reaction (qRT-PCR), which was carried out with an Exicycler 96 Real-Time Quantitative PCR System and AccuPower Dualstar qPCR PreMix Kit (Bioneer Co.). The qRT-PCR conditions used were as follows: 5 min at 95 ℃, initial denaturation for 5 s at 95 ℃, and 45 cycles of 5 s at 95 ℃ and 5 s at 60 ℃. The results were analyzed with Exicycler 96 3.0 software. The threshold cycle method ($2^{-\Delta\Delta CT}$) was used to analyze the changes in the expression of gene in a given sample relative to the control (cells cultured under the same conditions without the EtOAc fraction), using the method described previously[37]. For each sample, qRT-PCR was conducted three times. The primer sequences for the *mecA* and 16S rRNA genes are listed in Table 1.

2.10. Statistical analysis

The results are represented as the Mean±Standard deviation. Data were assayed using SPSS Statistics software (IBM, North Castle, NY, USA). We used an independent sample *T* test, and a value of $P<0.05$ was regarded as statistically significant.

Table 1. Primers used for the real time qRT-PCR.

Primer	Sequence (5′–3′)
16S rRNA of *Staphylococcus aureus*	Forward: ATACGTTCCCGGGTCTTGTA
	Reverse: CACCCCAATCATTTGTCCCA
mecA gene for PBP2a	Forward: GCACTTGTAAGCACACCTTCA
	Reverse: CTGGAACTTGTTGAGCAGAGG

PBP2a: penicillin-binding protein 2a.

3. Results

3.1. O. japonicus fractionation system

Since the MeOH extract showed the highest antibacterial activity among MeOH extract, ethanol extract, and hot water extract in a preliminary disk diffusion assayit was further systematically fractionated with *n*-hexane, DCM, EtOAc, *n*-butanol, and H_2O, according to polarity. Each of these fractions was again tested for antibacterial activity (data not shown), and among them, the EtOAc fraction demonstrating the highest antibacterial activity was used as the further research material.

3.2. HPLC analysis of the EtOAc fraction

In order to confirm the content of flavonoids and flavonoid glycosides such as kaempferol, quercetin, afzelin, astragalin, quercitrin, and isoquercitrin in the EtOAc fraction, kaempferol and quercetin, which are presumed to be the major two bioactive substances of this plant, were used as reference compounds in an HPLC analysis of the fraction. The results obtained (Supplementary Figure 1) clearly showed that the extracted EtOAc fraction contained quercetin and kaempferol, at 3.75% and 0.94%, respectively.

3.3. Biofilm inhibition assay

The biofilm inhibition assay was conducted to examine whether the EtOAc fraction of *O. japonicus* could inhibit MRSA biofilm formation (Figure 1). The MIC test was performed to determine the proper concentration for the experiment and obtained an MIC value of 8 mg/mL (data not shown). Therefore, the biofilm inhibition assay was performed at an appropriate concentration lower than this value. The data are the percentages of MRSA biofilm formation in the presence of different concentrations of the plant extract. The EtOAc fraction exhibited distinct inhibition activity against MRSA biofilm production in a dose-dependent manner. The rates of MRSA biofilm formation were (91.91±14.93)%, (67.66±7.67)%, (50.45± 9.11)%, and (38.91±4.77)% for the 0.5, 1.0, 2.0, and 4.0 mg/mL treatments, respectively.

3.4. Cell attachment assay

A cell attachment assay was conducted to observe the effect of the EtOAc fraction on the cell-surface attachment stage. The data are the percentages of MRSA cell-surface attachment in the presence of different concentrations of the plant fraction (Figure 2). The EtOAc fraction showed clear activity on MRSA cell-surface attachment in a dose-dependent way, up to the level of 1 mg/mL. However, increasing the concentration to more than 1 mg/mL did not further increase the inhibition effect. The rates of cell-surface attachment were (81.93±3.45)%, (44.14±6.15)%, (43.48±6.19)%, and (45.80±5.42)% for the 0.5, 1.0, 2.0, and 4.0 mg/mL treatments, respectively.

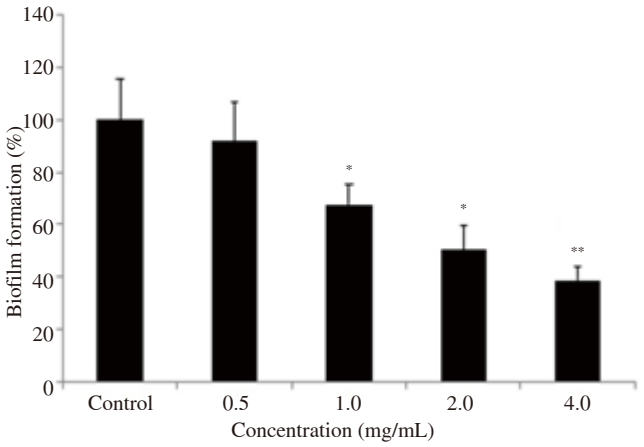

Figure 1. Percentage of MRSA biofilm formation in microtiter plate wells containing varying concentrations of the EtOAc fraction of *Orostachys japonicus*. Each treatment was done in triplicate. The experiment is representative of three independent experiments, and the error bars indicate standard deviation. The differences between the control and treatment (⩾1 mg/mL) were statistically significant (*P<0.05, **P<0.01).

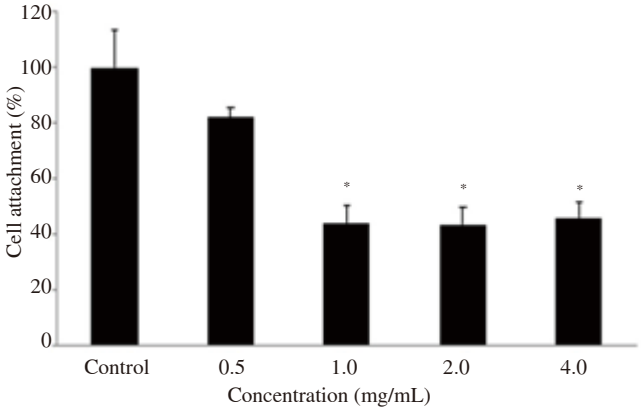

Figure 2. Percentage of MRSA cell-surface attachment in microtiter plate wells containing varying concentrations of the EtOAc fraction of *Orostachys japonicus*. Error bar indicates the standard deviation of three independent experiments. All differences between control and treatments, except that at 0.5 mg/mL, were statistically significant (*P<0.05).

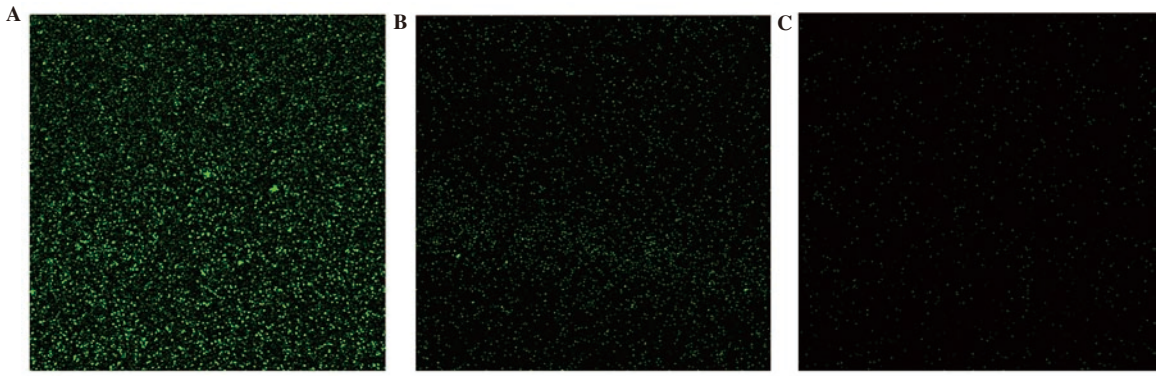

Figure 3. Confocal laser scanning microscopy images of the MRSA biofilms. The bacterial biofilms were exposed to (A) dimethyl sulfoxide (DMSO), (B) EtOAc fraction (0.5 mg/mL), and (C) EtOAc fraction (1.0 mg/mL). The magnification power is 400×.

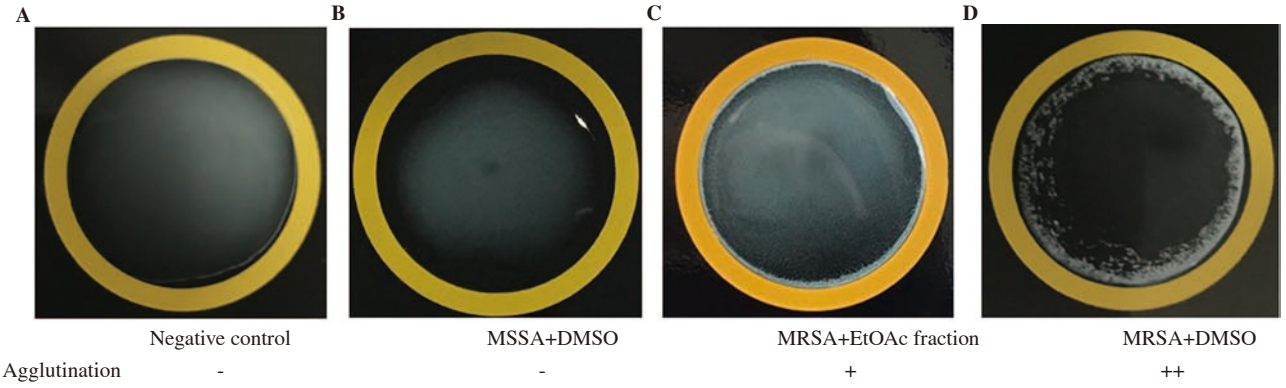

Figure 4. Representative images of penicillin-binding protein 2a (PBP2a) agglutination on MRSA biofilms. (A) Negative control, MRSA treated with latex without PBP2a antibody; (B) Methicilin sensitive *Staphylococcus aureus* (MSSA) treated with DMSO; (C) MRSA treated with EtOAc fraction (1 mg/mL); (D) MRSA treated with DMSO. (B), (C), and (D) were all treated with latex containing PBP2a antibody. (C) shows a lower level of agglutination than (D). ++, high; +, low; –, no agglutination.

3.5. Confocal laser scanning microscopy

To verify the biofilm inhibition assay results for the EtOAc fraction, the process was examined more clearly using confocal laser scanning microscopy (LSM 510 META; Carl Zeiss, Germany) under 400× magnification power. The results of the assay showed that when the concentration of the EtOAc fraction was increased to 1 mg/mL, the amount of green fluorescence was remarkably reduced relative to that in the untreated biofilm (Figure 3).

3.6. PBP2a latex agglutination test

In this test, a higher intensity of agglutination is observed in response to a high level of PBP2a expression in the biofilm. The dimethyl sulfoxide (DMSO)-treated MRSA control showed an intact intensity of agglutination, whereas EtOAc fraction-treated MRSA revealed a much lowered agglutination of PBP2a in all the plant fraction concentrations tested (Figure 4).

3.7. qRT-PCR analysis for the test of mecA gene inhibition

To investigate the effect of the EtOAc fraction on the PBP2a-encoding *mecA* gene, qRT-PCR analysis was performed to target the gene (Figure 5). The data showed that 1 mg/mL of the EtOAc fraction inhibited the expression of *mecA* by about 40%. However, when the concentration of the EtOAc fraction was doubled (2 mg/mL), the *mecA* gene expression increased unexpectedly and the inhibitory effect of the plant fraction disappeared.

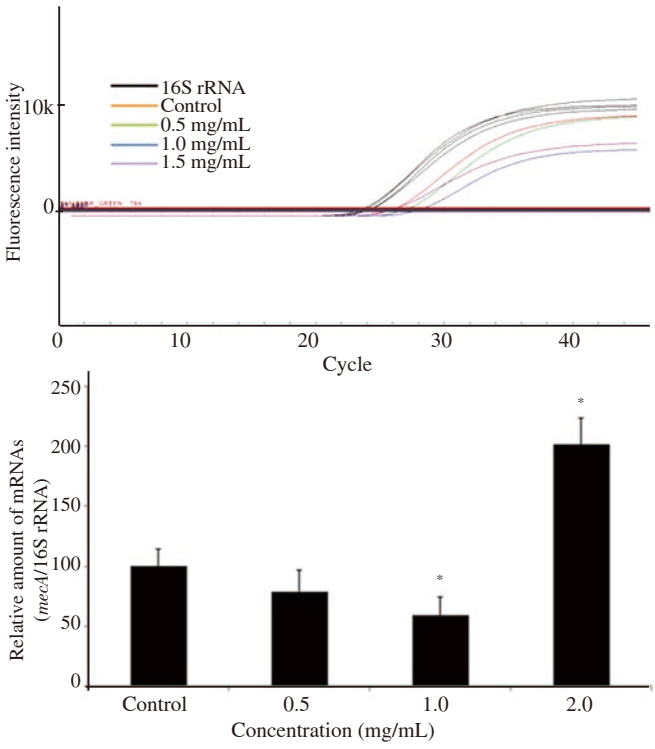

Figure 5. Representative real time qRT-PCR amplification curves of the MRSA *mecA* gene and 16S rRNA (A), and relative amount of *mecA* gene/16S rRNA mRNAs expressed in MRSA (B). Error bars indicate the standard deviation of three independent experiments. All differences between control and treatments (except that at 0.5 mg/mL) were statistically significant (*P<0.05).

4. Discussion

In several other studies, kaempferol and quercetin were reported to exhibit various biochemical activities, including antimicrobial activity[38–42]. The antimicrobial activity of flavonoids is known to be due to the numbers of hydroxyl groups that could have nonspecific interactions with proteins or cause inhibition of enzymes[43,44]. Since PBPs are a group of protein enzymes, flavonoids in the EtOAc fraction could form nonspecific interactions with them. These interactions can affect bacterial cell wall biosynthesis and eventually cause anti-MRSA activity. In addition, such nonspecific interactions might have resulted in the inhibition of PBP2a expression in this study. Meanwhile, although the DCM fraction was not used as an experimental material in this study, it is known to contain a large amount of sterols and glucosides[17,18]. Plant sterols have been proven to be antibacterial[45], and several glycosides have been reported to show antimicrobial activity in MRSA[46].

Biofilm formation in MRSA is known to be mediated by PBP2a[13]. PBP2a is an altered protein avoiding the antimicrobial activity of beta-lactam antibiotics as a result of its low binding affinity to such drugs. Previous study reported that PBP2a could raise cell-cell interactions in the biofilm development[14]. The *mecA* is a biomarker gene responsible for the bacterial resistance to methicillin and other beta-lactam antibiotics[4]. After acquisition of *mecA*, the gene is integrated into and localized in the chromosome of *S. aureus*. The *mecA* encodes PBP2a, which is different from other penicillin-binding proteins since its active site does not bind with methicillin or other beta-lactam antibiotics[5,6]. In this study, the EtOAc fraction inhibited *mecA* gene expression by around 40% at 1 mg/mL level. However, when the concentration of the EtOAc fraction was elevated to 2 mg/mL, *mecA* gene expression increased unexpectedly and the inhibition effect disappeared. In previous studies, similar results were reported and the phenomenon was explained as follows. When bacteria are confronted by an extremely adverse environment such as exposure to antibiotics, biofilms may form more vigorously as an urgent defense mechanism against the external stressor[5,6]. This means that if the concentration of the EtOAc fraction is too high, it could build the extremely unfavorable environment that triggers increased biofilm formation. There is a need to discuss these results in conjunction with those of the cell attachment assay, in which the EtOAc fraction exhibited definite inhibitory activity against MRSA cell-surface attachment in a dose-dependent manner up to the concentration of 1 mg/mL, but showed no further effect beyond this concentration. Considering these two results, an EtOAc fraction concentration of more than 1 mg/mL may be recognized by MRSA as absolutely unfavorable for its survival, triggering the bacterium to raise its level of *mecA* gene expression. Consequently, PBP2a protein expression is increased, which promotes cell-surface attachment, and thus the inhibition of MRSA biofilm formation is negated. However, the possibility that the inhibiting activity of the EtOAc fraction against biofilm formation of MRSA may be caused by other mechanisms cannot be absolutely ruled out, because this fraction contains a mixture of six flavonoids or flavonoid glycosides, such as kaempferol, quercetin, and their glycosides[18]. Further research is needed to confirm which plant constituents are exactly responsible for the antibacterial activity, and whether these compounds work alone or synergistically. However, in spite of these unexpected experimental results, at an appropriate level (in this case up to 1 mg/mL), the EtOAc fraction clearly shows anti-biofilm formation activity. It is suggested that the EtOAc fraction could suppress the expression of *mecA* and consequently the production of PBP2a, resulting in inhibition of MRSA biofilm formation. In conclusion, the flavonoid-rich EtOAc fraction from *O. japonicus* MeOH extract could be a potential resource for the development of new antibacterial agents without causing antibiotic resistance as well as showing anti-biofilm formation activity.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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Authors' contributions

JH Kim and SYH contributed to the design and perfomance of the experiment, and also drafted the manuscript. JH Kwon and DSL contributed to the final version of the manuscript. DSL supervised the project.

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