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SHORT COMMUNICATION

In Vitro Antibacterial Activity of Phlorotannins from Edible Brown Algae, *Eisenia bicyclis* Against Streptomycin-Resistant *Listeria monocytogenes*

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Abstract *Listeria monocytogenes* (LM) is an important food borne pathogen responsible for listeriosis. Further, LM is an etiological agent associated with life threatening conditions like meningitis and encephalitis. Biofilm forming and drug resistant LM may potentially become difficult to treat infections and hence effective controlling measures are required to prevent LM infections. In view of this, the present study evaluated an anti-listerial potential of edible brown seaweed, *Eisenia bicyclis*, by disc diffusion and micro-dilution methods. The results of the present study suggested that the anti-listerial activity of various phlorotannins isolated form *E. bicyclis* were in the range of 16–256 µg/ml. Among the phlorotannins isolated, fucofuroeckol-A (FAA) exhibited the highest anti-listerial potential (MIC range 16–32 µg/ml) against LM strains tested. Further, in checker board synergy assays, FFA-streptomycin combination exhibited significant synergy (fractional inhibitory concentration index, $\sum$FIC < 0.5) against aminoglycoside resistant clinical strains of LM. The results of the present study suggested the potential use of edible seaweed *E. bicyclis* as a source of natural phlorotannins to control food borne pathogenic infections.

Keywords Antibiotic resistance · Anti-listerial agents · *Eisenia bicyclis* · Phlorotannins

Listeria monocytogenes (LM), an important foodborne pathogen is responsible for listeriosis, meningitis and encephalitis. The ability of LM to produce virulence factors causes listeriosis a difficult to treat infection. Also listerial biofilms formed in food processing environment are resistant to antibiotics and disinfectants [1, 2]. Increased antimicrobial resistant pathogens in the food chain [3] demands novel food pathogen control strategies. *Eisenia bicyclis*, edible marine algae, is popular in Asian cuisine [4] and is known to exhibit antioxidant, anti-hypertension, anti-allergic, and anti-tumor activities [5]. In search of novel food-compatible anti-listerial compounds of seaweed origin, the present study evaluated the anti-listerial property of phlorotannins purified from *E. bicyclis*.

All chemicals, antibiotics, and reagents used were purchased form Sigma Chemical Co (St. Louis, MO, USA) unless or otherwise mentioned. *E. bicyclis* (Ulleung Trading Co. Ulleung-gun, Korea) was thoroughly washed with distilled water and dried at 60 °C in a hot air oven. A reference LM strain (ATCC 19112) was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Clinical strains of LM were obtained from Gyeongsang National University Hospital (GNUH; Jinju, Korea). All strains were aerobically cultured in brain heart infusion broth (BHI; Difco Inc., Detroit, MI), and incubated at 37 °C. In case of determining antibacterial properties, Mueller–Hinton broth and agar (MHB and MHA; Difco Inc.) were used. The extraction and purification of *E. bicyclis* was performed according to the method previously reported [6]. The crude and isolated compounds were dissolved in dimethyl sulfoxide (DMSO). All antimicrobial

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assays were performed according to the guidelines of Clinical and Laboratory Standards Institute [7]. For disc diffusion assays, MHA plates seeded with test strains (10^4 CFU/ml) and sterile paper discs (6 mm diameter) impregnated with crude extract, fractions or pure compounds and placed on the surface of MHA and incubated at 35 °C. Untreated and solvent treated (DMSO, 10%) discs served as blank and negative controls, respectively. After 24 h, the size of inhibitory zones was determined. In growth assays, MHB was adjusted with two fold serial dilutions of either fractions or pure compounds in DMSO (1% v/v in final volume) were inoculated with test strains (10^4 CFU/ml) and incubated at 35 °C. After 24 h, the optical density (OD_{610} nm) of the culture was determined using microplate reader. DMSO (1% v/v) served as negative controls. MIC is determined as the lowest concentration of test agent which cause > 90% inhibition in the growth of the test pathogen. For synergy testing, a checker board synergy testing was employed where a serial two-fold dilution of antibiotic as well as test compound was mixed to obtain different combinations of concentrations in MHB. The plates were inoculated with the test bacteria (10^4 CFU/ml) and incubated stationary at 35 °C for 24 h. The fractional inhibitory concentration index ($\sum$FIC) was determined according to the method previously reported [8]. A potential synergy was conferred when FIC index was < 0.5.

In disc diffusion assay, the methanolic extracts of *E. bicyclis* at 1 and 5 mg/disc exhibited a zone of inhibition (ZOI) in the range of 9.0 ± 0.5 to 14.0 ± 0.1 mm against LM strains (Table 1). These results are in agreement with the results of previous study [9] that methanolic extracts of seaweed plants were inhibitory against food borne pathogens including LM. A liquid–liquid extraction system selectively separates bioactive compounds based on their partition coefficient with different solvents. Using this approach we partitioned aqueous methanolic crude extracts of *E. bicyclis* with various non polar and polar solvents and the ZOI and MICs of each solvent soluble fraction was tested against LM strains. Among the solvent soluble fractions, the ethyl acetate fraction exhibited ZOI in the range of 9.5 ± 0.4 to 15.0 ± 0.1 mm against LM strains (Table 2). Further, ethyl acetate fraction exhibited significantly lower MIC values (MIC range 128–256 µg/ml) against test LM strains which is comparable to the MIC values obtained (128 µg/ml) for ethyl acetate fraction of *Ecklonia cava* against LM [10].

Usually ethyl acetate fractions of seaweeds were reported to contain antimicrobial phenolics, tannins and flavonoids [9]. The purification of ethyl acetate soluble fraction resulted in six phlorotannins (Fig. S1) namely eckol (EK), fucofuroeckol-A (FFA), 7-phloroeckol (7-P), dioxinodehydroeckol (DD), phlorofucofuroeckol (PFF), dieckol (DE) whose identity was confirmed in comparison with the proton and carbon NMR spectra of authentic samples [9, 11, 12]. Further, these pure phlorotannins were evaluated against LM. As shown in Table 3, it is evident that FFA exhibited potent anti-listerial activity (MIC range 16–32 µg/ml) followed by PFF (32–128 µg/ml) and DE (64–128 µg/ml). All other compounds exhibited moderate toxicity against LM strains. Phlorotannins isolated from *E. kurome* and *E. bicyclis* (phloroglucinol, EK, PFF, DE and 8,8′-bieckol) were reported to possess antibacterial activity against Gram-positive and Gram-negative pathogens [13, 14]. Although the ethyl acetate fraction of *E. cava* was reported to possess an anti-listerial activity (MIC, 256 µg/

Table 1 Disc diffusion assay of methanol extract and its solvent-soluble fractions from *Eisenia bicyclis* against *Listeria monocytogenes*

Strains	Conc. (mg/disc)	Zone of inhibition (mm)[a]					
		MeOH[b]	Hexane	DCM	EtOAc	BuOH	H_2O
KCTC 19112	1	9.5 ± 0.2[c]	8.0 ± 0.1	8.0 ± 0.3	9.5 ± 0.4	ND	ND
	5	13.0 ± 0.5	10.0 ± 0.4	10.0 ± 0.5	15.0 ± 0.1	9.0 ± 0.2	ND
Strain 2148	1	10.0 ± 0.2	7.5 ± 0.2	7.0 ± 0.3	10.0 ± 0.4	ND	ND
	5	14.0 ± 0.1	9.0 ± 0.1	8.5 ± 0.4	14.5 ± 0.3	10.0 ± 0.1	ND
Strain 2637	1	9.0 ± 0.5	7.5 ± 0.4	7.5 ± 0.5	9.5 ± 0.4	ND	ND
	5	13.0 ± 0.1	8.5 ± 0.1	10.0 ± 0.1	14.0 ± 0.2	9.5 ± 0.2	ND
Strain 2868	1	9.0 ± 0.2	ND	ND	9.0 ± 0.2	ND	ND
	5	12.0 ± 0.5	8.0 ± 0.1	8.5 ± 0.4	13.5 ± 0.2	8.0 ± 0.3	ND

ND no detected antibacterial activity

[a] Methanol extract and its fractions from *E. bicyclis* were loaded onto discs (6 mm in diameter)

[b] *MeOH* methanolic extract, *Hexane* *n*-hexane-soluble fraction, *DCM* dichloromethane-soluble fraction, *EtOAc* ethyl acetate-soluble fraction, *BuOH* *n*-butanol-soluble fraction, *H_2O* water-soluble fraction

[c] Data are the averages of triplicate experiments

Table 2 Minimum inhibitory concentration (MIC) of methanol extract and its solvent-soluble fractions from *Eisenia bicyclis* against *Listeria monocytogenes*

Strains	MIC (μg/mL)[a]					
	MeOH[b]	Hexane	DCM	EtOAc	BuOH	H_2O
KCTC 19112	512	512	512	256	1024	–[c]
Strain 2148	128	256	512	128	512	–
Strain 2637	256	512	512	256	512	–
Strain 2868	256	512	512	256	1024	–

–, No activity detected

EtOAc ethyl acetate-soluble fraction, *BuOH* *n*-butanol-soluble fraction, *H_2O* water fraction

[a] Methanol extract and its fractions from *E. bicyclis* were loaded onto discs (6 mm in diameter)

[b] *MeOH* methanolic extract, *Hexane* *n*-hexane-soluble fraction, *DCM* dichloromethane-soluble fraction

Table 3 Minimum inhibitory concentration (MIC) of isolated phlorotannins from *Eisenia bicyclis* against *Listeria monocytogenes*

Strains	MIC (μg/mL)						
	EtOAc fr.[a]	EK	FFA	7-P	DD	PFF	DE
KCTC 19112	256	128	16	64	64	32	64
Strain 2148	128	128	16	128	64	32	64
Strain 2637	256	256	32	128	128	64	128
Strain 2868	256	256	32	128	128	128	128

[a] *EtOAc fr.* ethyl acetate-soluble fraction, *EK* eckol, *FFA* fucofuroeckol-A, *7-P* 7-phloroeckol, *DD* dioxinodehydroeckol, *PFF* phlorofucofuroeckol, *DE* dieckol

ml), the active compound was not reported [10]. Additionally, this study suggested a potent antibacterial activity of FFA against LM strains.

Antibiotic-resistant LM strains are frequently observed in the retail food [15]. One of the effective ways to develop new antimicrobials is to repurpose the existing antimicrobials against drug-resistant pathogens [16]. The clinical strains of LM used in the present study exhibited border line resistance to aminoglycoside antibiotics especially streptomycin (MIC > 8 μg/ml). The checkerboard assay results suggested that FFA-streptomycin combination exhibited synergy against all LM strains. The combination of streptomycin and FFA resulted in a ΣFIC_{min} range of 0.18–0.26 and ΣFIC_{max} range of 0.53–0.56 against all strains (Table 4). The median ΣFIC against LM strains ranged from 0.32 to 0.37 suggesting FFA-streptomycin combination exhibited marked synergy (Table 4). Earlier, β-lactam adjuvant action of phlorotannin from *E. bicyclis* and *E. cava and* against methicillin-resistant *Staphylococcus aureus* was reported [17, 18]. However, in our best of knowledge, the anti-listerial activity of FFA from *E. bicyclis* has not been investigated. The concentration of the FFA used in the present study is low and is clinically achievable. Brown algae, *E. bicyclis* and its phlorotannins were reported to be safe when orally administered in mice and exhibits relatively less cytotoxicity [13]. Hence, development of seaweed based therapeutic intervention is expected to reduce the listerial infections. In conclusion, the findings obtained from this study suggest that the marine brown algae *E. bicyclis* could be novel natural materials for the control and spread of *L. monocytogenes* infections in food and humans.

Table 4 Checker board synergy of streptomycin in combination with fucofuroeckol-A (FFA) against *Listeria monocytogenes*

Strains	Test compound	MIC (μg/mL)	Median ∑FIC[a]	$\sum FIC^{b}_{max}$	$\sum FIC^{c}_{min}$	Minimum conc. for observing synergy
Listeria monocytogenes (KCTC 19112)	FFA	16	0.344[d]	0.531	0.266	0.25
	Streptomycin	8				2.0
L. monocytogenes isolate 2148	FFA	16	0.375	0.563	0.266	0.25
	Streptomycin	16				4.0
L. monocytogenes isolate 2637	FFA	32	0.375	0.563	0.266	0.5
	Streptomycin	16				4.0
L. monocytogenes isolate 2868	FFA	32	0.328	0.563	0.188	2.0
	Streptomycin	32				4.0

$\sum FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, 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

[a] ∑FIC, the sum of FICs

[b] $\sum FIC_{max}$, the maximum ∑FIC

[c] $\sum FIC_{min}$, the minimum ∑FIC

[d] The FIC index indicated synergistic; < 0.5, addictive; 0.5 to < 1.0, indifferent; > 1.0 to < 2.0, antagonistic; > 2.0

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Compliance with Ethical Standards

Conflict of interest None.

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