FAS http://e-fas.org
Fisheries and Aquatic Sciences

Original Article
Fish Aquat Sci 18(3), 241-247, 2015

In vitro Antibacterial and Synergistic Activity of an *Ecklonia cava* Extract against Antibiotic-Resistant *Streptococcus parauberis*

Sung-Hwan Eom[1], Jeniel A. Santos[2], Ji-Hoon Kim[3,4], Won-Kyo Jung[4,5], Do-Hyung Kim[2,6] and Young-Mog Kim[2,3,4]*

[1]Korea Food Research Institute, Sungnam 13539, Korea
[2]KOICA-PKNU International Graduate Program of Fisheries Science, Pukyong National University, Busan 48513, Korea
[3]Department of Food Science and Technology, Pukyong National University, Busan 48513, Korea
[4]Marine-Integrated Bionics Research Center, Pukyong National University, Busan 48513, Korea
[5]Department of Biomedical Engineering, Pukyong National University, Busan 48513, Korea
[6]Department of Aquatic Life Medicine, Pukyong National University, Busan 48513, Korea

Abstract

In an effort to discover alternative phytotherapeutic antimicrobial agents to combat *Streptococcus parauberis,* a fish pathogenic bacterium, we evaluated the antibacterial activity of seaweed extracts *in vitro*. A methanolic extract of *Ecklonia cava* exhibited strong antibacterial activity against *S. parauberis* isolated from olive flounder *Paralichthys olivaceus*. Furthermore, the *n*-hexane soluble (Hexane) fraction of the *E. cava* methanolic extract exhibited the greatest antibacterial effect on *S. parauberis* strains with a minimum inhibitory concentration (MIC) ranging from 256 to 1,024 μg/mL. In addition, the MIC values of oxytetracycline against antibiotic-resistant *S. parauberis* were markedly reduced up to 64-fold in combination with the Hexane fraction, suggesting that the antibacterial activity of the antibiotic was restored when combined with the Hexane fraction. The interaction between both antibiotics and the Hexane fraction was assessed by the fractional inhibitory concentration (FIC) index. The Hexane fraction and oxytetracycline combination against antibiotic-resistant *S. parauberis* strains resulted in a median ∑FIC range of 0.502 to 0.516. Thus, the synergistic ranges of median ∑FIC < 1 were observed for all combinations of the Hexane fraction and oxytetracycline against *S. parauberis*. To the best of our knowledge, this is the first report indicating the efficacy of an *E. cava* extract against fish pathogenic bacterium *S. parauberis*.

Key words: Antibiotic resistance, *Ecklonia cava*, Fish pathogen, *Streptococcus parauberis*

Introduction

Over the past several years, streptococcal infections have dramatically increased due to the growth of aquaculture (Baeck et al., 2006; Park et al., 2009). Streptococcosis is a common disease in fish caused by six different Gram-positive streptococcal species (Bercovier et al., 1997). One species, *Streptococcus parauberis* (formerly known as *S. uberis* type II), is an alpha hemolytic Gram-positive bacterium that causes mastitis in cows (Leigh, 2002). To date, this species has emerged as a causative agent of streptococcosis in several fish species, including turbot and olive flounder *Paralichthys*

Received 24 August 2015; **Revised** 5 September 2015
Accepted 8 September 2015

***Corresponding Author**
E-mail: ymkim@pknu.ac.kr

olivaceus (Domenech et al., 1996). In addition, this bacterium was reported to be a major cause of streptococcosis in Korea and the causative agent in an outbreak affecting olive flounder on Jeju Island, Korea in 2005 (Baeck et al., 2006). Streptococcosis caused by *S. parauberis* is becoming an endemic disease that results in external hemorrhaging, a pale friable liver with hepatomegaly in olive flounder (Kim et al., 2006). In trout, streptococcosis results in eye discharge, spleen and kidney congestion, and mucohaemorrhagic enteritis (Domenech et al., 1996).

Broad-spectrum antibiotics are commonly used to control streptococcal infections in aquaculture. Park et al. (2009) reported the isolation of antibiotic-resistant *S. parauberis* strains from olive flounder. Their study suggested the need for new approaches to control and overcome an increase in antibiotic-resistant *S. parauberis*. One alternative approaches is to use compounds that interact with antibiotics to improve their antibacterial efficacy. However, synthetic compounds can be toxic and cause several negative side effects when utilized in combination with antibiotics. Alternatively, phytochemicals from natural resources, including plants, have been reported to alter the efficacy of antibiotics against several pathogenic bacteria (Adwan and Mhanna, 2008; Gupta et al., 2009; Eom et al., 2014; Lee et al., 2014).

Seaweeds contain various metabolites that are capable of inhibiting bacterial growth (Rhimo et al., 2010; Cox et al., 2010; Eom et al., 2013). Among these seaweed species, edible brown alga *Ecklonia cava*, an organism native to the cost of Korea and Japan, has been utilized as an herbal remedy. Extracts from this alga, including fucoidan, fucan sulphate and phlorotannins, exhibit antitumor, anticoagulant, antioxidants and anti-inflammatory activity (Kim et al., 2006; Kang et al., 2012). In addition, this seaweed exhibits antibacterial activity against several human pathogenic bacteria (Hornsey and Hide, 2007; Kandhasamy and Arunachalam, 2008; Eom et al., 2013). Although various studies have shown the effectiveness of seaweed extracts in combating human pathogenic bacteria, there is less information on the antibacterial efficacy of this extract for fish pathogens. Thus, the objective of this study was to evaluate: (i) the antibacterial activity of *E. cava* extract toward the fish pathogenic bacterium *S. parauberis* and (ii) the synergistic effect of the extract and antibiotics in combination as an antibacterial agent against *S. parauberis*.

Materials and Methods

Bacterial strains and growth conditions

In this study, we used *S. parauberis* KSP strains (KSP2, 3, 5, 7, 8, 9, 26, 44, 46, and 47) provided by the Department of Aquatic Life and Medicine, Pukyong National University (Busan, Korea). The Korean Collection for Type Cultures (KCTC; Daejeon, Korea) provided the reference strain of *S. parauberis* (KCTC 3651). All strains were cultured in Brain Heart Infusion broth (BHI) (Difco, Sparks, MD, USA) and incubated at 25°C for 18-24 h.

Samples and extraction method

In total, we used thirteen seaweed species. Dried seaweed powder (1.0 kg) was extracted and fractionated using organic solvents as described by Lee et al. (2014). The methanolic (MeOH) extract of *E. cava* (101.3 g) was fractionated with *n*-hexane (Hexane; 1.0 L × 3), dichloromethane (1.0 L × 3), ethyl acetate (EtOAc) (1.0 L × 3), and *n*-butanol (BuOH) (1.0 L × 4). The water fraction was obtained by filtering the remaining materials from the BuOH fraction. Finally, each extract was evaporated using a rotary evaporator (Eyela, Tokyo, Japan) under a vacuum at 45°C. Another twelve methanolic extracts from various marine algae, previously prepared and stored under appropriate conditions (at -70°C), were used for the same purpose.

Antibacterial assay

We evaluated the antibacterial activity of seaweed extracts and antibiotics against *S. parauberis* strains using a disk diffusion assay as described by the Clinical Laboratory Standard Institute (CLSI, 2012). In brief, *S. parauberis* strains in 3 mL of BHI were grown at 25°C for 24 h. An aliquot of 100 μL of each strain was spread on Mueller Hinton agar (Difco) supplemented with 5% sheep blood. On the inoculated plates, we placed disks containing 5 mg of seaweed extract and disks with standard antibiotics. After incubation for 18-24 h at 25°C, antibacterial activity was measured by the diameter (mm) of the zone of growth inhibition.

The sensitivity of the *S. parauberis* strains to the *E. cava* extract and commercial antibiotics was determined by the two-fold serial dilution method using Cation Adjusted Muller-Hinton Broth (CAMHB) (MBCell, Seoul, Korea) supplemented with 5% lysed horse blood (MBCell) (CLSI, 2014). The minimum inhibitory concentration (MIC) was determined as the lowest concentration of *E. cava* extracts and antibiotics that resulted in the complete inhibition of visible growth in CAMHB after incubation at 25°C with shaking for 12 h.

Checkerboard method using a fractional inhibitory concentration (FIC) assay

Interactions between the *E. cava* EtOAc fraction and antibiotics were tested by the checkerboard method using an FIC assay (Hseish et al., 1993; Meletiadis et al., 2010). The sum of the FICs (∑FIC) was calculated for each well using the following equation: $\sum FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, where MIC_A and MIC_B are the MICs of drugs A and B, respectively, and C_A and C_B are the concentrations of the drugs in combination, respectively, in all wells corresponding to an

MIC (isoeffective combinations). Among the ∑FICs calculated for all isoeffective combinations, we reported the minimum ∑FIC ($\sum FIC_{min}$) and the maximum ∑FIC ($\sum FIC_{max}$) to capture synergistic and antagonistic interactions, respectively. Synergistic effects were evaluated on an FIC index as described by Lee et al. (2014). Interactions were defined as synergistic if the FIC index was < 1.0, additive if the FIC index equaled 1.0, subadditive if the FIC index was between 1.0 and 2.0, indifferent if the FIC index equaled 2, and antagonistic if the FIC index was > 2.0.

Results and Discussion

Antibacterial activity of MeOH extracts from thirteen marine algae

To evaluate the antibacterial activity of seaweed extracts toward *S. parauberis* strains, MeOH extracts from thirteen marine algae were utilized in a disk diffusion assay. The *E. cava* MeOH extract exhibited broad-spectrum antibacterial activity against all tested *S. parauberis* strains, expect for *S. parauberis* KCTC 3651. The diameters of the zones of inhibition were within the range of 8.0-12.0 mm at 5 mg/disk (Table 1). The greatest sensitivity was observed for *S. parauberis* KSP 8 and KSP 47, while strains KSP 3 and KSP 7 were the least affected. Kanagasabhapathy et al. (2006) classified the zones of inhibition as follows: weak (< 2 mm), moderate (2-5 mm) and strong (> 5 mm) antibacterial activity. Thus, the *E. cava* extract had a strong inhibitory effect on the *S. parauberis* strains. Furthermore, these results indicate that the *E. cava* extract had strong antibacterial activity against *S. parauberis* compared to the other seaweed extracts. These results are consistent with our previous study of antibacterial activities of an *E. cava* MeOH extract against *Listeria monocytogenes* (Nshimiyumukiza et al., 2015), *Staphylococcus aureus* (Eom et al., 2013) and *Enterococcus faecalis* (Kim et al., 2015).

To further investigate the antibacterial effects of *E. cava*, we fractionated the MeOH extract using several solvents. Of those, the Hexane fraction exhibited the greatest antibacterial activity against all *S. parauberis* strains (zone range: 6.0-11.0 mm at 5 mg/disk) compared to the other fractions (Table 2). Although the Hexane fraction had an inhibitory effect on the growth of *S. parauberis* KCTC 3651, the MeOH extract did not have any significant inhibitory effect.

From these results, we hypothesized that an antibacterial substance against *S. parauberis* could be abundant in the Hexane fraction of the *E. cava* extract. Interestingly, these results differed from our previous studies in which the *E. cava* EtOAC fraction exhibited the strongest antibacterial activity against human pathogenic bacteria (Eom et al., 2013; Kim et al., 2015; Nshimiyumukiza et al., 2015). Also, Rangaiaha et al. (2010) reported that seaweed extracts in different solvents exhibited different antimicrobial activities. The antimicrobial activity of organic solvent extracts against microorganisms could be related to the presence of different bioactive metabolites (Kolajinathan et al., 2009; Manivannan et al., 2011).

Thus, we performed a quantitative analysis using an MIC assay to precisely evaluate the antibacterial activity of the *E. cava* MeOH extract and its soluble fractions against *S. parauberis*. As shown in Table 3, the solvent-soluble fractions had broad-spectrum antibacterial activity against *S. parauberis* strains, with MIC values ranging from 256 to > 1,024 μg/mL. Among the solvent-soluble fractions, the Hexane fraction resulted in the lowest MIC values, by completely inhibiting the growth of *S. parauberis* strains (except KSP26) in the range

Table 1. Disc diffusion assay of seaweeds extract against *Streptococcus parauberis* strains

Seaweeds	Zone of inhibition (mm)*										
	KCTC 3651†	KSP 2	KSP 3	KSP 5	KSP 7	KSP 8	KSP 9	KSP 26	KSP 44	KSP 46	KSP 47
Ulva lactuca	-‡	-	-	-	-	-	-	-	-	-	-
Ishige okamurae	-	-	-	-	9.0	10.0	-	10.0	10.0	10.0	-
Codium fragile	-	-	-	-	-	-	-	-	-	-	-
Chondracanthus tenellus	-	-	-	-	-	-	-	-	-	-	-
Ecklonia cava	-	11.0	8.0	10.0	8.0	12.0	11.0	10.0	11.0	11.0	12.0
Ecklonia stolonifera	-	-	-	-	-	-	-	-	-	-	-
Hitzikia fucales	-	-	-	-	-	-	-	-	-	-	-
Undaria pinnatifida	-	-	-	-	-	-	-	-	-	-	-
Ulva perutsa	-	-	-	-	-	-	-	-	-	-	-
Eisenia bicyclis	-	-	8.0	-	-	10.0	-	-	9.0	-	-
Laminaria japonica	-	-	-	-	-	-	-	10.0	-	-	-
Carpopeltis prolifera	-	-	-	-	-	-	-	-	-	-	-
Sargassum fulvellum	-	-	-	-	-	-	-	-	-	-	-

S. parauberis KCTC 3651 is a reference strain from the Korean Collection for Type Cultures, Daejeon, Korea. *S. parauberis* KSP strains were provided by the Department of Aquatic Life and Medicine, Pukyong National University (Busan, Korea). *Seaweed methanolic extract (5 mg/disc) were loaded onto a disc (6 mm in diameter). †*S. parauberis* strains tested in this study. ‡No remarkable inhibition.

Table 2. Disc diffusion assay of methanol extract and its solvent-soluble extracts from *Ecklonia cava* against *Streptococcus parauberis* strains

Stains	Zone of inhibition (mm)					
	MeOH	Hexane	DCM	EtOAc	BuOH	H_2O
S. parauberis KCTC 3651	-*	8.0	-	9.0	-	-
S. parauberis KSP2	11.0	9.0	-	10.0	7.0	-
S. parauberis KSP3	8.0	6.0	-	8.0	-	-
S. parauberis KSP5	10.0	9.0	7.0	10.0	-	-
S. parauberis KSP7	8.0	6.0	-	8.0	-	-
S. parauberis KSP8	12.0	11.0	6.0	12.0	9.0	-
S. parauberis KSP9	11.0	10.0	-	11.0	7.0	-
S. parauberis KSP26	10.0	9.0	-	10.0	8.0	7.0
S. parauberis KSP44	11.0	10.0	-	10.0	7.0	-
S. parauberis KSP46	11.0	9.0	7.0	11.0	-	-
S. parauberis KSP47	12.0	11.0	6.0	12.0	9.0	-

*No remarkable inhibition. The methanolic extract and its fractions from *E. cava* were loaded onto a disk (6 mm in diameter). MeOH, methanolic extract; Hexane, *n*-hexane fraction, DCM, dichloromethane fraction; EtOAc, ethyl acetate fraction; BuOH, *n*-butanol fraction; H_2O, water fraction.

Table 3. Minimum inhibitory concentration (MIC) of the methanol extract and its soluble fractions from *Ecklonia cava* against *Streptococcus parauberis* strains

Strains	MIC (μg/mL)					
	MeOH	Hexane	DCM	EtOAc	BuOH	H_2O
S. parauberis KCTC 3651	1,024	512	1,024	512	1,024	> 1,024
S. parauberis KSP2	1,024	512	> 1,024	512	> 1,024	> 1,024
S. parauberis KSP3	1,024	1,024	> 1,024	1,024	1,024	> 1,024
S. parauberis KSP5	1,024	1,024	1,024	512	> 1,024	> 1,024
S. parauberis KSP7	1,024	1,024	> 1,024	1,024	> 1,024	> 1,024
S. parauberis KSP8	1,024	256	1,024	512	> 1,024	> 1,024
S. parauberis KSP9	1,024	512	> 1,024	1,024	1,024	> 1,024
S. parauberis KSP26	1,024	512	512	1,024	1,024	> 1,024
S. parauberis KSP44	1,024	512	> 1,024	1,024	> 1,024	> 1,024
S. parauberis KSP46	1,024	1,024	512	1,024	> 1,024	> 1,024
S. parauberis KSP47	1,024	256	1,024	512	1,024	> 1,024

MeOH, methanolic extract; Hexane, *n*-hexane fraction, DCM, dichloromethane fraction; EtOAc, ethyl acetate fraction; BuOH, *n*-butanol fraction; H_2O, water fraction.

Table 4. Antibiotic resistance of *Streptococcus parauberis* strains

S. parauberis strains	Zone of inhibition (mm)									
	AMC (≤ 16)*	AMP (≤ 20)	AMX (≤ 16)	GEN (< 14)	DOX (≤ 19)	ENR (≤ 16)	ERY (≤ 23)	FFC (≤ 16)	OTC (≤ 17)	SXT (≤ 10)
KCTC 3651	34	36	34	20	29	30	30	30	32	23
KSP2	33	30	30	16	12	28	6	31	6	24
KSP3	32	33	29	19	14	32	6	29	6	28
KSP5	33	30	30	16	32	20	21	35	30	25
KSP7	33	30	31	17	13	29	6	33	6	26
KSP8	33	31	32	18	12	30	9	32	6	25
KSP9	31	30	31	16	13	28	7	31	6	24
KSP26	40	39	41	18	32	30	35	35	31	16
KSP44	34	32	30	20	13	30	7	31	7	27
KSP46	32	37	31	21	13	32	9	32	6	30
KSP47	34	34	32	17	13	31	8	31	6	27

*Zone diameter of breakpoint of antibiotics (Gray and Shryock, 2005). AMC, 30 μg amoxicillin/clavulanic acid; AMP, 10 μg ampicillin; AMX, 25 μg amoxicillin; GEN, 10 μg gentamicin; DOX, 30 μg doxycycline; ENR, 5 μg enrofloxacin; ERY, 15 μg erythromycin; FFC, 30 μg florfenicol; OTC, 30 μg oxytetracycline; SXT, 23.75 μg trimethoprism/ sulfamethoxazole.

of 256 to 1,024 μg/mL (Table 3). Kanjana et al. (2011) reported the following MIC scale for plant extracts: < 100 μg/mL, strong inhibitors; 100-500 μg/mL, moderate inhibitors; 500-1,000 μg/mL, weak inhibitors; and > 1,000 μg/mL, not inhibitors. Thus, we observed moderate inhibition against *S. parauberis* strains KSP8 and KSP47 using the Hexane fraction. Meanwhile, weak inhibition was observed against *S. parauberis* strains KSP2, KSP9, and KSP44 using the Hexane fraction.

Antibiotic resistance of *Streptococcus parauberis* strains

To determine the antibiotic resistance of *S. parauberis*, ten commercial antibiotics were tested against several strains. As shown in Table 4, all *S. parauberis* strains tested in this study exhibited susceptibility to amoxicillin/clavulanic acid (AMC), ampicillin (AMP), amoxicillin (AMX), gentamycin (GEN), enrofloxacin (ENR), florfenicol (FFC), and trimethoprim/sulfamethoxazole (SXT), with zones of inhibition ranging in diameter from 16 to 40 mm. Alternatively, most of the *S. parauberis* strains (KSP2, 3, 7, 8, 9, 44, 46, and 47) were resistant to doxycycline (DOX), erythromycin (ERY), and oxytetracycline (OTC). However, *S. parauberis* strains KCTC 3651 (a nonpathogenic strain not isolated from fish), KSP5, and KSP26 did not exhibit resistance to these antibiotics. In addition, antibiotic susceptibility testing by disk diffusion assays revealed that the antibiotic-resistant *S. parauberis* strains (KSP2, 3, 7, 8, 9, 44, 46, and 47) exhibited the highest resistance to ERY and OTC compared to DOX. Thus, the *S.*

Table 5. Minimum inhibitory concentrations (MIC) and fractional inhibitory concentration (FIC) indices of the *n*-hexane (Hexane) fraction of *Ecklonia cava* in combination with erythromycin (ERY) and oxytetracycline (OTC) against *Streptococcus parauberis*

Stains	Test Compound	MIC (μg/mL)	Median ∑FIC*	$\sum FIC_{max}$†	$\sum FIC_{min}$‡	Minimum concentration for synergy
S. parauberis KSP2	Hexane	512	0.533§	1.016	0.375	16.0
	ERY	512				256.0
	Hexane	512	0.502	1.016	0.188	128.0
	OTC	512				16.0
S. parauberis KSP3	Hexane	1,024	1.001	1.016	0.188	64.0
	ERY	256				128.0
	Hexane	1,024	0.516	1.016	0.094	512.0
	OTC	1024				16.0
S. parauberis KSP7	Hexane	1,024	0.504	1.016	0.375	256.0
	ERY	1,024				128.0
	Hexane	1,024	0.502	1.004	0.188	256.0
	OTC	256				8.0
S. parauberis KSP8	Hexane	256	0.281	1.016	0.188	128.0
	ERY	512				8.0
	Hexane	256	0.508	1.004	0.250	32.0
	OTC	512				8.0
S. parauberis KSP9	Hexane	512	1.016	1.063	0.500	128.0
	ERY	1,024				256.0
	Hexane	512	0.504	1.016	0.141	128.0
	OTC	1,024				16.0
S. parauberis KSP44	Hexane	512	0.566	1.016	0.500	128.0
	ERY	1,024				256.0
	Hexane	512	0.502	1.016	0.313	64.0
	OTC	1,024				128.0
S. parauberis KSP46	Hexane	1,024	0.625	1.125	0.500	256.0
	ERY	1,024				256.0
	Hexane	1,024	0.502	1.016	0.313	32.0
	OTC	1,024				128.0
S. parauberis KSP47	Hexane	256	0.516	1.063	0.500	64.0
	ERY	512				128.0
	Hexane	256	0.502	1.004	0.094	32.0
	OTC	512				64.0

*∑FIC , the sum of FICs; †$\sum FIC_{min}$, the minimum ∑FIC; ‡$\sum FIC_{max}$, the maximum ∑FIC.

§The FIC index indicated synergistic; < 0.5, addictive; 0.5 to < 1.0, indifferent; > 1.0 to < 2.0, antagonistic; > 2.0. ∑FIC was calculated for each well with the equation: $\sum FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, 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.

parauberis strains were highly resistant to ERY (zone diameter range: 6.0-9.0 mm) and OTC (zone diameter range: 6.0-7.0 mm), which far exceeded the breaking point of resistance for these antibiotics (≤ 23 mm for ERY and ≤ 17 mm for OTC) (Gray and Shryock, 2005).

The results obtained in this study are consistent with those of previous reports indicating that ERY and OTC have limited efficacy toward *S. parauberis* isolated from olive flounder (Jeong et al., 2006; Park et al., 2009). Meng et al. (2009) reported that *S. parauberis* strains with high-level resistance to OTC are often associated with resistance to ERY. This is likely because most *erm* genes encoding ERY resistance proteins are carried on transposons, and through transposition become linked to *tet* genes encoding tetracycline or OTC resistance proteins.

ERY and OTC were used in an MIC assay with the antibiotic-resistant *S. parauberis* strains to verify the antibiotic concentrations needed to inhibit bacterial growth. As shown in Table 5, the MIC values for both antibiotics against the antibiotic-resistant strains ranged between 256 and 1,024 μg/mL. The MIC values for ERY and OTC against the *S. parauberis* strains were significantly higher than the MIC breakpoint values of 1 μg/mL for ERY and 16 μg/mL for OTC (Gray and Shryock, 2005). These results suggest that ERY and OTC are no longer effective for treating infections caused by antibiotic-resistant *S. parauberis*.

Synergistic effects of the *E. cava* Hexane fraction and antibiotics against *S. parauberis*

One effective strategy for overcoming antibiotic resistance is to restore the activity of the antibiotic. This has been demonstrated by the use of antibiotics in combination with antibacterial agents derived from natural resources (Eom et al., 2014; Lee et al., 2014; Nshimiyumukiza et al., 2015). Here, we evaluated the interaction between the Hexane fraction of an *E. cava* MeOH extract and commercial antibiotics (ERY and OTC) against antibiotic-resistant *S. parauberis* strains using an FIC assay.

As shown in Table 5, the MIC values for ERY and OTC in combination with the Hexane fraction were markedly reduced, with 64-fold inhibition against the antibiotic-resistant *S. parauberis* strains. Thus, the MIC for OTC against *S. parauberis* KSP9 was reduced from 1,026 to 16 μg/mL in combination with 126 μg/mL of the Hexane fraction. These results suggest that the antibacterial activity of the conventional antibiotics ERY and OCT was restored when used in combination with the Hexane fraction. The synergistic antibacterial activity between the antibiotics and the Hexane fraction of *E. cava* was assessed by FIC analysis.

FIC indices of ERY and OCT in combination with the Hexane fraction are presented in Table 5. For all strains, the Hexane fraction and ERY combination resulted in a $\sum FIC_{min}$ range of 0.118 to 0.500 and a $\sum FIC_{max}$ range of 1.016 to 1.125. The Hexane fraction and OTC combination resulted in a $\sum FIC_{min}$ range of 0.094 to 0.313 and a $\sum FIC_{max}$ range of 1.004 to 1.016 for all strains. These results suggest that the Hexane fraction had greater synergistic effects in combination with OTC than with ERY. In addition, the median ∑FIC of the OTC and Hexane fraction combination ranged from 0.502 to 0.516. As reported by Lee et al. (2014), the synergistic ranges of median $\sum FIC < 1$ were observed for all combinations of OTC and the Hexane fraction against the antibiotic-resistant *S. parauberis* strains.

Despite the multitude of studies on the synergistic effects of natural compounds in combination with antibiotics to treat human pathogenic bacteria, few studies have focused on using this therapy to treat fish pathogens. Although the use of antibiotics is an effective way to control fish pathogenic bacteria in aquaculture farms, it may result in increased antibiotic resistance (Eom et al., 2013). Therefore, restoring the antibacterial activity of conventional antibiotics by combining them with natural compounds is an attractive approach. Natural products can increase the antibacterial efficacy of antibiotics without increasing antibiotic resistance among the bacterial pathogens in aquatic animals. In this study, we demonstrated that an *E. cava* extract could inhibit *S. parauberis* and that the synergistic effect created by the *E. cava* extract and conventional antibiotics significantly increased the level of antibacterial activity against *S. parauberis*. Thus, the results obtained in this study will contribute to the development of an alternative phytotherapeutic agent for use against infections with the fish pathogenic bacterium *S. parauberis*.

Acknowledgments

This research was supported by Marine Biotechnology Program (20150220) funded by Ministry of Oceans and Fisheries, Republic of Korea. This research was also supported by a special fund of Pukyong National University donated by the SKS Trading Co. in Lynnwood,WA, USA in memory of the late Mr. Young Hwan Kang, who had deep concern for and inspiration by fishery science.

References

Adwan G and Mhanna M. 2008. Synergistic effect of plant extracts and antibiotics on *Staphylococcus aureus* strains isolated from clinical specimen. Middle-East J Sci Res 3, 134-139.

Baeck GW, Ji Hyung Kim JH, Gomez DK and Park SC. 2006. Isolation and characterization of *Streptococcus* sp. from diseased flounder (*Paralichthys olivaceus*) in Jeju Island. J Vet Sci 7, 53-58.

Bercovier H, Ghittino C and Eldar A. 1997. Immunization with bacterial antigens: infections with streptococci and related organisms. In: Gudding R, Lillehaugg R, Midtlying PJ, Brown F (Eds.), Fish Vaccinology. Dev Biol Stand. Karger, Basel, Switzerland, pp 153–160.

Choi JG, Kang OK, Brice OO, Lee YS, Chae HS, Oh YC, Sohn DH, Park Y, Choi HG, Kim SG, Shin DW and Kwon DY. 2010. Antibacterial activity of *Ecklonia cava* against methicillin-resistant *Staphylococcus aureus* and *Salmonella* spp. Foodborne Path Dis 7, 435-441.

Clinical and Laboratory Standards Institute (CLSI). 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Ninth Edition. CLSI document M07-A9. CLSI, Wayne, PA, USA.

Clinical and Laboratory Standards Institute (CLSI). 2014. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. CLSI document M100-S24. CLSI, Wayne, PA, USA.

Cox S, Abu-Ghannam N and Gupta S. 2010. An assessment of the antioxidant and antimicrobial activity of six species of edible Irish seaweeds. Int Food Res J 17, 205-220.

Domenech A, Fernandez-Garayzabal JF, Pascual C, Garcia JA, Cutuli MT, Moreno MA, Collins MD and Dominguez L. 1996. Streptococcosis in cultured turbot, *Scophthalmus maximus* (L.), associated with *Streptococcus parauberis*. J Fish Dis 19, 36-38.

Eom SH, Kim DH, Lee SH, Yoon NY, Kim JH, Kim TH, Chung YH, Kim SB, Kim YM, Kim HW, Lee MS and Kim YM. 2013. *In vitro* antibacterial activity and synergistic antibiotic effects of phlorotannins isolated from *Eisenia bicyclis* against methicillin-resistant *Staphylococcus aureus*. Phytother Res 27, 1260-1264.

Eom SH, Lee DS, Jung YJ, Park JH, Choi JI, Yim MJ, Jeon JM, Kim HW, Son KT, Je JY, Lee MS and Kim YM. 2014. The mechanism of antibacterial activity of phlorofucofuroeckol-A against methicillin-resistant *Staphylococcus aureus*. Appl Microbiol Biotechnol 98, 9795-9804.

Gray JT and Shryock TR. 2005. Antibiotic susceptibility testing of bacteria isolated from animals. Clin Microbiol Newsletter 27, 131-135.

Gupta S, Rajauria G, and Ghannam NA. 2009. Study of the microbial diversity and antimicrobial properties of Irish edible brown seaweeds. Int J Food Sci Technol 45, 482-489.

Hornsey IS and Hide D. 1974. The production of antimicrobial compounds by British marine algae I. Antibiotic-producing marine algae. Br Phycol J 9, 353-361.

Hsieh MH, Yu CM, Yu VL and Chow JW. 1993. Synergy assessed by checkerboard. A critical analysis. Diagn Microbiol Infect Dis 16, 343-349.

Jeong YU, Kang CY, Kim MJ, Heo MS, Oh DC and Kang BJ. 2006. Characterization of Streptococcosis occurrence and molecular identification of the pathogen of cultured flounder in Jeju Island. Kor J Microbiol 42, 199-204.

Kanagasabhapathy M, Sasaki H, Haldar S, Yamasaki S and Nagata S. 2006. Antibacterial activities of marine epibiotic bacteria isolated from brown algae of Japan. Ann Microbiol 56, 167-173.

Kandhasamy M and Arunachalam KD. 2008. Evaluation of *in vitro* antibacterial property of seaweeds of southeast coast of India. Afr J Biotechnol 7, 1958-1961.

Kang SM, Heo SJ, Kim KN, Lee SH , Yang HM , Kim AD and Jeon YJ. 2012. Molecular docking studies of a phlorotannin, dieckol isolated from *Ecklonia cava* with tyrosinase inhibitory activity. J Bioorg Med Chem 20, 311-316.

Kim JH, Gomez DK, Baeck GW, Shin GW, Heo GJ, Jung TS and Park SC. 2006. Pathogenicity of *Streptococcus parauberis* to olive flounder *Paralichthys olivaceus*. Fish Pathol 41, 171-173.

Kim KN, Heo SJ, Song CB, Lee J, Heo MS, Yeo IK and Jeon YJ. 2006. Protective effect of *Ecklonia cava* enzymatic extracts on hydrogen peroxide-induced cell damage. Process Biochem 41, 2393-2401.

Kim SY, Kim YM, Kim EJ and Lee MS. 2015. Synergistic antibacterial activity of *Ecklonia cava* extract against antibiotic resistant *Enterococcus faecalis*. Korean J Fish Aquat Sci 48, 51-57.

Kolanjinathan K, Ganesh P and Govindarajan M. 2009. Antibacterial activity of ethanol extract of seaweeds against fish bacterial pathogens. Eur Rev Med Pharmaco Sci 13, 173-177.

Lee JH, Eom Sh, Lee EH, Jung YJ, Kim HJ, Jo MR, Son KT, Lee HJ, Kim JH, Lee MS and Kim YM. 2014. *In vitro* antibacterial and synergistic effect of phlorotannins isolated from edible brown seaweed *Eisenia bicyclis* against acne-related bacteria, Algae 29, 47-55.

Leigh JA. 2002. *Streptococcus uberis*: A permanent barrier to the control of bovine mastitis? Vet J 157, 225-238.

Manivannan K, Thirumaran G, Devi GK, Anantharaman P and Balasubramanian T. 2009. Proximate composition of different group of seaweeds from Vedalai Coastal Waters (Gulf of Mannar): Southeast Coast of India. Middle East J Sci Res 4, 72-77.

Meletiadis J, Pournaras S, Roilides E and Walsh TJ. 2010. Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, Monte Carlo simulation analysis, and in vitro-in vivo correlation data for antifungal drug combinations against *Aspergillus fumigatus*. Antimicrob Agents Chemother 54, 602-609.

Meng F, Kanai K and Yoshikoshi K. 2009. Characterization of drug resistance in *Streptococcus parauberis* isolated from Japanese flounder. Fish Pathol 44, 40-46.

Nshimiyumukiza O, Kang SK, Kim HJ, Lee EH, Han HN, Kim Y, Kim DH, Kim JH, Eom SW and Kim YM. 2015. Synergistic antibacterial activity of *Ecklonia cava* (Phaeophyceae: Laminariales) against *Listeria monocytogenes* (Bacillales: Listeriaceae). Fish Aquat Sci 18, 1-6.

Park YK, Nho SW, Shin GW, Park SB, Jang HB, Cha IS, Ha MA, Kim YR, Dalvi RS and Kang BJ, Jung TS. 2009. Antibiotic susceptibility and resistance of *Streptococcus iniae* and *Streptococcus parauberis* isolated from olive flounder (*Paralichthys olivaceus*). Vet Microbiol 136, 76-81.

Rangaiah SG, Lakshmi P and Manjula E. 2010. Antimicrobial activity of seaweeds *Gracillaria, Padina* and *Sargassum* sps. on clinical and phytopathogens. Int J Chem Anal Sci 1, 114-117.

Rhimou B, Hanssane R, José M and Nathalie B. 2010. The antibacterial potential of the seaweeds (Rhodophyceae) of the strait of gibraltar and the Mediterranean Coast of Morocco. Afr J Biotechnol 9, 6365-6372.