Thesis for the Degree of Master of Engineering

Anti-MRSA (Methicillin Resistant *Staphylococcus aureus*) Activity of *Aspergillus oryzae* Fermentation Extract of *Ecklonia cava* Residue

by

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Pukyong National University

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(감태 부산물 황국균 발효 추출물의 항메티실린 내성 황색포도상구균에 대한 항균 효과)

Advisor : Dr. Young-Mog Kim

by

Hye-Jin Hwang

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요 약

다양한 해양유래 생물종을 대상으로 실시한 일련의 연구에서 해조류인 감태에서 가장 뛰어난 anti-MRSA 활성이 조사되었고, 또한 열수 추출 후의 감태 부산물에서도 상당 수준의 anti-MRSA 활성이 관찰되었다. 감태는 우리나라 제주도 지역에서 자생하는 대형 갈조류로서 그 열수 추출물은 식품과 화장품 등의 원료로 최근 주목을 받고 있다. 하지만 열수 추출 후에 대량으로 발생하는 부산물은 극히 일부분만 퇴비로 사용 될 뿐이고 대부분은 산업 쓰레기로 무단 방치 되거나 해양에 투기하고 있는 실정이다. 따라서, 다량의 생리활성 성분을 여전히 가지고 있는 것으로 기대되는 감태 부산물의 산업적 활용범위를 넓히기 위한 시도가 필요하다. 이에 본 연구에서는 감태 부산물의 산업적 이용도를 증대하기 위하여 추출공정에 미생물 발효공정을 도입하고 미생물의 발효 (즉 효소 분해 작용)에 의해 MRSA에 대한 항균활성이 어떻게 변화하는지를 연구하였다. 또한, 감태 부산물의 항균 특성에 대하여도 조사 하였다.

감태 부산물 추출액을 유기용매로 획분하고 각 획분의 MRSA에 대한 항

균활성 실험 결과 EtoAc 획분 > n-BuOH 획분 순으로 항균활성이 나타났으며 n-hexan 획분, CH_2Cl_2 획분, H_2O 획분에서는 항균활성이 나타나지 않았다. 감태 부산물을 황국균으로 액상 발효하였을 경우에도 감태 부산물의 추출물 처럼 EtoAc 획분에서 가장 높은 항균활성이 관찰되었다. 감태 부산물의 황국균 발효 추출물의 경우 EtoAc 획분의 MRSA에 대한 MIC 값이 64~256 μg/ml 으로 감태 부산물의 EtoAc 획분의 MIC 값(128~256 μg/ml) 보다 증가 하는 것으로 나타났다. 이러한 결과는, 유용 성분의 열수 추출 후에도 세포벽 등의 세포 조직에 다량으로 남아 있는 항균성 물질들이 황국균에 의해서 생산되는 다양한 효소들의 분해 작용에 의해 보다 효과적으로 추출된 것으로 사료 되었고, 실제 HPLC를 이용하여 분석한 결과 감태의 phlorotannins 중 MRSA에 대한 강한 항균 활성을 가지고 있는 것으로 알려진 dieckol의 함량이 발효에 의해 증가된 것으로 조사되었다. 이외에도 다른 세균들에 대한 항균활성도 발효에 의해 증가한 것으로 조사되어 대량으로 발생하는 감태의 열수 추출부산물에 대한 미생물 발효공정의 적용은 천연 항생제의 개발에 이용될 수 이용될 수 있을 것으로 기대된다.

Introduction

Emergence and spreading of drug resistance bacteria is a serious clinical problem in many country. The infectious disease have reduced extraordinary with an rapid improvement of new antimicrobial treatment in the last 60 years. Since antibiotics were initially used in the army during World War II, the improper use of penicillin resulted in the appearance of resistant mutant (Alanis A.J. 2005).

Methicillin-resistance *Staphylococcus aureus* (MRSA) is the most severe gram-positive bacterium in public health because it has become resistant to almost all available antibiotics, except vancomycin and teicoplanin, and the resurgence of multi-drug resistant (Lowry F. D. 1998; Liu *et al*., 2008). Vancomycin has been the drug of choice for the treatment of infections caused by MRSA, even though vancomycin-resistance *Staphylococcus aureus* recently has been reported in several countries. Therefore, alternative therapeutic agents are needed against MRSA. The development of new drugs or alternative therapeutics is urgently necessary.

Marine algae in human consumption has been documented since 600 BC. But marine algae have been the group of organisms that have received more attention in field of marine natural products over the last 25 years (Chapman *et al*., 1980; Rhu *et al*., 1989). Marine organisms have become an important source of pharmacologically

active metabolites. Many of published reviews have been reported the importance of these organisms as potential sources of pharmaceutical leads (Tim *et al.*, 2004). Among marine organisms, seaweeds are suitable for human food and animal feed as well as for fertilizer, fungicides, herbicides, and phycocolloids; algin, carrageenan, and agar (Chapman *et al.*, 1980). Seaweeds are also known to have some specific metabolites that are having such biologically activities as antifungal, antioxidation, antivirus and antitumor activity, and prevention of such geriatric diseases such as arteriosclerosis, myocardial infarction and hypertension (Kim *et al.*, 2006; Nagayama *et al.*, 2002; Chen *et al.*, 2002) and moreover seaweeds have abundant polysaccharides, various minerals and vitamins.

In an effort to discover an alternative therapeutic agent, seaweed extracts have been screened for antibacterial activity against MRSA. Among them, the extract from *Ecklonia cava* was exhibited an antibacterial activity against MRSA (in this study). *E. cava* is commonly used as foodstuff, pharmaceutical and nutraceutical materials (Bu *et al.*, 2006; Kim *et al.*, 2005). The residue of *E. cava*, which is a by-product of the seaweed products, has been disposed as a fertilizer or an industrial waste. However, it is expected that the seaweed residue still contains many of bioactive compounds in the tissues.

Fermentation is a biochemical reaction that splits complex organic compounds into relatively simple components. Also, microbial

fermentation results in the break-down of bioactive compounds or the production of bioactive compounds (Adewusi *et al.*, 1999; Achinewhu *et al.*, 1998). In order to reuse the *E. cava* residue, new approach using microbial fermentation was performed in this study.

Thus, the objective of this study was to identify antibacterial substances from *E. cava* against MRSA and to evaluate the possibility of the industrial reuse of *E. cava* residue, which has been massively produced after the extraction of the raw seaweed, by microbial fermentation.

Materials and Methods

1. Materials

1.1. Plant materials

Ecklonia cava and its residue, which was the by-product of hot-water extraction of the raw seaweed, were kindly provided by Professor Yu-Jin Jen (Jeju National University, Jeju, Korea). Other seaweeds were purchased at Gi-jang market, Busan, Korea in March 2008. The raw seaweeds were rinsed with fresh water to eliminate foreign materials such as sand, shells, and others. Then, the sample was soaked into two volumes of water for one hour to desalinize. This process of desalination was repeated 2-3 times and then dried by a frozen drier (Ilshin, No.TFD5503, Korea). The samples were cut with grinded mill to make it into an efficient size for extraction. The residue of *E. cava* residue obtained after hot-water extraction was also dried by the frozen-dryer.

1.2. Microorganism and culture

The strain used for microbial fermentation in this study was *Aspergillus oryzae*, which was isolated from Nuruk and kept at the Laboratory of Food Microbiology, Pukyong National University. The strain was grown aerobically at 30℃ in Yeast and Mold (YM) broth (Difco, USA). The medium was acidified to pH 4.0 by adding a sterile acidic solution [10% HCl (v/v) and 1.5% tartaric acid (w/v)] using aseptic technique. Mycelium was harvested after incubating for 5 days.

The bacterial strains used for evaluation of antibacterial activity in this study were *Staphylococcus aureus* (KCTC 1927), and two methicillin-resistance *Staphylococcus aureus* (MRSA; KCCM 40510 and KCCM 40511), 7 clinical MRSA strains isolated at Dong-A University Hospital (Busan, Korea), *Bacillus cereus* (KCTC 3624), *Bacillus subtilus* (KCTC 1028), *Staphylococcus aureus* (KCTC 1927), *Escherichia coli* (KCTC 1682), *Salmonella typhimurium* (KCTC 1925). These strains were from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea) and the Korea Culture Center of Microorganisms (KCCM; Seoul, Kore). All strains were grown aerobically at 37℃ in Mueller Hinton broth (MHB, Difco, USA) for minimal inhibitory concentration (MIC) assay and in Mueller-Hinton agar (MHA; Difco, USA) for the disc diffusion assay.

2. Methods

2.1. Fermentation

In order to ferment the residue, the mixture of *E. cava* residue was prepared with adding 20 folds of water and autoclaving. The fermentation was carried out in a flask (1 L) with 300 ml of mixture, which was previously inoculated with 1% mycelium of *A. oryzae* (W/V). Samples were incubated aerobically with agitating at 30℃ under 120 rpm and samples was taken at regular intervals (Oboh *et al.*, 2002).

2.2 Determination of cell growth

The growth of *A. oryzae* in the mixture was monitored to measure viable cell count during the periods of fermentation. The viable cell count was performed to follow the standard plate count agar method (Bertrand *et al.*, 1997).

2.3. Physiochemical analysis

During the fermentation of mixture, the changes of pH were also monitored. The pH was measured by a pH meter (Accumet model 15 pH meter; Denver Ins. Co., USA).

2.4. Extraction and fractionation of samples

Dried samples were grinded and finely powdered with food mixer (HMF-1000A, Hanil Electronics, Korea). The powdered samples (15 g) was extracted with 300 ml of methanol at 80℃ for 3 hrs.

The methanolic extract was concentrated by rotary evaporation at 40℃. A liquid - liquid solvent fractionation procedure was performed to fraction an antibacterial substance according to relative polarity. The methanolic extract was evaporated with a rotary evaporator and then dissolved in 2 L of distilled water. The hydrated solution was fractionated by the same volume of n-hexane (C_6H_{14}), dichloromethane (CH_2C_{l2}), ethyl acetate (EtOAc), n-buthanol (n-BuOH), and water (H_2O) in that order. After shaking vigorously and allowing the two phases to settle, the lower (aqueous) phase was removed. Then, additional 2 L of *n*-hexane was added into the previous aqueous phase, shaken, allowed to equilibrate, and then removed the aqueous phase at six times. The hexane phases were combined and evaporated with a rotary evaporator. Other organic solvents were also fractionated in the order of polarity and evaporated as descried above.

The scheme of extraction and solvent fractionation was illustrated in Fig. 1. Finally, *n*-hexane, dichloromethane, ethyl acetate, *n*-buthanol, and water fraction were obtained. The concentration of fractions were adjusted to 200 mg per ml by dissolving in dimethyl sulfoxide reagent and used for further study.

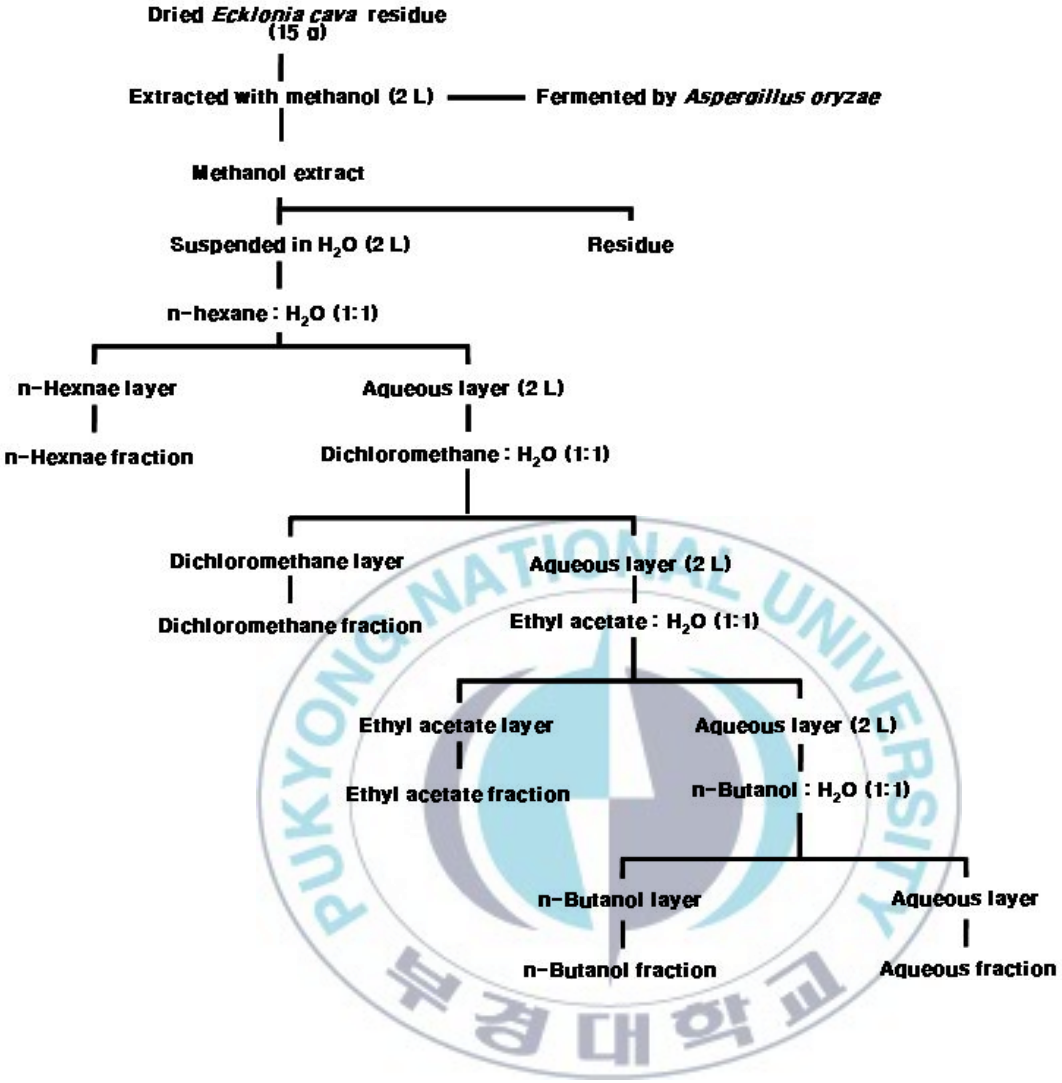

Fig. 1. Scheme of extraction and liquid-liquid solvent fraction.

After fungal fermentation of *E. cava* residue, the same volume of indicated solvent was added and extracted to shake at room temperature for 1 hrs. The extract was concentrated and fractionated as described above.

2.5. Polymerase chain reaction (PCR) amplification

In order to confirm a *mecA* gene from MRSA, MRSA strains were cultured 18 hr cuture in Tryptic Soy Broth (TSB, Difico, USA). Then, 3 ml of cell culture was collected and chromosomal DNA was prepared using a genomic
DNA extraction kit (*Acu-Prep*®; Bioneer, Daejeon, Korea). The chromosomal DNA were used as a template for PCR reaction. DNA was amplified through 30 cycles of denaturation (94℃, 30 s), annealing (50℃, 30 s), and polymerization (72℃, 60 s). A 10 μl aliquot of amplified DNA was examined by electrophoresis through a 1% agarose gel in 0.5 x TBE buffer (10 x TBE; 108 g Tris; 55.5 g boric acid; 9.3 g EDTA, pH 8.3; and adjusted to 1000 ml with H_2O). The amplified PCR product is expected to be about 500 bp (Eom *et al.*, 2008; Murakami *et al.*, 2002; Kim *et al.*, 1993).

2.6. Disk diffusion method

The antibacterial activity was evaluated by a growth inhibition assay. Test strains were cultured in TSB at 37℃ until the cell concentrations reached at 0.5 McFarland standard turbidity. One ml of bacterial culture containing approximately 10^4 CFU per ml were spread on MHA plate and a paper disc (6 mm in diameter) containing 1 mg of each extract was then placed in the plate. After incubating 24 hr at 37℃, the diameter of inhibition zone was measured.

2.7. Measurement of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) of the extracts and vancomycin was determined by the two-fold serial dilution method in MHB as described by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). MIC was defined as the lowest concentration of crude extract that inhibited the visual growth after incubation at 37℃ for 24 hr and was performed in triplicates.

2.8. Determination of an antibacterial substance against MRSA

2.8.1. Analytical methods

In order to identify an MRSA substance from the solvent soluble fractions of *E. cava* residue, a high performance liquid chromatography (HPLC) analysis was performed using a C_{18} reverse-phase column (ODS aqua 125A, 250 × 10 mm; Phenomenix, USA) with an Agilent 1100 HPLC system (Agilent Tech, USA). For the detection of a bioactive substance, a linear gradient elution of 90% water with 10% methanol (v/v) to 100% methanol was used at a flow rate of 0.8 ml per min for 45 min. The eluate was monitored at 230 nm (Aznar *et al.*, 2001).

2.8.2. Evaluation of anti-MRSA activity

In order to evaluate anti-MRSA activity of each fraction and putative anti-MRSA substance(s), the MIC test was performed as described above.

Results and Discussion

1. Detection of the *mecA* gene in MRSA strains

MRSA horizontally acquired *mecA* gene encoding a penicillin-binding protein, PBP2a, which is intrinsically insensitive to methicillin and all β-lactams (third-generation cephalosporins, cefamycins, and carbapenems) (Foster T.J., 2004). As antibiotic application has been increased, Staphylococcal resistance rapidly developed.

Since MRSA strains are important for staphylococcal infection, the presence of *mecA* gene in MRSA strains used in this study were identified by the PCR assay using the *mec*A gene specific primers as described in Materials and Methods. As shown in Table 1, the PCR primers were constructed based on the previous reports (Eom *et al.*, 2008; Murakami *et al.,* 2002; Kim *et al.,* 1993). The PCR amplification generated the same size of DNA products (about 500 bp) in a standard and 7 clinically isolated MRSA strains (Fig. 2). However, none of the specific PCR product was observed in the methicillin sensitive *S. aureus* (MSSA) under the same condition. It has been known that MRSA horizontally acquired *mecA* gene encoding a penicillin-binding protein, PBP2a, which is intrinsically insensitive to methicillin and all β-lactams (third-generation cephalosporins, cefamycins, and carbapenems) (Foster T.J., 2004). Considering above

results, it was concluded that the antibacterial resistance of the MRSA strains tested in this study was resulted from the existence of *mecA* gene.

2. Anti-MRSA activity of seaweed extracts

In preparing medicinal and physio-functional materials, it is common to soak them in alcohol or organic solvent. To screen natural resources exhibiting an antibacterial activity against MRSA, which is the most problematic gram-positive bacterium in public health, it was previously prepared methanol extracts of various seaweeds as described in Material and Methods. Antibacterial activity of methanol extracts against MRSA was qualitatively assessed by measuring inhibition zones (Table 2).

Among them, the extract from *E. cava* and its residue only showed antibacterial activity against both *S. aureus* and MRSA strains tested in this study, suggesting that *E. cava* contains an antibacterial substance against drug-resistant *S. aureus,* MRSA. Also, the extracts exhibited almost similar antibacterial activity against *S. aureus*, a food-borne pathogenic bacterium used as control.

It was also assessed that the antibacterial activity of the seaweed extracts against the bacterial pathogens associated with food poisoning

Table 1. Sequence of primers used for detection of *mecA* gene

Primer	Sequence	Map position
mecA-F	5′-AAAATCGATGGTAAAGGTTGGC-3′	1282-1303
mecA-R	5′-AGTTCTGCAGTACCGGATTTGC-3	1793-1814

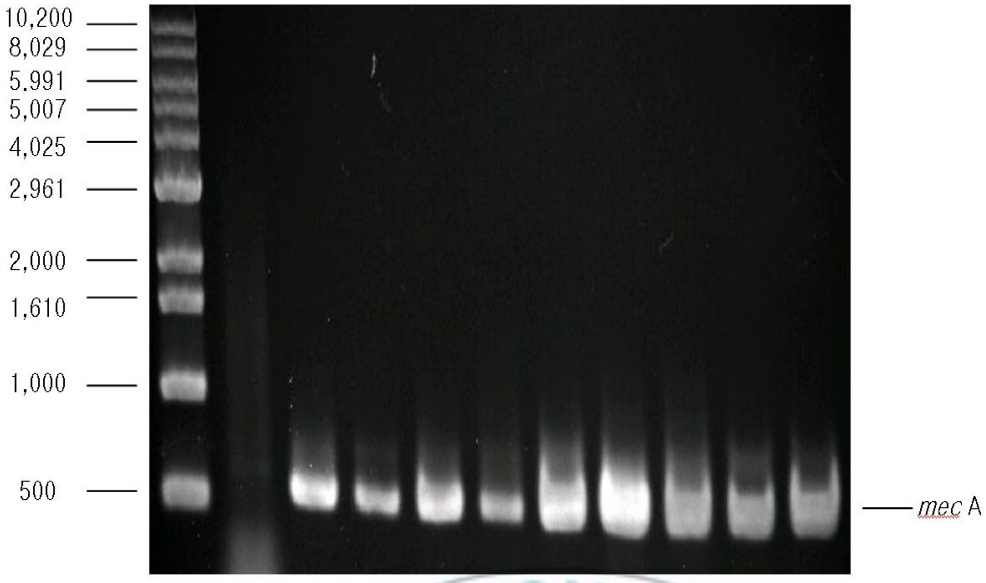

Fig. 2. PCR detection of *mecA* gene in methicillin resistant *Staphylococcus aureus* (MRSA). Two standard and 7 clinically isolated strains were used (Eom *et al.*, 2008). The PCR primers used were mecA-F/mecA-R which were specific to *mec*A gene only. lane 1, 1 kb ladder serving as molecular weight marker; lane 2, *S. aureus*; lane 3, MRSA (KCCM 40510); lane 4, MRSA(KCCM 40511); lane 5, DH 3; lane 6, DH 4; lane 7, DH 5; lane 8, DH 6 ; lane 9, DH 8; lane 10, DH 10; lane 11, DH 17; respectively.

Table 2. Antibacterial activity of methanolic extracts of various seaweeds against *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA)

Seaweeds	Zone of inhibition (mm)									
	MRSA (KCCM 40510)	MRSA (KCCM 40511)	MRSA (DH 70503)	MRSA (DH 70504)	MRSA (DH 70505)	MRSA (DH 70506)	MRSA (DH 70508)	MRSA (DH 70510)	MRSA (DH 70517)	*S. aureus* (KCTC 1927)
Ecklonia cava	18.0	15.0	14	13	18	12	14	13	11	12
Ecklonia cava residue	16.0	15.0	16	14	12	14	17	13	12	12
Laminaria japonica aresch	-	-	-	-	-	-	-	-	-	-
Undaria pinnatifida	-	-	-	-	-	-	-	-	-	-
Sargassum fulvellum	-	-	-	-	-	-	-	-	-	-
Hizikia fusiforme	-	-	-	-	-	-	-	-	-	-
Gracilaria verrucosa	-	-	-	-	-	-	-	-	-	-
Gelidium amansii	-	-	-	-	-	-	-	-	-	-

5 mg of methanol extract from various samples was loaded onto a disk (6 mm in diameter). Data are the averages of duplicate experiments. -, no detected antibacterial activity.

and spoilage (Table 3). *E. cava* and its residue also evidenced antibacterial activity against the Gram-positive and -negative bacteria tested in the present study. The clear zone of both extracts against bacterial pathogens were in the range of 7-19 mm, thus indicating that this compound is less effective against *E. coli*.

Interestingly, the methanolic extract of *E. cava* residue exhibited almost similar anti-MRSA activity compared to the extract of raw material. This result suggested that the seaweed residue still contains many of bioactive compounds in the tissues and will be useful as a valuable resource. In order to evaluate the possibility of the residue as a natural antibiotic or therapeutic substance against MRSA and to identify an anti-MRSA substance from the seaweed, further experiments were performed.

To perform more detailed investigation on antibacterial activity, the methanol extract of *E. cava* residue was further fractioned with organic solvents such as n-hexane (C_6H_{14}), dichloromethane (CH_2C_{l2}), ethyl acetate (EtOAc), n-buthanol (n-BuOH), and water (H_2O) as illustrated in Fig. 1. A lyophilized powder (15 g) of *E. cava* residue was percolated in methanol (3 times × 300 ml), followed by partitioning with several organic solvents to yield *n*-hexane (0.005 g), dichloromethane (0.01 g), ethyl acetate (0.295 g), *n*-butanol (0.36 g) and water fractions (0.33 g).

The anti-MRSA activity of n-hexane, dichloromethane, EtOAc, n-BuOH, and water from the methanol extract was evaluated by

Table 3. Antibacterial activity of methanolic extracts of various seaweeds against food-borne pathogenic or -spoilage bacteria

Seaweeds	Zone of inhibition (mm)			
	Bacillus cereus (KCTC 3624)	*Bacillus subtilis* (KCTC 1028)	*Escherichia coli* (KCTC 1682)	*Salmonella typhimurium* (KCTC 1925)
Ecklonia cava	16	15	9	19
Ecklonia cava residue	15	16	7	18
Laminaria japonica aresch	-	-	-	-
Undaria pinnatifida	-	-	-	-
Sargassum fulvellum	-	-	-	-
Hizikia fusiforme	-	-	-	-
Gracilaria verrucosa	-	-	-	-
Gelidium amansii	-	-	-	-

5 mg of methanol extract from various samples was loaded onto a disk (6 mm in diameter). Data are the averages of duplicate experiments. -, no detected antibacterial activity.

measuring inhibition zones. Among them, the ethyl acetate fraction showed the strongest anti-MRSA activity, which was even higher than that of the methanol extract (Table 4). Among other fractions, butanol fraction only showed anti-MRSA activity, even though the activity was less than that of ethyl acetate. No significant activity, however, was observed in the other fraction (Table 4).

Additionally, the antibacterial activity of the *E. cava* residue against the bacterial pathogens associated with food poisoning and spoilage was investigated (Table 5). The methanolic extract of *E. cava* residue exhibited antibacterial activity against bacteria expect *E. coli*. Among the solvent fraction of the extract, the ethyl acetate fraction showed the strongest antibacterial activity, which was even higher than that of the methanol extract (Table 5).

3. Change of general properties by *A. oryzae* fermentation in water extract of *E. cava* residue

3.1. Microbial growth

During fermentation of the residue mixture, the fungal cell growth of *A. oryzae* was monitored as described in Materials and Methods.

As shown in Fig. 3. the fungal cells were increased over the fermentation periods.

Table 4. Antibacterial activity of *Ecklonia cava* residue extracts against *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA)

Profile of solvent extract	Zone of inhibition (mm)									
	MRSA (KCCM 40510)	MRSA (KCCM 40511)	MRSA (DH 70503)	MRSA (DH 70504)	MRSA (DH 70505)	MRSA (DH 70506)	MRSA (DH 70508)	MRSA (DH 70510)	MRSA (DH 70517)	*S. aureus* (KCTC 1927)
Methanol extract	9	8	8	7	7	8	8	7	7	7
Hexane fraction	-	-	-	-	-	-	-	-	-	-
CH_2Cl_2 fraction	-	-	-	-	-	-	-	-	-	-
Ethyl acetate fraction	10	9	9	7	9	8	10	8	7	7
Buthanol fraction	8	7	7	7	7	7	7	7	7	-
Water fraction	-	-	-	-	-	-	-	-	-	-

Solvent fractions were obtain as described in materials and Methods. 1 mg of each sample was loaded onto a disk (6 mm in diameter). Data are the averages of duplicate experiments. -, no detected antibacterial activity.

Table 5. Antibacterial activity of *Ecklonia cava* residue extracts against food-borne pathogenic or -spoilage bacteria

Profile of solvent extract	Zone of inhibition (mm)			
	Bacillus cereus (KCTC 3624)	*Bacillus subtilis* (KCTC 1028)	*Escherichia coli* (KCTC 1682)	*Salmonella typhimurium* (KCTC 1925)
Methanol extract	8	8	-	7
Hexane fraction	-	-	-	-
CH_2Cl_2 fraction	-	-	-	-
Ethyl acetate fraction	9	10	-	11
Buthanol fraction	7	8	-	8
Water fraction	-	-	-	-

Solvent fractions were obtain as described in materials and Methods. 1 mg of each sample was loaded onto a disk (6 mm in diameter). Data are the averages of duplicate experiments. -, no detected antibacterial activity.

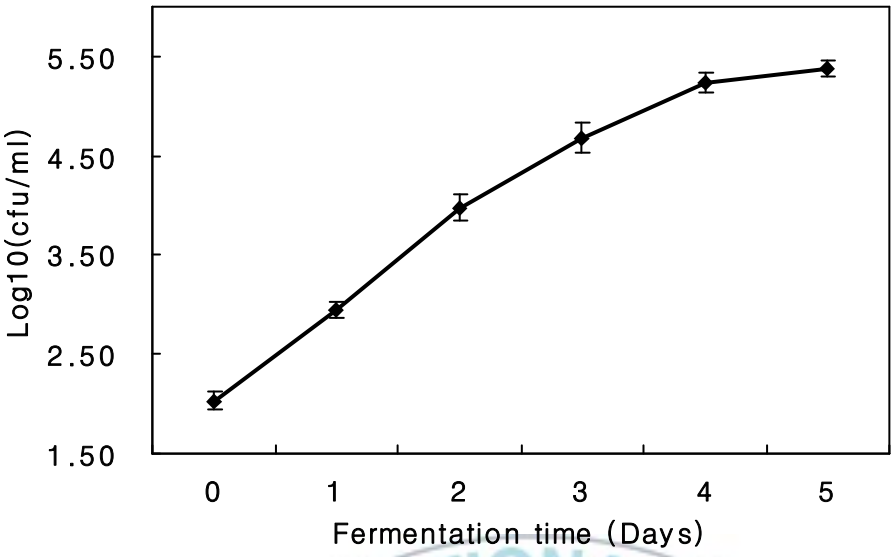

Fig. 3. Fungal cell growth in water extract of *Ecklonia cava* residue. *Aspergillus oryzae* was inoculated and the cell growth was and monitored as described in Materials and Methods.

The highest growth of *A. oryzae* was observed after 2-3 days of fermentation and the growth was apt to decrease afterward.

3.2. Changes of pH

Changes of pH in the mixture were investigated during fermentation. As shown in Fig. 4, pH was increasing as extended the periods of fermentation. However, no significant difference of pH value was observed, indicating that the growth of *A. oryzae* has little influence on pH value. The reason for increasing the pH value during fermentation was unclear because it has been known that the growth of *A. oryzae* results in acidification due to production of organic acids (Bertrand *et al.*, 1997).

3.3. Change of yield of the extracts after *A. oryzae* fermentation of *E. cava* residue

After fermentation of *E. cava* residue (15 g), the extract was percolated in methanol (3 times × 300 ml), followed by partitioning with several organic solvents to yield *n*-hexane, dichloromethane, ethyl acetate, *n*-butanol and water fractions.

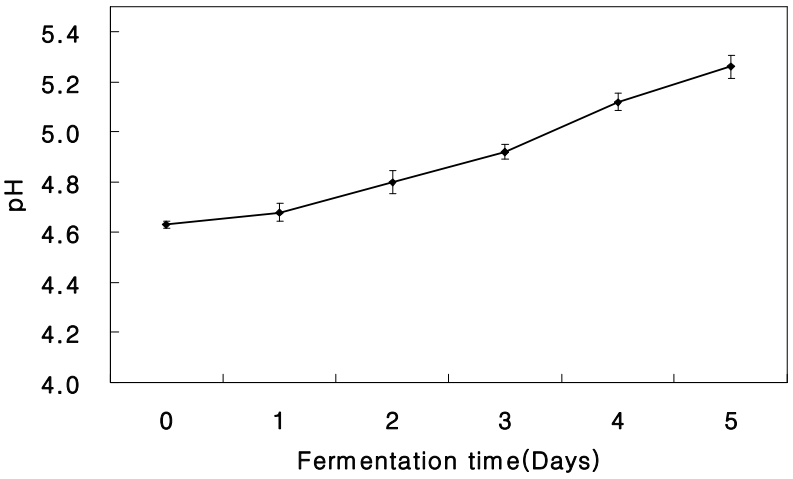

Fig. 4. Changes of pH in water extract of *Ecklonia cava* residue during *Aspergillus oryzae* fermentation.

As shown in Table 6, the yield of methanolic extract was increasing as extended the periods of fungal fermentation. Among solvent fraction of the extract, the yield of ethyl acetate fraction was dramatically increased. After 4-5 days of fermentation, the yield of ethyl acetate fraction was increase about 1.4 fold. The results obtained in this experiment suggested that the fungal fermentation mainly resulted in the break-down of ethyl acetate soluble compounds in the residue or in stimulating the solubility of compounds to ethyl acetate.

4. Change of antibacterial activity of *E. cava* residue by *A. oryzae* fermentation

As shown in Table 2, the methanolic extract of *E. cava* residue exhibited almost similar anti-MRSA activity compared to that of the raw material, suggesting that the seaweed residue still contains many of bioactive compounds such as anti-MRSA substance(s) and will be useful as a valuable resource to develope a natural antibiotic or therapeutic substance against MRSA.

It has been known microbial fermentation is a biochemical reaction that splits complex organic compounds into relatively simple components. As the result, the fermentation results in the break-down of bio-active compounds or the production of bio-active compounds (Adewusi *et al.*, 1999; Achinewhu *et al.*, 1998).

Table 6. Change of yield of *Ecklonia cava* residue extracts by *Aspergillus oryzae* fermentation

Extracts	Fermentation periods (days)					
	0	1	2	3	4	5
methanoic extract	1.0 g	1.05 g	1.03 g	1.048 g	1.131 g	1.130 g
n-hexane fraction	0.005 g	0.004 g	0.004 g	0.005 g	0.005 g	0.004 g
CH_2Cl_2 fraction	0.01 g	0.01 g	0.02 g	0.024 g	0.026 g	0.026 g
EtOAc fraction	0.295 g	0.296 g	0.306 g	0.329 g	0.378 g	0.396 g
n-BuOH fraction	0.36 g	0.38 g	0.35 g	0.36 g	0.355 g	0.355 g
H_2O fraction	0.33 g	0.33 g	0.35 g	0.35 g	0.345 g	0.345 g

* CH_2Cl_2 : dichloromethane EtOAc : ethyl acetate
n-BuOH : n-butanol H_2O : water

As mentioned above, it was expected that the application of microbial fermentation in the water extract of *E. cava* residue will cause any changes on the anti-MRSA activity whether the activity is incensing or decreasing.

The effect of fermentation on the anti-MRSA activity was investigated to evaluate the activity of *E. cava* residue before and after fermentation. As shown in Table 2, the anti-MRSA activity was only observed in ethyl actate and butanol fractions. After *A. oryzae* fermentation of the residue, the anti-MRSA activity was only observed in the both fractions. In case of ethyl acetate extract, the anti-MRSA activity was increased as extended the periods of fermentation (Table 7). It was observed that the antibacterial activity against food-borne pathogenic bacteria was also increasing. However, the butanol extract of the fungal ferment showed less activity than those of ethyl acetate extract over the periods of fermentation and the activity was apt to decrease against MRSA and other bacteria tested in this study (Table 8). These results was consistent with the result of Table 4 that the ethyl acetate fraction showed the strongest anti-MRSA activity.

5. Measurement of MIC value of *E. cava* residue extracts

To evaluate quantitatively antibacterial activity of *E. cava* residue against MRSA and other bacteria related in food borne and spoilage,

Table 7. Change of antibacterial activity in the ethyl acetate extract of *Ecklonia cava* residue fermented by *Aspergillus oryzae*

Strains	Zone of inhibition (mm)					
	Fermentation periods (days)					
	0	1	2	3	4	5
MRSA[a] (KCCM40510)	8	9	11	11	11	11
MRSA (KCCM40511)	8	8	9	10	8	8
MRSA (DH 70503)	9	10	10	11	8	9
MRSA (DH 70504)	7	8	9	9	7	7
MRSA (DH 70505)	9	9	11	11	11	10
MRSA (DH 70506)	8	8	10	10	8	7
MRSA (DH 70508)	10	10	11	11	8	7
MRSA (DH 70510)	8	9	9	10	9	8
MRSA (DH 70517)	7	7	9	9	8	7
Staphylococcus aureus (KCTC 1927)	7	8	9	10	9	9
Bacillus cereus (KCTC 3624)	8	8	9	8	7	7
Bacillus subtilis (KCTC 1028)	9	9	10	10	9	9
Escherichia coli (KCTC 1682)	-	-	-	-	-	-
Salmonella typhimurium (KCTC 1925)	8	9	9	9	8	8

[a]MRSA, methicillin resistant *Staphylococcus aureus*. The reside extract were obtain with ethyl acetate as described in Materials and Methods. 1 mg of each sample was loaded onto a disk (6 mm in diameter). Data are the averages of duplicate experiments. -, no detected antibacterial activity.

Table 8. Change of antibacterial activity in the butanol extract of *Ecklonia cava* residue fermented by *Aspergillus oryzae*

Strains	Zone of inhibition (㎜)					
	Fermentation periods (days)					
	0	1	2	3	4	5
MRSA[a] (KCCM40510)	9	8	8	7.5	7	8
MRSA (KCCM40511)	8	7.5	7.5	7.5	7	8
MRSA (DH 70503)	9	8	7.5	7.5	7.5	7
MRSA (DH 70504)	7	7	7.5	7.5	7.5	7
MRSA (DH 70505)	8	7.5	7	7	7	8
MRSA (DH 70506)	-	-	-	-	-	-
MRSA (DH 70508)	9	9	8	8	7.5	8
MRSA (DH 70510)	-	-	-	-	-	-
MRSA (DH 70517)	-	-	-	-	-	-
Staphylococcus aureus (KCTC 1927)	7	7	7	7	7	7
Bacillus cereus (KCTC 3624)	7	7	7	-	7	7
Bacillus subtilis (KCTC 1028)	8	7	7	7	7	7
Escherichia coli (KCTC 1682)	-	-	-	-	-	-
Salmonella typhimurium (KCTC 1925)	8	8	8	7	7	8

[a]MRSA, methicillin resistant *Staphylococcus aureus*. The reside extract were obtain with butanol as described in Materials and Methods. 1 mg of each sample was loaded onto a disk (6 mm in diameter). Data are the averages of duplicate experiments. -, no detected antibacterial activity.

its MIC value was determined by the two-fold serial dilution method (Table 10). MIC values of the ethyl acetate fraction against MRSA stains were in the ranges of 128-256 μg per ml (Table 9). The difference MIC values among MRSA strains were observed. The fraction also exhibited an antibacterial activity in the ranges of 256-512 μg per ml against other bacteria including Gram-negative strains. This result suggested that the ethyl acetate fraction may contain other antibacterial substances against Gram-negative bacteria and others. It is well known that vancomycin interferes with cell wall synthesis, as does penicillin, eventually leading to lysis of cell (Barna and Williams, 1984). As the result, this antibiotic is effective only Gram-positive bacteria not Gram-negative. Considering these results, it was supposed that *E. cava* contains a novel anti-MRSA substance and its anti-MRSA mechanism differs from that of vancomycin since vancomycin was not effective against gram-negative bacteria.

The butanol fraction also showed antibacterial activity in the ranges of 256->512 μg per ml against MRSA and other bacteria. However, the antibacterial activity of the fraction was inferior to ethyl acetate fraction (Table 10). These results were also consistent with the results obtained by disc diffusion assay.

In an effort to discover an alternative therapeutic agent against MRSA, several medicinal plants and seaweeds were screened. Among them, the methanolic extract of *E. cava* and its residue exhibited significant antibacterial activity.

Table 9. Minimum inhibitory concentrations (MICs) of *Ecklonia cava* extracts against methicillin-resistant *Staphylococcus aureus* (MRSA) and other strains

Strains	MIC (μg/ml)			
	Methanol extract	Ethyl acetate fraction	Butanol fraction	Vancomycin
MRSA (KCCM40510)	256	64	256	2
MRSA (KCCM40511)	128	64	256	2
MRSA (DH 70503)	128	64	256	2
MRSA (DH 70504)	256	128	512	2
MRSA (DH 70505)	128	64	256	1
MRSA (DH 70506)	128	64	256	2
MRSA (DH 70508)	256	128	512	2
MRSA (DH 70510)	128	64	256	1
MRSA (DH 70517)	256	128	256	1
Staphylococcus aureus (KCTC 1927)	256	128	256	0.5
Bacillus cereus (KCTC 3624)	512	256	512	0.5
Bacillus subtilis (KCTC 1028)	512	256	512	0.5
Escherichia coli (KCTC 1682)	512	256	512	512
Salmonella typhimurium (KCTC 1925)	512	256	512	512

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

Table 10. Minimum inhibitory concentrations (MICs) of *Ecklonia cava* residue extracts against methicillin-resistant *Staphylococcus aureus* (MRSA) and other strains

Strains	MIC (μg/ml)			
	Methanol extract	Ethyl acetate fraction	Butanol fraction	Vancomycin
MRSA (KCCM40510)	256	128	256	2
MRSA (KCCM40511)	256	256	512	2
MRSA (DH 70503)	128	128	256	2
MRSA (DH 70504)	256	256	512	2
MRSA (DH 70505)	256	128	256	1
MRSA (DH 70506)	256	256	>512	2
MRSA (DH 70508)	256	128	256	2
MRSA (DH 70510)	256	256	>512	1
MRSA (DH 70517)	256	256	>512	1
Staphylococcus aureus (KCTC 1927)	256	256	>512	0.5
Bacillus cereus (KCTC 3624)	512	512	512	0.5
Bacillus subtilis (KCTC 1028)	512	256	512	0.5
Escherichia coli (KCTC 1682)	512	512	>512	512
Salmonella typhimurium (KCTC 1925)	512	512	>512	512

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

Additionally, the activity of *E. cava* residue was seemed to enhance by *A. oryzae* fermentation (Table 7). To evaluate quantitatively the change of antibacterial activity of *E. cava* residue by fungal fermentation, its MIC value was determined by the two-fold serial dilution method (Table 11 and 12). MIC values of the ethyl acetate fraction against MRSA stains over the periods of fermentation were in the ranges of 64-256 μg per ml (Table 11). Also, the anti-MRSA activity was increased as extended the periods of fermentation and the activity was not reduced over the periods of fermentation in ethy acetate fraction. The highest anti-MRSA activity against most MRSA strains was determined after 2-3 days of fermentation (Table 11). However, no significant difference of antibacterial activity against other food-borne pathogenic bacteria tested in this study was observed (Table 11). In contrary to the ethyl acetate extract, the increase of anti-MRSA activity by fungal fermentation was not observed in butanol fraction of *E. cava* residue (Table 12). These results indicated that a anti-MRSA substance(s) of *E. cava* was increased by fungal fermentation and many of them will be ethyl acetate soluble compounds.

Table 11. Minimum inhibitory concentrations (MICs) of the ethyl acetate extract of *Ecklonia cava* residue fermented by *Aspergillus oryzae* against methicillin-resistant *Staphylococcus aureus* (MRSA) and other strains

Strains	MIC (μg/ml)					
	Fermentation periods (days)					
	0	1	2	3	4	5
MRSA (KCCM40510)	128	128	64	64	128	128
MRSA (KCCM40511)	256	128	128	64	128	128
MRSA (DH 70503)	128	128	64	64	128	128
MRSA (DH 70504)	256	128	64	64	128	128
MRSA (DH 70505)	128	128	64	128	128	128
MRSA (DH 70506)	256	256	128	128	256	256
MRSA (DH 70508)	128	128	64	128	128	128
MRSA (DH 70510)	256	128	64	64	128	128
MRSA (DH 70517)	256	256	128	128	256	256
Staphylococcus aureus (KCTC 1927)	256	256	128	128	256	256
Bacillus cereus (KCTC 3624)	512	512	512	512	512	512
Bacillus subtilis (KCTC 1028)	256	256	128	128	256	256
Escherichia coli (KCTC 1682)	512	512	512	512	>512	>512
Salmonella typhimurium (KCTC 1925)	512	512	512	512	512	512

The reside extract were obtain with ethyl acetate as described in Materials and Methods. MIC of each solvent extract and vancomycin was determined by the two-fold serial dilution method in Mueller Hinton broth.

Table 12. Minimum inhibitory concentrations (MICs) of the buthanol extract of *Ecklonia cava* residue fermented by *Aspergillus oryzae* against methicillin-resistant *Staphylococcus aureus* (MRSA) and other strains

Strains	MIC (μg/ml)					
	Fermentation periods (days)					
	0	1	2	3	4	5
MRSA (KCCM40510)	256	256	256	512	512	512
MRSA (KCCM40511)	512	512	512	512	>512	>512
MRSA (DH 70503)	256	256	512	512	512	512
MRSA (DH 70504)	512	512	512	512	512	512
MRSA (DH 70505)	256	256	256	512	512	512
MRSA (DH 70506)	>512	>512	>512	>512	>512	>512
MRSA (DH 70508)	256	256	256	512	512	512
MRSA (DH 70510)	>512	<512	>512	>512	>512	>512
MRSA (DH 70517)	>512	>512	>512	>512	>512	>512
Staphylococcus aureus (KCTC 1927)	512	512	512	512	512	>512
Bacillus cereus (KCTC 3624)	512	512	512	512	>512	>512
Bacillus subtilis (KCTC 1028)	512	512	512	512	512	512
Escherichia coli (KCTC 1682)	>512	>512	>512	>512	>512	>512
Salmonella typhimurium (KCTC 1925)	>512	>512	>512	>512	>512	>512

The reside extract were obtain with butanol as described in Materials and Methods. MIC of each solvent extract and vancomycin was determined by the two-fold serial dilution method in Mueller Hinton broth.

6. Isolation and characterization of anti-MRSA substances from *E. cava* residue

6.1 Identification of an antibacterial substance against MRSA

As described above, the methanolic extract of *E. cava* residue exhibited significant antibacterial activity against MRSA (Table 2). Additionally, ethyl acetate soluble fractions from the methanolic extract evidenced a profound anti-MRSA activity, which was even more prominent than that of the methanol extract (Table 4). Other fractions was demonstrated less pronounced anti-MRSA activity than that of the ethyl acetate fractions. Also, the anti-MRSA activity in the ethyl acetate extract of *E. cava* residue was enhanced by *A. oryzae* fermentation (Table 7 and 11). These results strongly suggested that ethyl acetate soluble compounds of *E. cava* would be mainly related with the anti-MRSA activity. In *E. cava*, phlorotannins has been known to be major ethyl acetate soluble compounds (Fig. 5 and 6). Phlorotannins are polyphenols formed via the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) units and are bio-active compounds known to function as antioxidant compounds (Taniguchi *et al.*, 1991). Among them, dieckol are recently known to function as an anti-MRSA compound (Lee *et al.,* 2008).

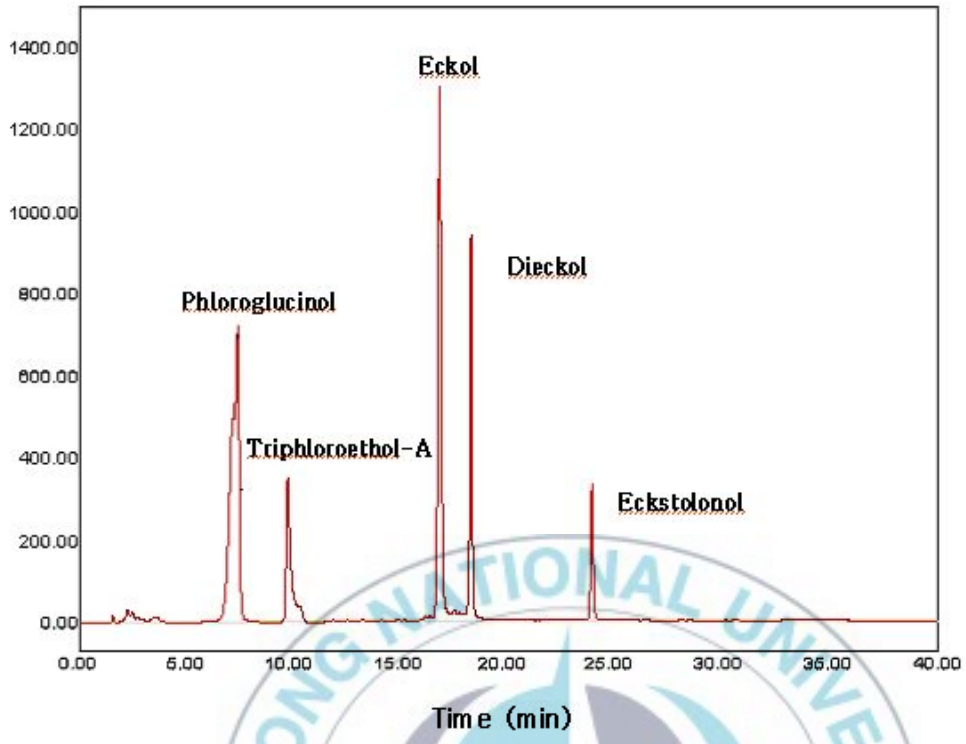

Fig. 5. HPLC elution profile of standard phlorotannins. Phlorotannins were isolated and analyzed by HPLC as described in Materials and Methods.

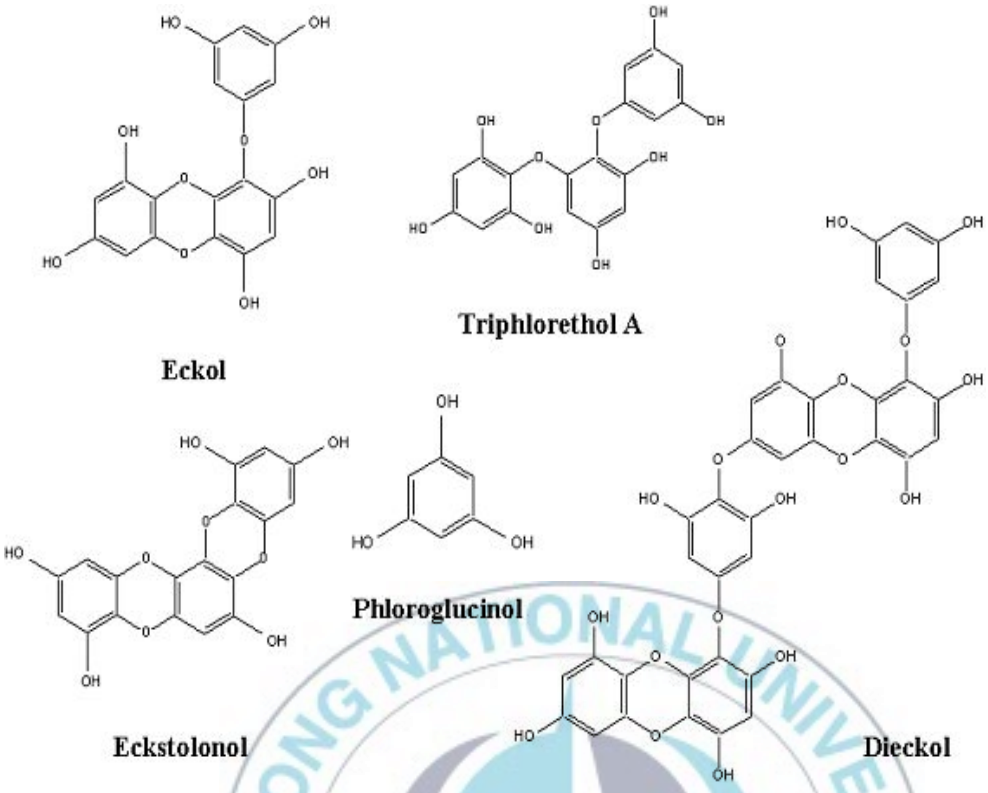

Fig. 6. Structures of phlorotannins isolated from *Ecklonia cava*.

From these results, it was hypothesized that the anti-MRSA activity observed in *E. cava* may have originated from ethyl acetate soluble compounds, mainly dieckol. In order to verify this hypothesis, HPLC analysis was conducted and the content of phlorotannins in ethyl acetate extract was investigated over the periods of fungal fermentation.

As has been expected, the total content of phlorotannins in the ethyl acetate extract were increased as extended the periods of fermentation. The highest content of phlorotannins was observed after 2 days of fermentation and then the content was apt to decreased afterward (Table 13). The dieckol was a major compounds compared to other phlorotannins over all periods of fermentation. The highest content of dieckol was observed after 2 days of fermentation that is consistent with the results of anti-MRSA activity of ethyl acetate extract against MRSA (Table 10). Thus, dieckol is one of major anti-MRSA substance in *E. cava*.

6.2. Measurement of MIC of phlorotannins against MRSA

As mentioned above, dieckol is verified to be one of major anti-MRSA substance in *E. cava*. However, there is no information about antibacterial or anti-MRSA activity of phlorotannins.

Table 13. Change of phlorotannins contents in the ethyl acetate extract of *Ecklonia cava* residue fermented by *Aspergillus oryzae*

Fermentation periods (days)	Phlorotannins contents (mg/g dry extract)					
	PG	TA	EK	DK	ES	Total
0 days	0.62	1.04	45.99	131.93	1.13	180.71
1 days	12..3	1.60	61.82	139.42	4.43	219.40
2 days	21.46	1.48	72.72	162.26	10.64	268.55
3 days	34.33	3.13	107.30	119.34	2.64	266.74
4 days	24.98	2.87	89.10	89.21	1.90	208.06
5 days	22.66	2.39	69.39	86.91	3.32	184.67

Phlorotannins were isolated and identified by HPLC analysis. DK, Dieckol; EK, eckol; ES, eckstonol; PG, phloroglucinol; TA, triphlorethol A.

To quantitatively evaluate anti-MRSA activity of phlorotannins, therefore, its MIC value was determined (Table 14). All of phlorotannins exhibited significant anti-MRSA activity. As expected, the dieckol showed the strongest activity against MRSA among phlorotannins. The MICs of dieckol in the ranges of 32-128 μg per ml were equal to or less than those of other phlorotannins against the MRSA strains (Table 14).

Considering all above results, the *A. oryzae* fermentation of *E. cava* residue resulted in the increase of the content of phlorotannins, which is related with the anti-MRSA activity in *E. cava*. As the result, the highest anti-MRSA activity was observed after 2-3 days of fungal fermentation, at which the content of phlorotannins was determinded to be the highest. Thus, the fungal fermentation would expected to stimulate a break-down of ethyl acetate soluble compounds in the residue.

Table 14. Minimum inhibitory concentrations (MICs) of phlorotannins originated from *Ecklonia cava*

Strains	MIC (μg/ml)				
	Phloroglucinol	Triphlorethol A	Eckol	Dieckol	Eckstolonol
Staphylococcus aureus (KCTC 1927)	256	256	256	128	528
MRSA[a] (KCCM40510)	128	64	64	32	64
MRSA (KCCM40511)	512	256	128	64	128
MRSA (DH 70503)	512	512	64	64	128
MRSA (DH 70504)	512	512	64	128	128
MRSA (DH 70505)	512	128	256	128	128
MRSA (DH 70506)	256	512	128	64	256
MRSA (DH 70508)	512	256	128	64	256
MRSA (DH 70510)	512	512	128	128	256

[a]MRSA, methicillin resistant *Staphylococcus aureus*. MIC of each solvent extract and vancomycin was determined by the two-fold serial dilution method in Mueller Hinton broth.

Summary

In an effort to discover an alternative therapeutic agent against MRSA, several medicinal plants and seaweeds were screened. Among them, the extract of *E. cava* residue exhibited significant antibacterial activity against MRSA. To perform more detailed investigation on antibacterial activity, the extract was further fractioned with organic solvents such as n-hexane (C_6H_{14}), dichloromethane (CH_2C_{12}), ethyl acetate (EtOAc), n-buthanol (n-BuOH), and water (H_2O). Among them, the ethyl acetate fraction showed the strongest anti-MRSA activity and MIC values of the fraction against MRSA stains were in the ranges of 128-256 μg per ml. Interestingly, the anti-MRSA activity of *E. cava* residue was seemed to enhance by *A. oryzae* fermentation. After 2-3 days of fungal fermentation of *E. cava* residue, the MIC values of ethyl acetate extract against MRSA stains in the ranges of 64-128 μg per ml.

Interestingly, the yield of solvent soluble compounds was also increased as extended the periods of fungal fermentation. Among the solvent extracts, the yield of ethyl acetate fraction was dramatically increased. From these results, it was supposed that the fungal fermentation mainly resulted in the break-down of ethyl acetate soluble compounds in the *E. cava* residue and the ethyl acetate soluble compounds of *E. cava* would be mainly related with the

anti-MRSA activity.

In *E. cava*, phlorotannins has been known as major ethyl acetate soluble compounds. All of phlorotannins exhibit significant anti-MRSA activity. Among them, dieckol showed the strongest activity against MRSA. The MICs of dieckol in the ranges of 32-128 μg per ml were equal to or less than those of other phlorotannins against the MRSA strains. The total content of phlorotannins was increased as extended the periods of fermentation. The highest content of phlorotannins was observed after 2 days of fermentation and then the content was apt to decreased afterward. The dieckol was a major compound over all periods of fermentation compared to other phlorotannins. The highest content of dieckol was observed after 2 days of fermentation and the strongest anti-MRSA activity was also observed after 2 days of fermentation. Considering above results, dieckol seems to be one of major anti-MRSA substances in *E. cava*.

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2년 동안의 대학원 생활은 학문적인 면뿐만 아니라 인생에 있어서도 많은 배움의 시간이었습니다. 힘들고 어려웠던 시간도 많았지만 그때마다 힘이 되어준 분들 덕분에 무사히 마칠 수 있었던 것 같아 이렇게 감사의 마음을 전합니다.

본 논문이 완성되기까지 부족하고 못난 제자를 언제나 변함없는 사랑으로 가르침과 격려를 주시고, 학문과 인생의 길에서 많은 것을 보고 배우고 느낄 수 있게 해주신 김영목 교수님께 진심으로 감사드리며, 그 고마움을 평생토록 간직하려 합니다. 그리고, 저의 미흡한 논문을 자상하게 심사해 주시고 유익한 지도편달을 해주셨던 조영제 교수님, 양지영 교수님, 학부 때부터 많은 가르침을 주셨던 이근태 교수님, 김선봉 교수님, 이양봉 교수님, 전병수 교수님, 안동현 교수님께도 감사의 마음을 전하고자 합니다.

본 논문이 완성될 수 있기까지 주위에 계신 많은 분들의 도움이 없었다면 이룰 수 없는 결실이라 생각합니다. 특히, 본 연구를 수행함에 있어 적극적인 지원과 지도를 해주신 제주대학교 전유진 교수님과 미생물학과 이명숙 교수님, 학문적 성장의 지표가 되셨던 동의과학대학 임상병리학과의 허성호 교수님께도 깊은 감사의 말씀을 드리며, 학업의 길을 걸을 수 있도록 도와주신 양식환경연구소 이태식 소장님, 부족한 저에게 따뜻한 격려와 용기와 희망을 주시는 국립수산과학원 식품안전연구과 김지회 연구관님, 이희정 연구관님, 오은경 연구사님, 많은 도움과 격려를 주신 김풍호 연구사님, 목종수 연구사님, 유홍식 연구사님, 조미라 연구사님, 손광태 연구사님, 실험실에 들어와서 아무것도 몰랐던 저에게 학문적 지식과 더불어 삶에 있어서 새로운 방향을 제시해준 저의 정신적 지주이자 사랑하는 이가정 연구사님과 진이언니, 보미언니에게 무엇보다도 고마움을 전합니다. 늘 따뜻한 마음으로 격려해주셨던 신순범 연구사님, 박큰바위 연구사님, 심길보 연구사님, 항상 고마운 김주경님, 함께 울고, 웃고, 어깨를 두드려주

며 격려해준 내 친구 선경이, 정화, 수선이, 지혜언니, 전라도에서 열심히 학생을 가르치고 있을 진순이, 은진이, 영원한 우리의 공익 희성이 에게도 진심으로 감사의 마음을 전합니다. 대학원 생활을 하면서 물신양면으로 많은 도움을 준 식품미생물 실험실의 파파라치 근식이, 마음 넓은 송원선배, 엄삼촌 성환선배, 식품공학과 윤지후 태영, 최강동안 사랑스런 조포비 현아, 말보다 마음이 더 잘 통하는 민승 언니, 넌 어느 별에서 왔니 금잔디 윤경, 베트남 힘쎈 공주 향남, 참 좋은 인연으로 만난 사랑스러운 알 경란, 늘 든든한 우리 실험실 보거스 동원, 간지남 대웅, 날쌘돌이 이광덕, 명품남 해원, 귀여운 슈렉 공룡 재홍, 몸짱 명철, 식품공학과 구준표 형훈, 소이정 호준, 송우빈 연중, 포항공주 은영, 변리사를 꿈꾸는 은주, 이쁜 밥순이 현주, 꽃사슴 선영, 포항 지주 지일, 울산 일진 민정, 날다람쥐 은주와 김보살 선경 후배님들에게도 고마운 마음을 전하고 싶습니다. 또한, 항상 변치 않은 우정을 나눠준 나의 대학동기 혜민, 해성, 정화, 힘들 때 마다 지쳐 쓰러지지 않게 도와준 하늬, 혜연, 묘해 에게도 고마움을 전합니다. 또한, 늘 곁에서 묵묵히 바라봐주며 격려해준 내 사랑하는 보석반지 상희, 연희 그리고 언제나 환한 미소로 기댈 수 있는 어깨가 되어준 주형오빠에게도 너무나도 감사한 마음을 전합니다. 작은 공간에 다 열거하지 못한 그 밖의 소중한 인연을 맺은 분들 모두에게 고마움을 전합니다. 그리고 언제나 힘이 되어주고 든든한 버팀목이 되어준 나의 둘도 없는 동생 장호와 큰딸을 믿어주시고 의견을 존중해주시며 늘 사랑을 아끼지 않으셨던 부모님과 마음으로 걱정해주시는 할머니께 이루 말할 수 없는 감사와 사랑을 전하며 이 논문을 바칩니다. 제가 사랑하고 저를 사랑해주신 모든 사람들 덕분에 힘든 시기를 잘 마칠 수 있었습니다. 진심으로 감사드리며, 항상 모든 일에 최선을 다하고 노력하는 모습으로 살아가도록 하겠습니다.

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