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Synergistic Antibacterial Activity of *Ecklonia cava* (Phaeophyceae: Laminariales) against *Listeria monocytogenes* (Bacillales: Listeriaceae)

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Abstract

In an effort to discover alternative antimicrobials against *Listeria monocytogenes*, several marine algae were screened. The methanolic extract of *Ecklonia cava* exhibited the highest antibacterial activity against *L. monocytogenes*, with the ethyl acetate (EtOAc) soluble fraction of *E. cava* methanolic extract having a MIC value of 256 μg/mL and a MBC value of 512 μg/mL. The MIC values of streptomycin in combination with the EtOAc fraction were markedly reduced up to 64-fold, suggesting that the antibacterial activity of the antibiotic was restored when combined with the EtOAc fraction. The interaction between streptomycin and the EtOAc fraction was assessed by fractional inhibitory concentration (FIC) indices. The combination of streptomycin and the EtOAc fraction against *L. monocytogenes* resulted in $\sum FIC_{min}$ range of 0.141 to 0.266 and $\sum FIC_{max}$ of 0.531 for all strains. The median $\sum FIC$ against *L. monocytogenes* strains ranged from 0.172 to 0.344. Thus, synergistic ranges of FIC <1 were observed for all combinations of streptomycin and the EtOAc fraction against *L. monocytogenes* strains. Indeed, the median $\sum FIC$ of streptomycin–EtOAc fraction ranged from 0.172 to 0.344, suggesting a marked synergy.

Key words: Antibacterial activity, *Ecklonia cava*, *Listeria monocytogenes*, Synergistic effect

Introduction

Listeria monocytogenes is a foodborne pathogen that causes listeriosis, a severe invasive infection in humans with a particularly high case-fatality rate. Listeriosis is a major public health concern in all regions of the world due to the severity of manifestations (i.e., septicemia, meningitis and fetal death), with a case-fatality rate ranging from 20% to 50% (Denny and McLauchlin, 2008). Despite efficient antibiotic therapy, listeriosis represents a public health problem because it is frequently fatal. To overcome these problems, a wide range of synthetic antimicrobial agents (sodium benzoate, calcium benzoate, sorbate) has been used as food preservatives (Sherwin, 1990). However, antibiotic resistance has been described among *Listeria* spp., particularly *L. monocytogenes* strains isolated from food, the environment, or sporadic human listeriosis cases (Poyart-Salmeron, et al., 1990; Facinelli et al., 1991; Charpentier et al., 1995).

Recently, consumers have begun demanding foods that are fresh, natural, and minimally processed, along with the requirement for enhanced safety and quality. This perspective has put pressure on the food industry for progressive removal

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of chemical preservatives, and has fuelled research into alternative natural antimicrobials (Lanciotti et al., 2004). Additionally, increased public awareness of the negative effects caused by synthetic chemicals has led to the search for "green solutions," such as organic and synthetic chemical-free food products (Abutbul, 2004).

In that regard, seaweeds have become an important source of pharmacologically active metabolites, with a broad spectrum of biological activities with possible applications in food production to prevent bacterial and fungal growth. In fact, compounds with antioxidant, antiviral, antifungal, and antimicrobial activities have been detected in brown, red, and green algae (Eom et al., 2011). *Ecklonia cava*, of the family Laminariaceae, ranges along the eastern coast of Korea and Japan. It is utilized as a food ingredient, animal feed, fertilizer, and medicine. Additionally, *E. cava* was reported to harbor compounds with various biological activities, including carotenoids, fucoidan, and phlorotannins (Kang et al., 2012). *E. cava* extracts exhibit various biological functions, including antioxidant, antibacterial, protease inhibitory, immunomodulatory, antiasthmatic, and tyrosinase inhibitory activity (Kim et al., 2006; Kang et al., 2012). However, the antibacterial activity of *E. cava* against *L. monocytogenes* has not been reported to date. The present study aimed to investigate the antibacterial activity of *E. cava* against *L. monocytogenes*.

Materials and Methods

Samples and extraction

Dried *E. cava* powder (1.0 kg) was extracted and fractionated using organic solvents as described in Lee et al. (2014). The methanolic extract of *E. cava* (101.3 g) was partitioned in turn with *n*-hexane (Hexane; 1.0 L × 3), dichloromethane (DCM; 1.0 L × 3), ethyl acetate (EtOAc; 1.0 L × 3), and *n*-butanol (*n*-BuOH; 1.0 L × 4). The water fraction was obtained by filtering the remaining materials from the *n*-BuOH partition. Finally, each extract was evaporated using a rotary evaporator (Eyela, Tokyo, Japan) under vacuum at 45°C. Another 12 methanol extracts from various marine algae, previously prepared and stored under appropriate conditions (refrigerated at -70°C), were used for the same purpose.

Total phenolic contents

The contents of total phenolic (TP) compounds in the fractionated *E. cava* extracts were evaluated using the modified Folin–Ciocalteu method (Eom et al., 2013), taking phloroglucinol as a standard and with results expressed as phloroglucinol equivalents (PGE). The concentration of TP compounds was 241.0 ± 1.6 mg PEG/g in the MeOH extract, 26.8 ± 6.9 mg PEG/g in the hexane fraction, 83.3 ± 1.0 mg PEG/g in the DCM fraction, 556.8 ± 2.7 mg PEG/g in the EtOAc fraction, 416.3 ± 18.4 mg PEG/g in the BuOH fraction, and 34.3 ± 4.1 mg PEG/g in the water fraction.

Microbial strains and cultures

The bacterial strains used in this study were *L. monocytogenes* KCTC 3710 from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea) and three clinical isolates provided by the Gyeongsang National University Hospital (Jinju, Korea), a member of the National Biobank of Korea. All strains were cultured aerobically at 37°C in Brain Heart Infusion broth (BHI; Difco, USA).

Disk diffusion assay

The antibacterial efficacy of marine algae extracts was evaluated by disk diffusion assay followed by the CLSI (2009), as described in our previous reports (Lee et al., 2014; Eom et al., 2011). In brief, bacterial strains were cultured in BHI at 37°C until an OD at 600 nm of 0.5. One hundred microliters of bacterial culture containing approximately 10^4 - 10^5 CFU/ mL were spread on Mueller Hinton agar (MHA; Difco,

Table 1. Disk diffusion assay of methanolic extract and its fractions from *Ecklonia cava* against *Listeria monocytogenes* strains

*S*trains	Concn. (mg/disk)	Zone of inhibition (mm)[a]					
		MeOH[b]	Hexane	DCM	EtOAc	BuOH.	H_2O
L. monocytogenes KCTC 3710	1	13.0 ± 2.0	10.0 ± 2.0	8.7 ± 0.6	13.0 ± 3.0	10.0 ± 1.7	10.0 ± 2.8
	5	18.0 ± 2.0	13.0 ± 2.0	14.0 ± 2.0	23.0 ± 2.0	16.3 ± 1.2	14.8 ± 2.1
L. monocytogenes isolate P2148	1	9.0 ± 0.0	9.0 ± 1.7	8.0 ± 0.0	10.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0
	5	10.0 ± 1.0	10.7 ± 2.3	8.0 ± 0.0	14.0 ± 2.0	9.3 ± 0.6	8.0 ± 0.0
L. monocytogenes isolate P2637	1	7.3 ± 5.5	9.7 ± 1.5	7.0 ± 0.0	8.3 ± 0.6	7.7 ± 0.6	8.0 ± 0.0
	5	8.0 ± 1.0	10.3 ± 2.1	8.0 ± 0.0	13.0 ± 0.0	8.3 ± 1.5	8.0 ± 0.0
L. monocytogenes isolate P2868	1	7.7 ± 0.6	10.0 ± 1.7	7.3 ± 0.6	8.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	5	9.0 ± 1.7	11.3 ± 0.6	8.0 ± 0.0	13.0 ± 0.0	8.0 ± 0.0	6.0 ± 4.0

[a]Methanol extract and its fraction from *E. cava* was loaded onto a disk (6 mm in diameter).
[b]MeOH, methanolic extract; DCM, dichloromethane fraction; EtOAc, ethyl acetate fraction; BuOH, butanol fraction; H_2O, water fraction.

USA) plates. Paper discs (6 mm in diameter) containing 1 and 5 mg of extract were placed on the MHA plate. After incubating for 24 h at 37°C, the diameter of the inhibition zone was measured. The experiment was carried out three times and the mean values were calculated.

Measurement of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial that inhibits visual growth of microorganisms after 20 - 24 h of incubation at 37°C (Gierson and Afolayan, 1999). The MICs of marine algae extracts and some commercial antibiotics were determined by a twofold serial dilution method in Mueller–Hinton broth (MHB; Difco) (CLSI, 2008).

Minimum bactericidal concentration (MBC) is defined as the lowest concentration of an antimicrobial required for a 99.9% reduction in the viable cell population (NCCLS, 2003). For MBC determination, an aliquot of inoculums was taken from a MIC test well that did not show turbidity and was poured onto BHI agar plates. The agar plates were incubated at 37°C until growth was seen in the growth control plates. The number of colonies on each agar plate was counted. The MIC and MBC experiments were repeated in triplicate.

Antibiotic susceptibility test

Sensitivity and/or resistance of *L. monocytogenes* KCTC 3710 and clinical isolates was evaluated using the disk diffusion method with 16 antibiotics, either individually or in combination.

Synergic effects between EtOAc fraction of *E. cava* and antibiotics

The interaction between the EtOAc fraction of *E. cava* and antibiotics containing streptomycin (Sigma Chemical Co., St. Louis, MO, USA) against *L. monocytogenes* strains was tested by the checkerboard method using fractional inhibitory concentration (FIC) (Hsieh et al., 1993; Meletiadis et al., 2010). The sum of the FICs (∑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, in all of the wells corresponding to an MIC (isoeffective combinations). Among all of 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. The synergistic effect was evaluated as a fractional inhibitory concentration (FIC) index as described by Lee et al. (2014). The interaction was defined as synergistic if the FIC index was <1.0, additive if the FIC index was 1.0, subadditive if the FIC index was between 1.0 and 2.0, indifferent if the FIC index was 2, and antagonistic if the FIC index >2.0. Synergy was further sub-classified as either marked (FIC index, ≤0.5) or weak (FIC index, between 0.5 and 1.0).

Statistical analysis

Data are reported as means ± standard errors of the mean. Differences at $P<0.05$ were considered statistically significant. SPSS 12.0 (SPSS Inc., Chicago, IL) was used to perform the statistical analysis.

Results and Discussion

Antibacterial activity of MeOH extracts from 13 marine algae

MeOH extracts from 13 marine algae, including *E.* cava, were screened for their antibacterial activity against *L. monocytogenes* by disk diffusion assay as described in the Materials and methods. *E. cava* methanolic extract exhibited the greatest antibacterial activity against *L. monocytogenes* KCTC 3710 and all three isolates of *L. monocytogenes* used in this study (data not shown). The diameters of inhibition zone ranged from 7.3 mm to 13 mm for 1 mg/disk, and 9 mm to 18 mm for 5 mg/disk (Table 1). These results indicate that *E. cava* con-

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the MeOH extracts and its soluble fractions from *Ecklonia cava* against *Listeria monocytogenes* strains

Unit : μg/mL

Strains	MeOH		Hexane		DCM		EtOAc		BuOH		H_2O	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
L. monocytogenes KCTC 3710	256	512	512	1,024	512	1,024	256	512	512	1,024	1,024	>1,024
L. monocytogenes isolate P2148	512	1,024	512	1,024	512	1,024	256	512	512	1,024	512	1,024
L. monocytogenes isolate P2637	256	512	512	1,024	512	1,024	256	512	512	1,024	512	1,024
L. monocytogenes isolate P2868	256	512	512	1,024	512	1,024	256	512	1,024	>1,024	1,024	>1,024

MeOH, methanolic extract; DCM, dichloromethane fraction; EtOAc, ethyl acetate fraction; BuOH, butanol fraction; H_2O, water fraction.

tains higher levels of antibacterial compounds active against *L. monocytogenes* than do other seaweeds. Also, these results are in line with the study by Cox et al. (2010) of the antimicrobial properties of several Irish edible brown seaweeds and reported good antibacterial activities against *L. monocytogenes* for MeOH extracts of the seaweed *Laminaria digitata.*

To further investigate the mechanism underlying the antibacterial effect against *L. monocytogenes*, *E. cava* methanolic extract was fractioned using several solvents. Of those, the EtOAc fraction exhibited the greatest antibacterial activity against *L. monocytogenes* (the diameter of the clear zone around the disk ranged from 13 to 23.0 mm for 5 mg/disk) as compared to other fractions (clear zone diameters ranged from 6.0 to 16.3 mm for 5 mg/disk) (Table 1).

These results suggested that a substance with antibacterial activity against *L. monocytogenes* was present in the EtOAc soluble fraction of the *E. cava* extract, therefore supporting the first hypothesis. According to Choi et al. (2010), marine-derived polyphenols (phlorotannins) are the predominant EtOAc-soluble compound in brown algae (Choi et al. 2010). In this study, it was also found that the EtOAc fraction of *E. cava* methanolic extract contained the highest amount of TP compounds, as described in the Materials and Methods. Thus, on the basis of the aforementioned results, *E. cava* was subjected to further investigation of its antibacterial activity against *L. monocytogenes*.

Table 3. Minimum inhibitory concentration for streptomycin and gentamycin against *Listeria monocytogenes* strains

Strains	MIC values (μg/mL)	
	Gentamycin	Streptomycin
L. monocytogenes KCTC3710	8	8
L. monocytogenes isolate P2148	2	16
L. monocytogenes isolate P2637	2	16
L. monocytogenes isolate P2868	2	32

Determination of MIC and MBC of *E. cava* extract

The MIC assay was carried out to quantitatively evaluate the antibacterial activity of *E. cava* methanolic extract and its soluble fractions. MIC values of solvent fractions against *L. monocytogenes* varied depending on the polarity of the solvent. In general, the MIC values were in the range 256 to 1,024 μg/mL (Table 2). Among the solvent-soluble fractions, the EtOAc-soluble fraction evinced the lowest MIC values, as it completely inhibited the growth of *L. monocytogenes* KCTC 3710 and three clinical isolates (P2148, P2637 and P2868) at 256 μg/mL. This indicated that the EtOAc solvent-soluble fraction had the highest antibacterial activity. No marked difference between the standard strain and *L. monocytogenes* clinical isolates was observed (Table 2).

The antibacterial activities of *E. cava* extracts were also quantitatively evaluated by MBC assay. The MBC values—the lowest concentration that killed 99.99% of the initial inoculum—were slightly higher than MIC values, but in all cases did not exceed twice the MIC value. The MBC values for the EtOAc fraction were 512 μg/mL for all strains used, while MBC values of the other fractions ranged from 512 to >1,024 μg/mL for all strains (Table 2). These results are in accordance with those reported by Rahman et al. (2013) that, at 400 μg/disc, an EtOAc extract of *Poncirus trifoliata* seeds showed the greatest antibacterial activity against four of five *L. monocytogenes* strains. A similar result was reported by Bajpai and Kang (2009); i.e., the EtOAc fraction of the plant *Metasequoia glyptostroboides* showed the strongest antibacterial effect against four *L. monocytogenes strains*.

Antibiotic resistance of *L. monocytogenes* strains

Recent reports show an increased rate of resistance to one or more clinically relevant antibiotics in environmental *L. monocytogenes* isolates (Charpentier et al., 1995; Conter et al., 2009) and, less frequently, in clinical strains (Safdar and

Table 4. Minimum inhibitory concentrations (MIC) and fractional inhibitory concentration (FIC) indices of the ethyl acetate (EtOAc) fraction of *Ecklonia cava* in combination with streptomycin against *Listeria monocytogenes* strains

Strains	Test compound	MIC (μg/mL)	Median ∑FIC [a)]	$\sum FIC_{max}$ [b)]	$\sum FIC_{min}$ [c)]	Minimum concentration for observing synergy
L. monocytogenes KCTC 3710	EtOAc streptomycin	256 8	0.172 [d)]	0.531	0.141	32 0.125
L. monocytogenes isolate P2148	EtOAc streptomycin	256 16	0.297	0.531	0.250	32 2
L. monocytogenes isolate P2637	EtOAc streptomycin	256 16	0.344	0.531	0.266	4 4
L. monocytogenes isolate P2868	EtOAc streptomycin	256 32	0.297	0.531	0.250	32 4

[a)] ∑FIC , the sum of FICs; [b)] $\sum FIC_{min}$, minimum ∑FIC; [c)] $\sum FIC_{max}$, and the maximum ∑FIC.;

[d)] 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.

Armstrong, 2003). However, few studies have evaluated antimicrobial resistance in *Listeria* spp. (Hansen, 2005; Safdar and Armstrong, 2003). Thus the present study used both the disk diffusion and minimum inhibitory concentration assays to evaluate the antibacterial susceptibility of *L. monocytogenes* against several commercial antibiotics.

The antibiotic resistance patterns of the type strain and three clinical isolates of *L. monocytogenes* were assessed by a disk diffusion assay using 16 commercial antibiotic kits. In the disk diffusion assay, no clear zones were found around the disks containing either gentamycin or streptomycin for all three clinical isolates used in this study (data not shown). The failure of streptomycin and gentamycin to inhibit the growth of all three clinical *L. monocytogenes* isolates suggests resistance to these agents (data not shown).

The MIC value for streptomycin against *L. monocytogenes* KCTC 3710 was 8 μg/mL, compared to 16 μg/mL for P2148 and P2637, and 32 μg/mL for P2868. On the other hand, the MIC values of gentamycin against the *L. monocytogenes* strains tested in this study were 2 to 8 μg/mL (Table 3). The MIC values of streptomycin against *L. monocytogenes* strains were equal or higher than the generally accepted Soussy's MIC breakpoint values, ranging from 8 to 16 μg/mL (Aureli et al., 2003). In contrast, the MIC values of gentamycin against *L. monocytogenes* strains, with the exception of the type strain KCTC 3710, were lower than the generally accepted Soussy's MIC breakpoint values, ranging from 4 to 8 μg/mL (Aureli et al., 2003). These results indicate that streptomycin is no longer useful for treating *Listeria* infections. This adds to other reports, including Charpentier et al. (1995), Facinell et al. (1991), and Poyart-Salmeron et al., (1990), of cases of clinical isolates of *L. monocytogenes* resistant to multiple antibiotics, including gentamycin, streptomycin, erythromycin, kanamycin, sulfamethoxazole, rifampin, and others.

Synergistic effect between EtOAc fraction of *E. cava* and streptomycin against *L. monocytogenes*

It has been demonstrated that one of the more effective strategies in developing new drugs or alternative therapies is the restoration of antibiotic activity, in combination with antibacterial materials derived from natural products and traditional medicines, against drug-resistant bacteria (Eom et al. 2014; Lee et al., 2014). In this study, the interaction between the EtOAc fraction of *E. cava* and the commercial antibiotic streptomycin against *L. monocytogenes* strains was evaluated by FIC assay. Gentamycin was not assayed since most of the *L. monocytogenes* strains tested failed to exhibit resistance to this agent.

As shown in Table 4, the MIC values of streptomycin in combination with the EtOAc fraction of *E. cava* (at concentrations of 2 to 32 μg/mL) were markedly reduced up to 64-fold for KCTC 3710, 8-fold for P2148 and P2868, and 4-fold for P2637. Thus, the antibacterial activity of a traditional antibiotic, streptomycin, was restored by combination with the EtOAc fraction, since the minimum concentrations that inhibited growth with streptomycin were 0.125 to 4 μg/mL.

The synergistic antibacterial activity between streptomycin and the EtOAc fraction of *E. cava* was assessed by the FIC analysis. The FIC indices of streptomycin in combination with the EtOAc fraction are presented in Table 4. The combination of streptomycin and the EtOAc fraction resulted in a $\sum FIC_{min}$ range of 0.141 to 0.266 and $\sum FIC_{max}$ of 0.531 for all strains (Table 4). The median ∑FIC against *L. monocytogenes* strains ranged from 0.172 to 0.344. As described by Lee et al. (2014), the synergistic ranges of FIC <1 were observed for all combinations of streptomycin and the EtOAc fraction against *L. monocytogenes* strains. Indeed, the median ∑FIC of the streptomycin–EtOAc fraction ranged from 0.172 to 0.344, suggesting marked synergy (Table 4). These results suggest that *E. cava* extracts showed synergistic effects in combination with streptomycin. In addition, Choi et al. (2010) reported that the combination of *E. cava* extracts with ampicillin improved the inhibition of *S. aureus* and *Salmonella* spp.

In conclusion, this study evaluated the antibacterial activity of the edible marine brown alga *E. cava* against *L. monocytogenes*. The EtOAc fraction of *E. cava* extract showed the strongest antibacterial activity against *L. monocytogenes* among the solvent fractions tested, suggesting that the antibacterial activity of *E. cava* against *L. monocytogenes* may be related to the phlorotannin or marine-derived polyphenolic contents. Additionally, streptomycin in combination with the EtOAc fraction restored antibacterial activity against *L. monocytogenes* in a synergistic manner. Thus the results of the present investigation will contribute to the development of an alternative phytotherapeutic agent against *Listeria* infection.

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