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Synergistic Antibacterial Effect and Antibacterial Action Mode of Chitosan–Ferulic Acid Conjugate against Methicillin-Resistant *Staphylococcus aureus*

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We evaluated the synergistic antibacterial effect in combination with the chitosan–ferulic acid conjugate (CFA) and β-lactam antibiotics, such as ampicillin, penicillin, and oxacillin, against methicillin-resistant *Staphylococcus aureus* (MRSA) using fractional inhibitory concentration (FIC) indices. CFA clearly reversed the antibacterial activity of ampicillin, penicillin, and oxacillin against MRSA in the combination mode. Among these antibiotics, the combination of oxacillin-CFA resulted in a ΣFIC_{min} range of 0.250 and ΣFIC_{max} of 0.563, suggesting that the oxacillin-CFA combination resulted in an antibacterial synergy effect against MRSA. In addition, we determined that CFA inhibited the mRNA expression of gene *mecA* and the production of PBP2a, which is a key determinant for β-lactam antibiotic resistance, in a dose-dependent manner. Thus, the results obtained in this study supported the idea on the antibacterial action mechanism that oxacillin will restore the antibacterial activity against MRSA through the suppression of PBP2a production by CFA.

Keywords: Antibacterial activity, chitosan–ferulic acid conjugate, fractional inhibitory concentration, *mecA* gene, methicillin-resistant *Staphylococcus aureus*, synergy effect

Introduction

The improper use of antibiotics in the treatment of bacterial infections has resulted in the appearance of spreading resistant strains [12]. Among them, *Staphylococcus aureus* has been recognized as an important pathogen both in community-acquired and healthcare-associated infections. *S. aureus* has successfully become resistant to practically all antibiotics, and this is a serious clinical problem in the world today [10]. Methicillin-resistant *S. aureus* (MRSA) infections occur widely in both hospitals and communities. Of further concern, MRSA strains are now evolving additional resistance against standard types of antibiotics [5]. The resistance mechanism against methicillin is mediated via the *mec* operon, which is a part of the staphylococcal cassette chromosome *mec* (SCC*mec*) [8]. Therefore, MRSA exhibits resistance to β-lactam antibiotics by the acquisition of the *mecA* gene, which encodes penicillin binding protein 2a (PBP2a) [20]. The β-lactam groups of antibiotics are derived from a β-lactam structure. The major properties of β-lactam antibiotics inhibit several enzymes associated with the final step of peptidoglycan synthesis [6]. β-Lactam antibiotics preferentially bind to penicillin binding proteins in the cell wall and inactivate their transpeptidase and carboxypeptidase

activities [6]. Since PBP2a has a lower affinity for binding to β-lactam antibiotics (penicillins, cephalosporins, and carbapenems), MRSA is resistant to all β-lactam agents. Despite an urgent need for new antibiotics, the number of newly approved drugs is decreasing continuously [7]. Thus, the development of new drugs or alternative therapies is truly needed to treat MRSA.

Chitosan is a naturally occurring mucopolysaccharide, and it has low toxicity as well as being biodegradable and biocompatible. Several bioactivities of chitosan and chitosan derivatives, such as antioxidant, anticancer, antimicrobial, and enzyme inhibitory effects, have also been reported, and its unique bioactivities have been leading to its applications in the pharmaceutical industry [2, 11, 13]. We recently evaluated the antibacterial activity of chitosan-hydroxycinnamic acid conjugates against food-borne pathogenic bacteria and MRSA [13]. However, there is no further information on the antibacterial mechanism of the conjugates against MRSA. In the present work, we reported the in vitro antibacterial activity of the chitosan–ferulic acid conjugate (CFA) in combination with antibiotics against MRSA. In addition, we investigated the effect of CFA on the expression of the *mecA* gene and the production of PBP2a, which is a key determinant for β-lactam antibiotic resistance [20].

Materials and Methods

Preparation of the Chitosan–Ferulic Acid Conjugate

Chitosan (average molecular mass 310 kDa and 90% degree of deacetylation) was kindly donated by Kitto Life Co. (Seoul, Korea). The CFA, which exhibited the highest anti-MRSA activity, was prepared according to our previous method [13] (Fig. 1). The molar ratio of chitosan residue to ferulic acid is 1:0.1. After the preparation of CFA, ^{1}H nuclear magnetic resonance and differential scanning calorimetry analysis were conducted and compared with the results of the previous report by Lee *et al*. [13].

The ferulic acid content in the CFA was determined by using the Folin-Ciocalteau method. The CFA contains 7.08 ± 0.14 mg of ferulic acid/g CFA, and the average molecular mass of the CFA is 312 kDa, which is calculated by the content of ferulic acid in the CFA. The CFA is well soluble in water up to 10 mg/ml.

Bacterial Strains and Medium

The methicillin-susceptible *S. aureus* (MSSA) standard bacterial strain KCTC 1927 (ATCC 6538P, penicillin-resistant strain) was obtained from the Korea Collection of Type Culture (Daejeon, Korea) to assure reliability of the research results. Two standard MRSA strains, KCCM 40510 (ATCC 33591) and KCCM 40511 (ATCC 33593), were purchased from the Korea Culture Center of Microorganisms (KCCM, Seoul, Korea). These strains were grown aerobically at 37°C in Mueller-Hinton broth (MHB; Difco, Detroit, USA) and were subsequently used in experiments to measure the antibacterial activity.

Synergy Test by Combination with Commercial Antibiotics

The interactions between CFA and β-lactam antibiotics, including ampicillin, penicillin, and oxacillin, against MRSA were tested by the checkerboard method [15]. The synergy effect between CFA and the antibiotics was evaluated as a fractional inhibitory concentration (FIC) index [15]. The FIC was calculated as the minimum inhibitory concentration (MIC) of an antibiotic or CFA in combination divided by the MIC of the antibiotic or CFA alone.

RNA Isolation and RT-PCR Analysis

The effect of CFA on expression of the *mecA* gene in MRSA KCCM 40511 cells was determined using the reverse transcriptase polymerase chain reaction (RT-PCR). To investigate the mechanism of anti-MRSA activity of CFA, MRSA cells were grown aerobically at 37°C in MHB, in the presence of CFA at the concentrations of 4, 8, 16, and 32 μg/ml for 16 h. After harvesting, total RNA was isolated with zirconia beads and an RNAwiz kit (Ambion Inc., Austin, USA), according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from the isolated RNA (1 μg) through a reaction catalyzed by SuperScript II reverse transcriptase (Life Technologies Inc., Gaithersburg, USA) with random hexanucleotides, according to the manufacturer's instructions. The synthesized cDNA was qualified and quantified by a VERSA Max microplate reader, and the cDNA (200 ng) was used as template for PCR. Sequence-specific primers of each gene were designed from a previous result: *mecA* (527 bp PCR product: annealing temperature 54.4°C) [14]. The cycling conditions (33 cycles) using a Thermal cycler (Takara Bio Inc., Shiga, Japan) were

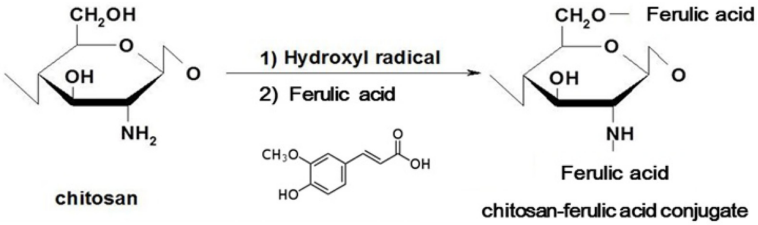

Fig. 1. Preparation of the chitosan–ferulic acid conjugate.

as follows: denaturation at 94°C for 30 sec, annealing at the indicated temperature of each primer for 30 sec, and extension at 72°C for 30 sec. The amplified PCR products were analyzed electrophoretically on 1.5% agarose gels and visualized with ethidium bromide.

Western Blot Analysis

The western blot technique was performed according to the standard procedures to measure the translated protein level [19]. Briefly, harvested cells were suspended in lysis buffer (20 mM Tris-HCl, 2 mM ethyleneglycoltetraacetic acid, 2 mM ethylenediaminetetraacetic acid, and 0.25 M sucrose, pH 7.5) and sonicated three times at 95 μA for 30 sec each time with an ultrasonicator (Ultrasonic Ltd., Hants, UK). Following 10 min of centrifugation at 13,000 ×*g*, the supernatant was obtained as the cell lysate. Protein concentration was measured with the Bradford protein assay method [1]. Cell lysates (10 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech., Rainham, UK), blocked with 10% skim milk, and hybridized with MRSA PBP2a monoclonal antibody (diluted 1:1,000; Abnova, Taipei, Taiwan). After incubation with horseradish peroxidase-conjugated anti-mouse immunoglobulin G secondary antibody (diluted 1:1,000; Santa Cruz Biotechnology, Santa Cruz, USA) at room temperature, immunoreactive proteins were detected using a chemiluminescent ECL assay kit (Amersham Pharmacia Biotech.) according to the manufacturer's instructions. Western blot bands were visualized using a LAS3000 Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

Statistical Analysis

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

Results

Synergy Effects between CFA and β-Lactams against MRSA

In this experiment, we evaluated the combined effects of CFA and β-lactam antibiotics, such as ampicillin, penicillin, and oxacillin, against MRSA. The MIC values of ampicillin, penicillin, and oxacillin against MRSA strains ranged from 128 to 256 μg/ml (Table 1). These results imply that β-lactam antibiotics (ampicillin, penicillin, and oxacillin) are no longer effective against the resistant bacteria [16]. However, the MICs of ampicillin against two standard MRSA strains (KCCM 40510 and KCCM 40511) were reduced from 128 to 32 μg/ml, when it was administered in combination with 32 μg/ml of CFA (KCCM 40510) and with 16 μg/ml of CFA (KCCM 40511), respectively (Table 1). The MICs of penicillin against MRSA strains were also reduced from 128 to 32 μg/ml, when it was administered in combination with 32 μg/ml of CFA (KCCM 40510) and with 16 μg/ml of CFA (KCCM 40511), respectively. In addition, the MIC value of oxacillin was considerably reduced from 256 to 32 μg/ml against MRSA (KCCM 40510) and from 128 to 16 μg/ml against MRSA (KCCM 40511), when it was in combination with CFA. In order to evaluate a synergy effect of β-lactam antibiotics in combination with CFA against MRSA, we performed the analysis using fractional inhibitory concentration indices (Table 1) [15]. A combination of CFA-ampicillin resulted in a median ΣFIC range of 0.500 against the two MRSA strains, suggesting that the combination would cause an addictive antibacterial effect against MRSA strains tested in this study (Table 2). An addictive antibacterial effect of CFA-penicillin combination against the MRSA

Table 1. Minimum inhibitory concentrations (MIC) and fractional inhibitory concentration (FIC) indices of the CFA (chitosan–ferulic acid) in combination with ampicillin against MSSA and MRSA strains.

Strains	Test compound	MIC (μg/ml)	Median ΣFIC[a]	ΣFIC_{max}[b]	ΣFIC_{min}[c]	Minimum concentration for observing synergy
MSSA (KCTC 1927)	CFA	64	1.125	1.500	0.625	16
	Ampicillin	2				1
MRSA (KCCM 40510)	CFA	128	0.500	0.675	0.375	32
	Ampicillin	128				32
MRSA (KCCM 40511)	CFA	64	0.500	1.125	0.375	16
	Ampicillin	128				32

[a]ΣFIC , the sum of FICs; [b]ΣFIC_{min}, minimum ΣFIC; [c]ΣFIC_{max}, maximum ΣFIC; The FIC index indicated; synergistic, <0.5; addictive, 0.5 to <1.0; indifferent, >1.0 to <2.0; antagonistic, >2.0. ΣFIC was calculated for each well with the equation $\Sigma FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, where MIC_A and MIC_B are the MICs of drugs A and B alone, respectively, and C_A and C_B are the concentrations of the drugs in combination, respectively.

Table 2. Minimum inhibitory concentrations (MIC) and fractional inhibitory concentration (FIC) indices of the CFA (chitosan–ferulic acid) in combination with penicillin against MSSA and MRSA strains.

Strains	Test compound	MIC (μg/ml)	Median ΣFIC[a]	ΣFIC_{max}[b]	ΣFIC_{min}[c]	Minimum concentration for observing synergy
MSSA (KCTC 1927)	CFA	64	1.250	1.500	1.000	32
	Penicillin	16				4
MRSA (KCCM 40510)	CFA	128	0.563	0.750	0.375	32
	Penicillin	128				32
MRSA (KCCM 40511)	CFA	64	0.500	0.750	0.375	16
	Penicillin	128				32

[a]ΣFIC, the sum of FICs; [b]ΣFIC_{min}, minimum ΣFIC; [c]ΣFIC_{max}, maximum ΣFIC; The FIC index indicated; synergistic, <0.5; addictive, 0.5 to <1.0; indifferent, >1.0 to <2.0; antagonistic, >2.0. ΣFIC was calculated for each well with the equation $\Sigma FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, where MIC_A and MIC_B are the MICs of drugs A and B alone, respectively, and C_A and C_B are the concentrations of the drugs in combination, respectively.

strains was also observed. Moreover, we observed a synergistic antibacterial activity in the combination of oxacillin and CFA, with a ΣFIC_{min} range of 0.250 against MRSA strains.

Inhibitory Activity of Chitosan–Ferulic Acid on the Expression of Gene *mecA* and the Production of PBP2a Related to Drug Resistance

As shown in Fig. 2A, the expression of gene *mecA* was inhibited by CFA in a dose-dependent manner. This result indicated that CFA inhibited the mRNA expression of the key antibiotic-resistant gene, *mecA*. To further elucidate the inhibitory effect of CFA on the production of PBP2a, western blot analysis was performed and its efficiency was compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The presence of the PBP2a band was detected in the western blot for MRSA cultures that were grown at sub-inhibitory concentrations of CFA (1/2, 1/4, 1/8, and 1/16× MIC). As shown in Fig. 2B, the PBP2a production was gradually attenuated or inhibited in a dose-dependent manner.

Discussion

Our group has previously reported about chitosan–hydroxycinnamic acid (caffeic acid, ferulic acid, and sinapic acid) conjugates, which have a high antioxidant and antimicrobial activity compared with unmodified chitosan [13]. The MIC patterns of the conjugates were similar to the previous results. Among these, CFA showed the highest

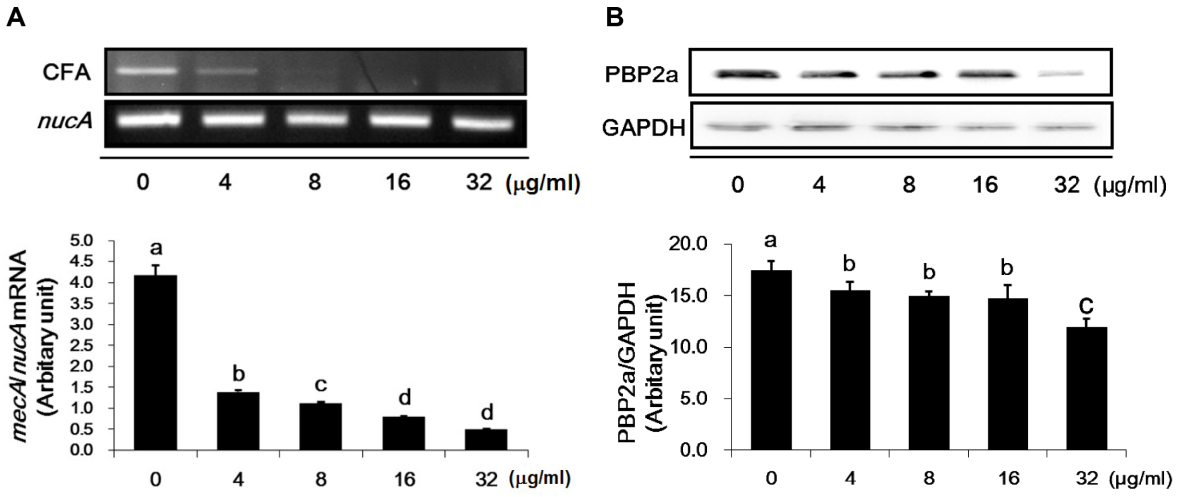

Fig. 2. Effect of the chitosan–ferulic acid conjugate (CFA) on the mRNA expression of the *mecA* gene (**A**) and on the expression of penicillin binding protein 2a (PBP2a) against an MRSA strain (**B**).
Methicillin-resistant *Staphylococcus aureus* (MRSA) KCCM 40511 was treated with the indicated concentrations of CFA.

Table 3. Minimum inhibitory concentrations (MIC) and fractional inhibitory concentration (FIC) indices of the CFA (chitosan–ferulic acid) in combination with oxacillin against MSSA and MRSA strains.

Strains	Test compound	MIC (μg/ml)	Median ΣFIC[a]	ΣFIC_{max}[b]	ΣFIC_{min}[c]	Minimum concentration for observing synergy
MSSA (KCTC 1927)	CFA	64	1.125	1.250	0.500	32
	Oxacillin	128				32
MRSA (KCCM 40510)	CFA	128	0.375	0.563	0.250	32
	Oxacillin	256				32
MRSA (KCCM 40511)	CFA	64	0.375	0.563	0.250	16
	Oxacillin	128				16

[a]ΣFIC, the sum of FICs; [b]ΣFIC_{min}, minimum ΣFIC; [c]ΣFIC_{max}, maximum ΣFIC; The FIC index indicated; synergistic, <0.5; additive, 0.5 to <1.0; indifferent, >1.0 to <2.0; antagonistic, >2.0. ΣFIC was calculated for each well with the equation $\Sigma FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, where MIC_A and MIC_B are the MICs of drugs A and B alone, respectively, and C_A and C_B are the concentrations of the drugs in combination, respectively.

antibacterial activity against the MRSA strains (Table 1). Therefore, as a part of our ongoing investigation on the alternative ways of overcoming MRSA, we investigated the anti-MRSA mechanism of CFA against MRSA.

The combination of CFA and oxacillin was more effective against the MRSA strains than the combination of CFA with ampicillin or penicillin. The analysis of FIC indices revealed that the antibacterial activity against MRSA is restored when using the old-fashioned β-lactams (ampicillin, penicillin, and oxacillin) in combination with CFA, suggesting that CFA might have potential to be used as an adjunct in the treatment for antibiotic-resistant bacteria. These results are in accordance with the study by Lee and Je [11] that the combination of gallic acid-grafted-chitosan conjugates with ampicillin, penicillin, and oxacillin restored the antibacterial activity of β-lactams against MRSA.

The FIC indices in Table 2 suggested that oxacillin can synergistically restore the antibacterial activity against MRSA, when it is combined with CFA. The susceptibility of MRSA to β-lactam antibiotics will be restored if the PBP2a production is suppressed. Therefore, we investigated the inhibitory effect of CFA on the expression of the *mecA* gene and the production of PBP2a in MRSA. Among the two MRSA strains, KCCM 40511 was selected for further study since the strain demonstrated less MIC values against CFA and a higher synergy effect in combination with β-lactams, compared with those of KCCM 40510. The inhibitory effect of CFA on the expression of *mecA* was monitored by RT-PCR.

The *mecA* gene encodes the 78 kDa protein called PBP2a [3]. The *mecA* gene is located on a mobile element, SCC*mec*, which is horizontally transferable among staphylococcal species. Resistance to the β-lactam antibiotics, including ampicillin, penicillin, and oxacillin, is principally mediated by the production of PBP2a encoded by the *mecA* gene [6, 12]. Whereas the PCR result consistently showed the band for the *mec* regulatory gene, *mecI*, between the MRSA KCCM 40510 (ATCC 33591) and 40511 (ATCC 33591) strains, the *mecI* gene was not detected in MRSA KCCM 40510 by PCR [5, 8]. In addition, the SCC*mec* element in MRSA KCCM 40510 was identified as SCC*mec* type I, whereas MRSA KCCM 40511 has SCC*mec* type II [5, 18]. According to Dutton *et al*. [4], the *nucA* gene is expressed in both *S. aureus* and MRSA. Therefore, in this present research, the *nucA* was used for the detection of *S. aureus* and MRSA, which served as an internal control [9].

Thus, CFA inhibited the mRNA expression of the *mecA* gene and eventually led to the reduction or inhibition of the resistance protein production, PBP2a, in MRSA cells. The inhibition or suppression of *mecA* gene expression and PBP2a production is a promising approach to restore the susceptibility of MRSA to old-fashioned β-lactam antibiotics [17].

In conclusion, we demonstrated an antibacterial synergy effect with the combination of CFA and β-lactams in this research. The antibacterial activity of β-lactams against MRSA was restored when they were combined with CFA, which led to the restoration of susceptibility of MRSA to old-fashioned β-lactams (ampicillin, penicillin, and oxacillin). In addition, CFA could suppress the expression of the *mecA* gene and the production of PBP2a related to β-lactam antibiotics resistance in a dose-dependent manner. However, there still remain some issues about whether CFA can directly inactivate PBP2a. Since a limited amount of information is available on the ability of plant and herbal extracts to regulate drug-resistant properties at the molecular level, these results suggest that CFA mediates the suppressive effects on the methicillin resistance-associated genes, showing its tremendous potential as a promising candidate for a

variety of therapeutic applications.

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References

1. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
2. Cho YS, Kim SK, Ahn CB, Je JY. 2011. Preparation, characterization, and antioxidant properties of gallic acid-grafted-chitosans. *Carbohydr. Polym.* **83:** 1617-1622.
3. De Lencastre H, Wu SW, Pinho MG, Ludovice AM, Filipe S, Gardete S, *et al*. 1999. Antibiotic resistance as a stress response: complete sequencing of a large number of chromosomal loci in *Staphylococcus aureus* strain COL that impact on the expression of resistance to methicillin. *Microb. Drug Resist.* **5:** 163-175.
4. Dutton EK, Ottum SA, Bolken TC, Franke CA, Hruby DE. 2000. Expression of active monomeric and dimeric nuclease A from the gram positive *Streptococcus gordonii* surface protein expression system. *Protein Expr. Purif.* **19:** 158-172.
5. Eom SH, Lee DS, Jung YJ, Park JH, Choi JI, Yim MJ, *et al*. 2014. The mechanism of antibacterial activity of phlorofucofuroeckol-A against methicillin-resistant *Staphylococcus aureus*. *Appl. Microbiol. Biotechnol.* **98:** 9795-9804.
6. Foster TJ. 2004. The *Staphylococcus aureus* "superbug". *J. Clin. Invest.* **114:** 1693-1696.
7. Gould FK, Brindle R, Chadwick PR, Fraise AP, Hill S, Nathwani D, *et al*. 2009. Guidelines (2008) for the prophylaxis and treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the United Kingdom. *J. Antimicrob. Chemother.* **63:** 849-861
8. Lemaire S, Van Bambeke F, Mingeot-Leclercq MP, Glupczynski Y, Tulkens PM. 2007. Role of acidic pH in the susceptibility of intraphagocytic methicillin-resistant *Staphylococcus aureus* strains to meropenem and cloxacillin. *Antimicrob. Agents Chemother.* **51:** 1627-1632
9. Kateete DP, Kimani CN, Katabazi FA, Okeng A, Okee MS, Nanteza A, *et al*. 2010. Identification of *Staphylococcus aureus*: DNase and mannitol salt agar improve the efficiency of the tube coagulase test. *Ann. Clin. Microbiol. Antimicrob.* **9:** 1-7.
10. Lee DS, Eom SH, Kim YM, Kim HS, Yin MJ, Lee SH, *et al*. 2014. Antibacterial and synergic effects of gallic acid-grafted-chitosan with β-lactams against methicillin-resistant *Staphylococcus aureus* (MRSA). *Can. J. Microbiol.* **60:** 629-638.
11. Lee DS, Je JY. 2013. Gallic acid-grafted-chitosan inhibits foodborne pathogens by a membrane damage mechanism. *J. Agric. Food Chem.* **61:** 6574-6579.
12. Lee DS, Kang MS, Hwang HJ, Eom SH, Yang JY, Lee MS, *et al*. 2008. Synergistic effect between dieckol from *Ecklonia stolonifera* and β-lactams against methicillin-resistant *Staphylococcus aureus*. *Biotechnol. Bioprocess Eng.* **13:** 758-764
13. Lee DS, Woo JY, Ahn CB, Je JY. 2014. Chitosan-hydroxycinnamic acid conjugates: preparation, antioxidant and antimicrobial activity. *Food Chem.* **148:** 97-104.
14. Lee JW, Ji YJ, Lee SO, Lee IS. 2007. Effect of *Saliva miltiorrhiza* bunge on antimicrobial activity and resistant gene regulation against methicillin-resistant *Staphylococcus aureus* (MRSA). *J. Microbiol.* **45:** 350-357.
15. Norden CW, Wentzel H, Keleti E. 1979. Comparison of techniques for measurement of in vitro antibiotic synergism. *J. Infect. Dis.* **140:** 629-633.
16. Nshimiyumukiza O, Kang SK, Kim HJ, Lee EH, Han HN, Kim Y, *et al*. 2015. Synergistic antibacterial activity of *Ecklonia cava* (Phaeophyceae: Laminariales) against *Listeria monocytogenes* (Bacillales: Listeriaceae). *Fish. Aquat. Sci.* **18:** 1-6.
17. Olajuyigbe OO, Coopoosamy RM. 2014. Influence of first-line antibiotics on the antibacterial activities of acetone stem bark extract of *Acacia mearnsii* de Wild against drug-resistant bacterial isolates. *Evid. Based Complement. Alternat. Med.* **2014:** 423751.
18. Rebollo-Pérez J, Ordoñez-Tapia C, Herazo-Herazo C, Reyes-Ramos N. 2011. Nasal carriage of Panton Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* in healthy preschool children. *Rev. Salud Pública* **13:** 824-832.
19. Sambrook J, Russell DW. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
20. Vouillamoz J, Entenza JM, Hohl P, Moreillon P. 2004. LB11058, a new cephalosporin with high penicillin-binding protein 2a affinity and activity in experimental endocarditis due to homogeneously methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **48:** 4322-4327.