In Vitro Antibacterial and Synergistic Effect of Chitosan-Phytochemical Conjugates Against Antibiotic Resistant Fish Pathogenic Bacteria

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Abstract Chitosan-phytochemical conjugates exhibited significant antibacterial effect with minimum inhibitory concentration (MIC) ranging from 128 to 2048 µg/ml against antibiotic-resistant fish pathogenic bacteria such as *Edwardseilla tarda*, *Vibrio harveyi* and *Photobacterium damsela* which were isolated from Korean cultured fish. Furthermore, the MIC values of old-fashioned antibiotics such as erythromycin and oxytetracycline drastically reduced in combination with chitosan-phytochemical conjugates against the fish pathogenic bacteria. The combination of conjugates with erythromycin and oxytetracycline gave median Σ FIC results ranging from 0.281 to 0.625 and 0.312 to 0.625, respectively. This result indicates the synergistic antibacterial effects and an increased susceptibility against the antibiotics.

Keywords Antibacterial effect · Chitosan-phytochemical conjugate · Fish pathogenic bacteria · Synergy effect

Edwardseilla tarda, *Vibrio harveyi* and *Photobacterium damsela* are facultative anaerobic Gram-negative bacteria which have been reported to cause putrefactive diseases in most of the commercially farmed eels, shrimps and olive flounders in Korea, Japan and China [1, 2]. It also causes huge economic loss to aquaculture farms globally [3]. Broad spectrum antibiotics such as oxytetracycline (OTC) and erythromycin (ERY) have been used to treat these Gram negative pathogenic bacteria. However, these drugs have become ineffective due to their frequent misuse. In recent years, combination therapies of antibiotics have been perceived as a way to minimize the emergence of drug resistant bacteria. Unexpectedly this combination therapy approach has also contributed to the increased rate of the resistance organisms. In a natural environment, the evolution of bacteria occurs to survive against the unfavorable condition such as antibiotic exposure by forming biofilms (an structural and functional component), which is coordinated by quorum sensing (QS) signaling molecules produced by themselves [4]. In an effort to develop alternative therapies against antibiotic resistant pathogenic bacteria, several studies have been reported on restoring antibiotic effect by combining antibacterial substances with natural products that acts as QS inhibitors [4–6].

Chitosan is a naturally occurring linear polysaccharide that has been reported as an effective antioxidant, antitumor, anti-inflammatory and enzyme inhibitor, which is used in several pharmaceutical and food industries [7, 8]. The phenolic phytochemical compounds such as ferulic, sinapic and caffeic acids have been recognized as naturally occurring antioxidants and are widely present in food items

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such as apple, coffee, olive oil, and vinegar [9]. Chitosan derivatives prepared by conjugating with these phytochemicals showed increased antioxidant activities in comparison to the unconjugated chitosan and also possess good antimicrobial effect against several foodborne pathogens and methicillin-resistant *Staphylococcus aureus* (MRSA) [9, 10]. The aim of present study is to evaluate the antibacterial effects of chitosan conjugates and also synergistic effect of combination of chitosan conjugates with antibiotics against fish pathogenic bacteria.

Chitosan-phytochemical conjugate was prepared followed by the previous reports [5, 7, 8]. The resulting chitosan-phytochemical conjugates were named as chitosan-ferulic acid (CFA), chitosan-sinapic acid (CSA), and chitosan-cafeic acid (CCA), respectively. The bacterial strains used in this study like *V. harveyi*, *E. tarda*, and *P. damsela* were isolated from black rockfish (*Sebastes schlegeli*), Japanese eel (*Anguilla japonica*), and olive flounder (*Paralichthys olivaceus*), and are preserved in the Department of Aquatic Life and Medicine, Pukyong National University, Busan, Korea. All bacterial strains were anaerobically cultured in Tryptic Soy Broth (TSB; Difco Detroit, MI) and incubated at 37 °C for 24 h. *V. harveyi* and *P. damsela* were cultured in TSB supplemented with 1% NaCl.

The antibacterial effect was determined by minimum inhibitory concentration (MIC) assay using two-fold serial dilution method. The overnight cultures of the strains were adjusted to 10^4 CFU per ml by using phosphate-buffered saline (PBS) and the inoculum was prepared in Muller-Hinton Broth (MHB, Difco) with 1% NaCl supplementation for *V. harveyi* and *P. damsela*. MIC values were recorded after 24–48 h incubation at 37 °C in a shaking incubator. Antibiotic susceptibilities of the fish pathogenic bacteria were tested against two antibiotics (ERY and OTC) which are commonly used in aquaculture using disc diffusion assay. The synergistic effect of phytochemical conjugates and antibiotics was determined by the fractional inhibitory concentration (FIC) index as described earlier [6].

Chitosan conjugates exhibited broad spectrum antibacterial effects against the fish pathogenic bacterial strains with MIC values in the range of 128–2048 µg/ml. However, weak antibacterial effect was observed in the unmodified chitosan (control) and the conjugates against two strains of *E. tarda* (EET 34 and EET 53) and all strains of *P. damsela* ranging 1024–2048 µg/ml (Table 1). The chitosan conjugates also act as quorum sensing inhibitor, which cannot eradicate bacteria and functionally active at lower concentration than that of antibiotics [11]. It has been reported that chitosan increase its antimicrobial activity after conjugating a hydroxycinnamic phytochemicals on its backbone [10]. Among all conjugates, CCA

Table 1 Minimum inhibitory concentration (MIC) of chitosan-phytochemical conjugates against fish pathogenic bacteria

Strains	MIC (µg/ml)			
	CCA	CFA	CSA	UM
<i>Edwardseilla tarda</i> EET 34	1024	1024	1024	1024
<i>E. tarda</i> EET 53	1024	1024	1024	1024
<i>E. tarda</i> EET 54	128	128	128	128
<i>Photobacterium damsela</i> FP 2137	2048	2048	2048	2048
<i>P. damsela</i> FP 2261	2048	2048	2048	2048
<i>P. damsela</i> FP 4137	2048	2048	2048	2048
<i>Vibrio harveyi</i> RFHW 9L	256	256	512	256
<i>V. harveyi</i> RFHW 1KA	256	256	256	256
<i>V. harveyi</i> RFHW 3KA	128	256	256	128

UM chitosan (control), CCA chitosan-cafeic acid, CFA chitosan-ferulic acid, CSA chitosan-sinapic acid

Table 2 Antibiotic susceptibilities of fish pathogenic bacteria isolated from Korean cultured fish

Strains	Zone of inhibition (mm)	
	ERY	OTC
<i>Edwardseilla tarda</i> EET 34	6	28
<i>E. tarda</i> EET53	10	22
<i>E. tarda</i> EET 54	12	22
<i>Photobacterium damsela</i> FP 2137	20	30
<i>P. damsela</i> FP 2261	16	6
<i>P. damsela</i> FP 4137	12	8
<i>Vibrio harveyi</i> RFHW 9L	14	26
<i>V. harveyi</i> RFHW 1KA	30	34
<i>V. harveyi</i> RFHW 3KA	30	34
Zone diameter of resistant breakpoint ^a	≤14	≤21

AMP 10 mg ampicillin, ERY 5 mg erythromycin, OTC 30 mg oxytetracycline, STR 30 mg streptomycin

^aZone diameter of breakpoint of antibiotic [16]

exhibited a better antibacterial effect ranging from 128 to 256 µg/ml against a strain of *E. tarda* (EET 54) and all strains of *V. harveyi*. Kwon et al. (2007) reported that caffeic acid can quench free electrons from the electron transport chain which changes the electric potential of the bacterial membrane, and also it can reduce or completely inhibit the bacterial growth by interfering with the proton efflux pump as a results of interaction with dehydrogenase enzyme [12]. These results strongly suggest that the chitosan conjugates possess better antibacterial effect than unconjugated chitosan. Therefore, it is imperative to say that the antibacterial effect of chitosan increased by

Table 3 Minimum inhibitory concentrations (MIC) and fractional inhibitory concentration (FIC) indices of chitosan-phytochemical conjugates in combination with antibiotics against antibiotic-resistant fish pathogenic bacteria

Strains	Test compound	MIC ($\mu\text{g/ml}$)	Median $\sum\text{FIC}$	$\sum\text{FIC}_{\text{max}}$	$\sum\text{FIC}_{\text{min}}$	Minimum concentration for synergy
<i>Edwardseilla tarda</i> EET 34	UM	1024	1.062	1.062	0.312	64
	ERY	2048				2048
	CCA	1024	0.281	0.531	0.187	32
	ERY	2048				512
	CFA	1024	0.312	0.562	0.250	64
	ERY	2048				512
	CSA	1024	0.281	0.312	0.187	32
	ERY	2048				512
<i>E. tarda</i> EET 53	UM	1024	0.281	0.562	0.250	32
	ERY	2048				512
	CCA	1024	0.281	1.031	0.250	4
	ERY	2048				512
	CFA	1024	0.281	0.562	0.187	4
	ERY	2048				512
	CSA	1024	0.312	1.031	0.250	8
	ERY	2048				512
<i>E. tarda</i> EET 54	UM	128	0.281	0.351	0.25	4
	ERY	2048				512
	CCA	128	0.281	1.031	0.25	4
	ERY	2048				512
	CFA	128	0.281	0.562	0.187	4
	ERY	2048				512
	CSA	128	0.312	1.031	0.250	8
	ERY	2048				512
<i>Photobacter-ium damsela</i> e FP 2261	UM	2048	1.125	1.125	0.75	256
	OTC	16				16
	CCA	2048	0.625	1.062	0.250	1024
	OTC	16				2
	CSA	2048	0.531	1.125	0.500	128
	OTC	16				8
	CFA	2048	0.312	1.062	0.25	128
	OTC	16				4
<i>P. damsela</i> e FP 4137	UM	2048	0.531	0.562	0.25	64
	ERY	2048				1024
	CCA	2048	0.515	1.031	0.265	32
	ERY	2048				1024
	CFA	2048	0.562	1.125	0.375	128
	ERY	2048				1024
	CSA	2048	0.312	0.562	0.250	128
	ERY	2048				512
	UM	2048	1.125	1.031	0.562	256
	OTC	32				32
	CCA	2048	0.562	1.125	0.500	128
	OTC	32				16
	CFA	2048	0.625	1.5	0.500	256
	OTC	32				16
	CSA	2048	0.562	1.125	0.500	128
	OTC	32				16

Table 3 continued

Strains	Test compound	MIC ($\mu\text{g/ml}$)	Median $\sum\text{FIC}$	$\sum\text{FIC}_{\text{max}}$	$\sum\text{FIC}_{\text{min}}$	Minimum concentration for synergy
<i>Vibrio harveyi</i> RFHW 9L	UM	256	0.562	1.062	0.500	16
	ERY	2048				1024
	CCA	256	0.562	1.062	0.500	16
	ERY	2048				1024
	CFA	256	0.625	1.062	0.500	32
	ERY	2048				1024
	CSA	512	0.562	1.062	0.375	32
	ERY	2048				1024

UM chitosan (control), CCA chitosan-cafeic acid, CFA chitosan-ferulic acid, CSA chitosan-sinapic acid, ERY erythromycin, OTC oxytetracycline, $\sum\text{FIC}$ the sum of FICs, $\sum\text{FIC}_{\text{min}}$ minimum $\sum\text{FIC}$, $\sum\text{FIC}_{\text{max}}$ the maximum $\sum\text{FIC}$; The FIC index indicated synergistic; < 0.5 , additive; 0.5 to < 1.0 , indifferent; 1.0 to < 2.0 , antagonistic; ≥ 2.0 [5]. $\sum\text{FIC}$ was calculated for each well with the equation: $\sum\text{FIC} = \text{FIC}_A + \text{FIC}_B = (C_A/\text{MIC}_A) + (C_B/\text{MIC}_B)$, 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

conjugating with phytochemicals (sinapic acid, cafeic acid, and ferulic acid) in a synergistic way.

The strain *P. damsela* FP 4137, *V. harveyi* RFHW 9L and all strains of *E. tarda* exhibited antibiotic resistance against ERY. Two strains of *P. damsela* (FP2261 and FP 4137) exhibited resistance against OTC (Table 2). MIC test was further performed using the antibiotics exhibiting resistance to *E. tarda* strains (EET 34, 53, and 54), *V. harveyi* RFHW 9L strain, and *P. damsela* strains (FP 2261 and 4137) to verify the MIC concentration of the antibiotics that inhibit the growth of the bacterial sample (Table 3). As shown in Table 2, *E. tarda* was found to be susceptible to OTC, which is similar to other studies [13]. However, it exhibited resistance to ERY as previously reported [14]. Interestingly, all strains showed high-leveled resistance to ERY with the MIC value (2048 $\mu\text{g/ml}$) (Table 3). The MIC value for ERY against *E. tarda* was 2048 $\mu\text{g/ml}$, in contrast to the earlier reported result of value 8–64 $\mu\text{g/ml}$ [15]. MIC for OTC against two strains of *P. damsela* ranged from 16 to 32 and 2048 $\mu\text{g/ml}$ for ERY. *V. harveyi* RFHW 9L strain showed resistance to ERY but was susceptible to OTC, which is similar to the previous studies [16]. Higher MIC values indicates an acquired resistance of fish pathogenic bacteria against antibiotics indicating that they are no longer effective in their treatment and hence an alternative treatment is needed [17, 18].

The MIC values of ERY alone against *E. tarda* strains were 2048 $\mu\text{g/ml}$, were further reduced to 512 $\mu\text{g/ml}$ when in combination with chitosan conjugates that exhibits a synergistic effect (Table 3). *V. harveyi* RFHW 9L strain only exhibited drug resistance to ERY and the MIC values from 0.562 to 0.625 of median $\sum\text{FIC}$ index. The

combinations of OTC and ERY with the conjugates against *P. damsela* strains showed a synergy effect of 0.312–0.625 median $\sum\text{FIC}$ index. The combinations with CFA-OCT and CSA-ERY resulted in a synergistic antibacterial effect with 0.312 of median $\sum\text{FIC}$ index against *P. damsela* FP 2261 and FP 4137 strain, respectively. Interestingly, it has been observed that *P. damsela* strain revealed less synergy effects in the combination of antibiotics and chitosan as compared to the combination of antibiotics with chitosan conjugates against the fish pathogenic bacteria. These results indicate that the bioactive compounds present in the chitosan conjugates reversed the high-leveled resistance properties against the antibiotics and also improved its efficiency on administration in combination [5, 8]. In conclusion, this study has demonstrated that chitosan-phytochemical conjugates can inhibit the growth of the fish pathogenic bacteria and the combination therapy also showed that the conjugates can reverse a high level of resistance of the antibiotics, restoring its antibacterial effects. However, further research is needed to evaluate its synergistic effect in vivo for the effective treatment of these pathogenic bacteria.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Xu T, Zhang XH (2014) *Edwardsiella tarda*: an intriguing problem in aquaculture. *Aquaculture* 431:129–135. <https://doi.org/10.1016/j.aquaculture.2013.12.001>
- Hervio-Heath D, Colwell RR, Derrien A, Robert-Pillot A, Fournier JM, Pommepuy M (2002) Occurrence of pathogenic vibrios in coastal areas of France. *J Appl Microbiol* 92:1123–1135. <https://doi.org/10.1046/j.1365-2672.2002.01663.x>
- Sawabe T, Fujimura Y, Niwa K, Aono H (2007) *Vibrio comitans* sp. nov., *Vibrio rarus* sp. nov. and *Vibrio inusitatus* sp. nov., from the gut of the abalones *Haliotis discus discus*, *H. gigantea*, *H. madaka* and *H. rufescens*. *Int J Syst Evol Microbiol* 57:916–922. <https://doi.org/10.1099/ijs.0.64789-0>
- Koul S, Prakash J, Mishra A, Kalia VC (2016) Potential emergence of multi-quorum sensing inhibitor resistant (MQSIR) bacteria. *Indian J Microbiol* 56:1–18. <https://doi.org/10.1007/s12088-015-0558-0>
- Kim JH, Yu D, Eom SH, Kim SH, Oh J, Jung WK, Kim YM (2017) Synergistic antibacterial effects of chitosan-cafeic acid conjugate against antibiotic-resistant acne-related bacteria. *Mar Drugs* 15:167. <https://doi.org/10.3390/md15060167>
- Kim KH, Yu D, Eom SH, Kim HJ, Kim DH, Song HS, Kim DM, Kim YM (2018) Fucofuroeckol: a from edible marine alga *Eisenia bicyclis* to restore antifungal effect of fluconazole against fluconazole-resistant *Candida albicans*. *J Appl Phycol* 30:605–609. <https://doi.org/10.1007/s10811-017-1232-1>
- Cho YS, Kim SK, Ahn CB, Je JY (2011) Preparation, characterization, and antioxidant properties of gallic acid-grafted-chitosans. *Carbohydr Polym* 83:1617–1622. <https://doi.org/10.1016/j.carbpol.2010.10.019>
- Eom SH, Kang SK, Lee DS, Myeong JI, Lee JH, Kim HW, Kim KH, Je JY, Jung WK, Kim YM (2016) Synergistic antibacterial effect and antibacterial action mode of chitosan-ferulic acid conjugate against methicillin-resistant *Staphylococcus aureus*. *J Microbiol Biotechnol* 26:784–789. <https://doi.org/10.4014/jmb.1511.11046>
- Aytekkin AZ, Morimura S, Kida K (2011) Synthesis of chitosan-cafeic acid derivatives and evaluation of their antioxidant activities. *J Biosci Bioeng* 111:212–216. <https://doi.org/10.1016/j.jbiosc.2010.09.018>
- Lee DS, Woo JY, Ahn CB, Je JY (2014) Chitosan–hydroxycinnamic acid conjugates: preparation, antioxidant and antimicrobial activity. *Food Chem* 148:97–104. <https://doi.org/10.1016/j.foodchem.2013.10.019>
- Koul S, Kalia VC (2017) Multiplicity of quorum quenching enzymes: a potential mechanism to limit quorum sensing bacterial population. *Indian J Microbiol* 57:100–108. <https://doi.org/10.1007/s12088-016-0633-1>
- Kwon YI, Apostolidis E, Labbe RG, Shetty K (2007) Inhibition of *Staphylococcus aureus* by phenolic phytochemicals of selected clonal herbs species of *Lamiaceae* family and likely mode of action through proline oxidation. *Food Biotechnol* 21:71–89. <https://doi.org/10.1080/08905430701191205>
- Lim J, Hwang Y, Park B, Yun H (2003) Combination effects of cephalixin and gentamicin on *Edwardsiella tarda* and *Streptococcus iniae*. *Int J Antimicrob Agents* 22:67–69. [https://doi.org/10.1016/S0924-8579\(03\)00086-4](https://doi.org/10.1016/S0924-8579(03)00086-4)
- Xiao BZ, Chen X, Xiang CB, Tang N, Zhang QF, Xiong LZ (2009) Evaluation of seven function-known candidate genes for their effects on improving drought resistance of transgenic rice under field conditions. *Mol Plant* 2:73–83. <https://doi.org/10.1093/mp/ssn068>
- Stock I, Wiedemann B (2001) Natural antibiotic susceptibilities of *Edwardsiella tarda*, *E. ictaluri*, and *E. hoshinae*. *Antimicrob Agents Chemother* 45:2245–2255. <https://doi.org/10.1128/AAC.45.8.2245-2255.2001>
- Scarano C, Spanu C, Ziino G, Pedonese F, Dalmaso A, Spanu V, Virdis S, De Santis EP (2014) Antibiotic resistance of *Vibrio* species isolated from *Sparus aurata* reared in Italian mariculture. *New Microbiol* 37:329–337
- Choi MS, Park KH, Choi SH (1995) Drug resistance and R plasmids in *Edwardsiella tarda* from cultured eel. *Int J Aquac Fish Sci Res* 11:141–150
- Miller RA, Harbottle H (2017) Antimicrobial drug resistance in fish pathogens. *Microbiol Spectr*. <https://doi.org/10.1128/microbiolspec.ARBA-0017-2017>