Thesis for the Degree of Master of Fisheries Science

In Vitro Antibacterial Activity of *Eisenia bicyclis* Extract against Fish Pathogenic Bacteria

By

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February 2020

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어류 병원성 박테리아에 대한 대황 추출물(*Eisenia bicyclis*)의 *In Vitro* 항균 활성

Advisor: Prof. Wongyu Park

By

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A thesis submitted in partial fulfillment of the requirement

for the degree of

Master of Fisheries Science

in KOICA-PKNU International Graduate Program of Fisheries Science,

Graduate School of Global Fisheries,

Pukyong National University

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February 21, 2020

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Abstract

The present study discovered antibacterial activity of the Phaeophyceae *Eisenia bicyclis* methanolic extract and its organic soluble fractions against Gram-negatives fish pathogenic bacteria such as *Edwardsella tarda, Photobacterium demselae* and *Vibrio harveyi*. Extract showed a significant bactericidal effect when tested by standardized disc diffusion assay, minimum inhibitory concentration (MIC), and fractional inhibitory concentration (FIC). *E. bicyclis* was found to be a source of an alternative medicine and potency regenerator for erythromycin (ERY) as a non-effective antibiotic widely used in aquaculture. The largest diameter of the inhibition zone was 12 mm at 1 mg/disc and 20 mm at 5 mg/disc by ethyl

acetate soluble fraction ($EtOA_C$) and MIC ranged from 128 to 1,024 μg/mL. Furthermore, the combination of old fashion erythromycin and EtOAc lowered MIC dosage of the resistant strains and resulted in a synergistic way. Contrary, the combination of oxytetracycline (OTC) and EtOAc mainly resulted in antagonism. The present findings suggest that EtOAc of *E. bicyclis* restore the antibacterial activity of erythromycin, a 50S inhibitor antibiotic, and it can be providing a clue to fighting to spread antibiotic resistance in aquaculture.

Keywords: *Eisenia bicyclis,* Fish pathogenic bacteria, Antibacterial activity, Synergistic effect

Introduction

Fish is expected to become the first source of animal protein worldwide by the next decade (Monteiro, 2018). Besides, these animals are being farmed in the intensive systems that allow high stocking density (Mood and Brooke, 2012). It results in the unhealthy environment of cultivation and excessive stress, sensitivity, and susceptibility to the attack of pathogens (Jobling, 1994; Austin and Austin, 2007). Bacteria are the most common pathogenic threats and the environment easily adapt themselves through mutations, or amplification of the genome and horizontal genes transference (Pridgeon and Klesius, 2012). Additionally, many antibacterial agents are used to control diseases and hold productivity high. All these genetic and physiologic phenomena of mutation result in structure alteration of the target cell, bacteria resistance, antibiotic inefficiency, and consequent diseases outbreaks (Kohansky et al., 2010; Scarano et al., 2014).

The use of conventional drugs is progressively becoming unsustainable due to the loss of affectivity on fish and for the danger that they represent for Humans (O'Neill, 2014). Current bactericidal has been since early 1990s (Grossman, 2016). *De facto*, old fashion antibiotics are less effective on the farmed fish (Eid, 2016), and harmful to public

health as the resistance is transferred from aquaculture products to human (Burridge, 2010). A study from the UK government shows that until 2050, 10 million people are expected to die due to infection caused by multidrug-resistant bacteria (O'Neill, 2014).

Fish outbreak diseases caused by pathogenic bacteria are among global concerns due to the numerous reports. *Edwardsella tarda* is a species of bacteria that causes diseases on freshwater and marine fish, whether farmed or wild population. The mortality of infected fish can go up to 90% (Garcia, 2012). A second pathogen is *Photobacterium demselae* and it has been a major limiting factor in the culture of Seabream (*Sparus aurata)* (Romalde, 2002). Nevertheless, it affects crustaceans, mollusks, and cetaceans, and is considered as an emerging pathogen in the marine aquaculture (Rivas et al., 2013). Another important species is *Vibrio harveyi*, a classic a fish marine pathogen that can cause 100% of mortality in larval stages (Soto-Rodriguez et al., 2010).

It is a global task to search for alternative substances with strong antibiotic properties (Grossman, 2016). Using this information, plants have been screened searching for alternative antibiotics or active metabolites that naturally enhance drug potency to fight against emergent resistant bacteria in aquaculture. Nowadays, more attention is given to the screening of marine organisms. Seaweed is more adequate than chemotherapeutic and cheaper than synthetic drugs (Adaikalaraj et al., 2012). *Eisenia bicyclis* contains polysaccharides of unique structure which give them antioxidant, antineoplastic, anti-

Alzheimer, anticancer, anti-atherosclerosis, anti-inflammatory, ant-allergic and anticoagulant activities (Menshova et al., 2013; Manshova et al., 2014; Lee et al., 2014). It was hypothesized that this marine algae has active metabolites that naturally present antibiotic activity and enhance drug potency to fight against resistant strains. Therefore, this research aims to evaluate an antibiotic potential of brown seaweed *E. bicyclis* and its synergistic effect with erythromycin (ERY) and oxytetracycline (OTC) against fish pathogenic bacteria.

Material and Methods

1. Source of sample

Eisenia bicyclis was selected as a source of extract with potential antibiotic activity. This brown seaweed ranges along the temperate waters at the Pacific Ocean, particularly in the coastal region of the East Sea between Korea and Japan. A fresh sample was purchased from Ulleung Trading Co. (Ulleung-gun, Korea), in late June 2019.

2. Preparation of extract and fractionation

A voucher specimen of 6 kg of *E. bicyclis* was immediately cleaned two times with groundwater to remove physical, chemical or biological potential contaminators. Furthermore, the seaweed was dried under the shade at environmental temperature. The dried status was completed in a dry oven, Thermostable Vacuum Ovens (DAIHAN; Cheras, Malaysia) at 60°C for 24 h. The resultant dried seaweed was smoothly powdered to 4.026 kg using Auto-iQ Nutri Ninja Blender Duo (Shenzhen, China). The fine and smooth powder were contained in vacuum plastic and kept at -79°C. Then, the powders was extracted, suspense and fractionated with organic solvents.

The seaweed powder sample of 300 g was extracted with 70% methanol (MeOH) to increase the soluble phenolic as demonstrated by Menshova et al. (2013), 3 times at 70°C for 3 h in Digital-Heating Mantle, (MS-DM608/10L; LKLAB Inc.; Namyangju, Korea) with a

rotation between 290 to 300 rpm. Afterwards, the product of extraction was concentrated to 125.86 g of crude extract in a vacuum, employing a rotational evaporator device (EYELA Co., Tokyo, Japan) at 45°C, 60 to 70 cmHg of a pressure gauge. The obtained crude extract was suspended in 1 L of 10% MeOH overnight, sequentially, fractioned with *n*-Hexane, dichloromethane (DCM), ethyl acetate (EtOAc), and *n*-Butanol (BuOH) by the proportion (1 g: 1 mL) in triplicate. The sequence of the solvent fraction was conducted following eluotropic series, from less to extremely polar.

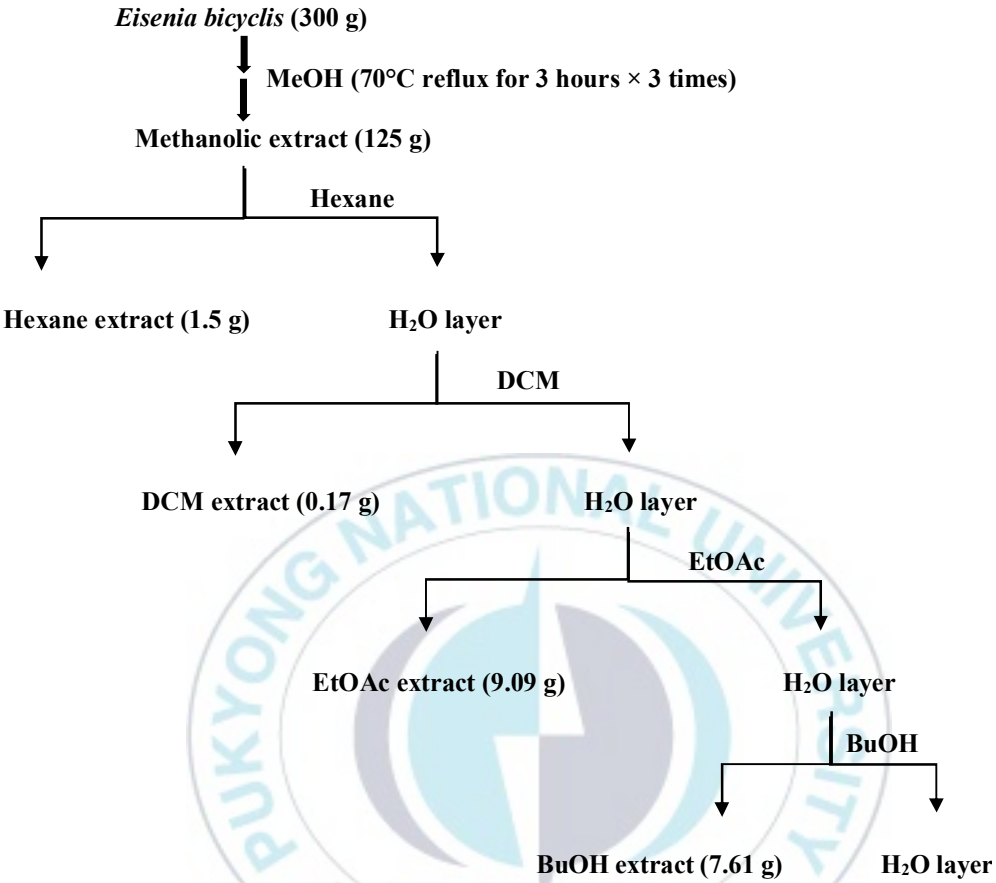

Figure 1. Scheme of methanolic extraction and the organic solvent fractions of *Eisenia bicyclis* dried powder. BuOH extract, n-Butanol soluble extract; DCM, dichloromethane soluble extract; EtOAc extract, ethyl acetate soluble extract; Hexane extract, n-Hexane soluble extract; and, H_2O extract, distilled water soluble extract.

3. Bacterial culture

The stock of bacteria was acquired from the Gyeongsang National University Hospital, Branch of National Culture Collection for pathogens (GNUH-NCCP) and preserved in the Fish Disease Prevention Lab of the Department of Aquatic Life Medicine, Pukyong National University (Table 1). The strains were cultivated in Tryptic Soy Broth (TSB; Difco Laboratories Inc., MI) supplemented with 1% sodium chloride (NaCl) and incubated anaerobically in DW-IB-210, Dongwon Scientific System (Seoul, Korea). Table 2 summarizes the culture conditions and media for bacterial growth and antibacterial activity assay performed using chemicals purchased from Sigma-Aldrich (St. Louis, MO).

Table 1. List of studied fish pathogenic bacteria

Bacterial species	Strain code	Source	Isolation year
Edwardseilla tarda	EET 34	*Anguilla japonica*	2011
	EET 53	*Anguilla japonica*	
	EET 54	*Anguilla japonica*	
Photobacterium damselae	FP 2137	*Paralichthys olivaceus*	2004
	FP2261	*Paralichthys olivaceus*	2005
	FP 4137	*Paralichthys olivaceus*	2004
Vibrio harveyi	FRHW 1KA	*Sebastes melanops*	2012
	RFHW 3KA	*Sebastes melanops*	
	RFHW 9L	*Sebastes melanops*	

Anguilla japonica, Japanese eel; *Paralichthys olivaceus,* Oliver flounder*; Sebastes melanops,* Black rockfish. All are marine fish species.

Table 2. Growth media and culture conditions of indicator fish pathogenic bacteria strains used for antibacterial activity and synergistic assay

Bacterial strain	Culture media	Incubation temperature	Antibacterial assay media	
			Disc Diffusion	MIC[b] and FIC[a]
Edwardsiella tarda EET 34	TSB[g] + 1% NaCl[e]	35°C	TSA[f] + 1% NaCl[e]	MHB[d] + 1% NaCl[d]
Edwardsiella tarda EET53				
Edwardsiella tarda EET 54				
Photobacterium damselae FP 2137				
Photobacterium damselae FP 2261	TSB[g] + 1% NaCl[e]	35°C	MHA[c] +1% NaCl[e]	MHB[d] + 1% NaCl[d]
Photobacterium damselae FP 4137				
Vibrio harveyi RFHW 9L				
Vbrio harveyi RFHW 1KA	TSB[g] + 1% NaCl[e]	35°C	TSA[f] + 1% NaCl[e]	MHB[d] + 1% NaCl[d]
Vibrio harveyi RFHW 3KA				

[a] FIC, fractional inhibitory concentration; [b] MIC, minimum inhibitory concentration; [c] MHA, Mueller Hinton Agar; [d] MHB, Mueller Hinton Broth; [e] NaCl, Sodium chloride; [f] TSA, Tryptic Soy Agar; [g] TSB, Tryptic Soy Broth.

4. Antibacterial assay of *E. bicyclis* extract

4.1. Disc diffusion assay

The disc diffusion assay was performed to determine qualitatively the susceptibility of fish pathogenic bacteria testing the effectiveness of known dosages of extract (Hadzicki, 2009). This standardized test was carried out following the procedures described in the international protocol of the Clinical and Laboratory Standard Institute (CLSI, 2012). Summarily, 100 µL of TSB containing 10^5 CFU/mL of individual strain obtained from the dilution in dimethyl-sulfoxide (DMSO) were seeded into MHA growth medium. Sequentially, 6 mm paper discs were soaked with fractions at the concentrated of 1 mg/10 µL and 5 mg/50 µL, and dried to allow the discs to absorb drug solution in the Forced Convection Dry Oven (DW-FO-121; Dongwon Scientific System, Busan, Korea) at 55°C. The dried disc was placed into the agar plate and incubated at 35°C for 24 h. Subsequently, the inhibition zone diameter was measured for judgements.

4.2. Minimum inhibitory concentration (MIC) assay

MIC is the lowest concentration of a particular active therapeutic drug/extract/combination of substances that prevents bacterial growth in approximately 90% (IDEXX, 2019). Its determination was performed following the standard assay protocol (CLSI, 2012). Briefly, 1,024 µg/mL of extract/fraction obtained from the dilution in DMSO was applied two-fold

serial dilution in 96-well microtiter plates containing 10^4 CFU/mL and incubated at 35°C for 24 h. Furthermore, results were accessed and interpreted according to the optical density ($OD_{600\ nm}$) of experimental samples read by Synergy™ HTX Multi-Mode Microplate Reader (Biotec, Korea). Therefore, the MIC indicated in the microtiter plate was considered as the clinical concentration of extract/fraction, required for a therapeutic effectivity.

5. Antibiotic susceptible test (AST)

AST is an *in vitro* response to any administration of bactericidal concentration with a known dose of the agent to blood or a tissue level (CLSI, 2012). Is used to know the adequate drug needed in response to the certain and diagnosed pathogen (Hadzicki, 2019). Its objective was to attain know the most successful antibiotic to combat specific strain by testing drugs from two classes; erythromycin (ERY) and oxytetracycline (OTC), as 50S and 30S ribosome inhibitors, respectively.

AST was performed by following the Kirby-Bauer method (KB test) in the MHA growth medium. The processes were carried out following the antibiogram assay described in (CLSI, 2012). Briefly, 5 and 30 mg of ERY and OTC was diluted in H_2O and dampened to the 6 mm paper disc. Furthermore, the disc was placed into MHA Petri dish previously seeded with 100 µL of 10^5 CFU/mL and incubated at 35℃ for 24 h. Lastly, the diameter of the inhibition zone

was measured to determine whether the bacteria are susceptible, intermediate or resistant to the application of each antibiotic following the guideline of WHO (2003) (Table 3).

Table 3. Inhibition zone diameter interpretation of disc diffusion assay (WHO, 2003)

Antibiotic	**Inhibition zone**		
	Susceptible	Intermediate	Resistant
Erythromycin Oxytetracycline	≥26	23 - 25	≤22

6. Synergy determination by fractional inhibitory concentration (FIC) assay

FIC is the comparison between the individual and the combined effect on the potency index value (Lee et al. 2014). The test was undertaken to find out the way to enhance drug potency of ERY and OTC against strains of fish pathogenic bacteria that previously demonstrated resistance to its application in the AST. The FIC index was calculated from known MIC_A and MIC_B following the equation utilized by Kim (2016). The FIC index was classified as claimed by Charway et al. (2018). Synergistic if the value of the FIC index was <1, additive if the FIC index was 1.0, sub-additive 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.

FIC index / $\Sigma FIC = FIC_A + FIC_B$

$FIC_A = MIC_{\text{COMBINATION}} / MIC_{A\ \text{ALONE}}$; and, $FIC_B = MIC_{\text{COMBINATION}} / MIC_{B\ \text{ALONE}}$

7. Statistical analysis

Data were averaged from the triplicate experiment. One way ANOVA was accessed by IBM SPSS Statistics Version 25. The significant difference was determined using the Tukey test for $P < 0.05$ and the standard deviation was measured for a spread of data around the mean.

Results and Discussion

1. Antibacterial activity of *E. bicyclis* extract against fish pathogenic bacteria by disc diffusion assay

The performance of qualitative and standard disc diffusion technic found a wide range of antibiotic activity naturally occurring in *E. bicyclis* against fish pathogenic bacteria. As shown in Table 4, the use of MeOH for extraction and *n*-Hexane, DCM, EtOAc and BuOH organic solvents for fractionation issued a broad range of antibiotic effect. It emitted 12 mm of the largest diameter of the inhibition zone from the application of EtOAc against *V. harveyi* AP9L and *V. harveyi* RFHW 3KA at the concentration of 1 mg/disc. Furthermore, it issued 20 mm against *V. harveyi* AP9L at the concentration of 5 mg/disc. The diameter of the inhibition zone was larger than the found by Kim (2015) and Kim et al. (2017) in opposition to the *Listeria monocytogenes* and Lee et al. (2014) against Staphylococcus genera. It indicates that fucofuroeckol-A is easily extracted at the polarity of ethyl acetate solvent because is the metabolite responsible for high antibiotic action and low toxicity (Lee et al., 2014).

The application of BuOH also exhibited significant affectivity in opposition to the growth of bacteria. The spectrum of this bactericidal activity was directly related to the biological

activities of the weed and its (Lin et al., 2008; Eom et al., 2011). Besides, eckol, 7-phloroeckol, dioxinodehydroeckol, phlorofucofuroeckol-A, and dieckol are bio-active compounds responsible for the bactericidal expression of *E. bicyclis* (Nagayama et al., 2002; Eom, et al., 2011; Kim et al., 2017). These findings support the increase of the solubility of extract components in BuOH soluble fraction when the extraction is performed above 50 and belong 100% (Zvyagintseva, et al., 1994; Menshova et al., 2013).

Although not statistically significant, MeOH soluble extract revealed the third-best effect, followed by *n*-Hexane, H_2O and DCM. Besides, it is the first report that the H_2O soluble fraction of *E. bicyclis* was able to inhibit the growth of more than 70% of tested strains. It was previously reported that this *Phaeophyceae macroalga* contains Laminarans polysaccharides, who can be soluble in water (Menshova, 2013). These single polymers are 1,3 and 1,6 attached β-d-glucose residues (Menshova et al., 2014), often associated with any important function on the bactericidal capability of a natural and therapeutic drug.

Table 4. Disc diffusion assay from methanol extract and its soluble fractions against fish pathogenic bacteria

Bacterial strain	Extract concentration	Zone of inhibition (mm)					
		MeOH	Hexane	DCM	EtOAc	BuOH	H_2O
E. tarda EET 34	1 mg/disc	-	7	7	8	7	-
	5mg/disc	7	8.6	8	11	11.3	7
E. tarda EET 53	1 mg/disc	-	-	7	7.3	7	-
	5mg/disc	-	7	8	9.6	12	7
E. tarda EET 54	1 mg/disc	-	-	-	7	7	-
	5mg/disc	7	7.6	8	10	12	-
P. damselae FP 2137	1 mg/disc	-	-	-	8	8	-
	5mg/disc	10	7	-	14	12	9
P. damselae FP2261	1 mg/disc	-	-	-	9	8	-
	5mg/disc	10	9	8	14	13	8.3
P. damselae FP 4137	1 mg/disc	-	-	-	-	8	-
	5mg/disc	7	-	-	9.6	7.3	-
V. harveyi AP9L	1 mg/disc	7	7	-	11.3	9	-
	5mg/disc	12.6	9	9.6	19.6	16	8.6
V. harveyi FRHW 1KA	1 mg/disc	-	-	-	8	7.6	-
	5mg/disc	10	7	7	12	14.3	8
V. harveyi RFHW 3KA	1 mg/disc	-	-	-	8	10	-
	5mg/disc	8.6	7.6	7	13.6	14	7.6

E. tarda, Edwardsiela tarda; P. damselae, Photobacterium demsielae; and, *V. harveyi, Vibrio harveyi.* BuOH, *n*-Butanol soluble extract; DCM, dichloromethane soluble extract; EtOAc, ethyl acetate soluble extract; Hexane, *n*-Hexane soluble extract; H_2O, distilled water soluble extract; "-", no detected antibacterial activity.

2. Antibacterial activity of *E. bicyclis* extract against fish pathogenic bacteria by MIC assay

The quantitative access of the antibacterial assay of *E. bicyclis* methanolic extract and its fractions against fish pathogenic bacteria was performed trough MIC, following procedures described in CLSI (2012). MIC experiment resulted in concentration value ranging from 128 to 1,024 µg/mL (Table 5). Among tested compounds, the EtOAc soluble faction demonstrated the strongest antibacterial activity ranged from 128 to 256 µg/mL. Naturally, drug affectivity was different in some microbes due to the biochemistry and molecular structure of the pathogens (Kohanski et al., 2008).

Another significant antibiotic activity was resultant of BuOH fraction application which exhibited the second broad-spectrum of effectivity against fish pathogenic bacteria. Over and above that, it is the first time that this fraction exhibit a wide range of activity in opposition to fish pathogenic bacteria. Perhaps, it means that the active compounds from the used seaweed are highly soluble in this organic solution (Menshova, 2013). Others extract also showed antibacterial action with MIC value ranging from 128 to 1,024 µg/mL. But, they were not statistically different. The fact suggests that fucofuroeckol-A has low solubility in those fractions showing less boarded antibacterial activity (Lee et al., 2014).

Except for EtOAc that exhibited broad and significant 256 µg/mL, all others soluble fractions showed ineffective MIC of 1,024 µg/mL against *P. demselae* FP413. Nevertheless, it is not the first report of resistance since it has presented a constant MIC value of 2,048 µg/mL to the application of chitosan-caffeic acid, chitosan-ferulic acid, chitosan-sinapic acid, and chitosan -garlic acid (Charway et al, 2018). The main active compounds with broad-spectrum of antibiotic of EtOAc soluble fraction is fucofuroeckol-A. It can boost the potency, although other fractions also contain other bioactive substances with bactericidal (Eom et al., 2011).

Table 5. MIC from methanol extraction and its soluble fractions against fish pathogenic bacteria

Bacterial Strain	MIC (μg / mL)					
	MeOH	**Hexane**	**DCM**	**EtOAc**	**BuOH**	**DW**
E. tarda EET 34	256	512	512	128	256	512
E. tarda EET 53	256	512	256	128	256	512
E. tarda EET 54	256	512	256	128	256	512
P. damselae FP 2137	256	512	512	256	256	512
P. damselae FP2261	256	512	512	256	256	512
P. damselae FP 4137	512	1,024	1,024	128	1,024	1,024
V. harveyi AP9L	512	128	512	128	256	512
V. harveyi FRHW 1KA	256	512	512	128	256	1,024
V. harveyi RFHW 3KA	256	512	512	256	256	1,024

E. tarda, Edwardsiela tarda; P. damselae, Photobacterium demsielae; and, *V. harveyi, Vibrio harveyi.*MIC, minimum inhibitory concentration. BuOH, *n*-Butanol soluble extract; DCM, dichloromethane soluble extract; EtOAc, ethyl acetate soluble extract; Hexane, *n*-Hexane soluble extract; H2O, distilled water soluble extract.

3. Antibiotic resistance profiles of fish pathogenic bacteria

Antibacterial susceptibility test is the result of the *in vitro* disc diffusion method carried out using antimicrobial agents against bacteria (WHO, 2003). Per that, outcomes from the application of ERY showed a difference in susceptibility. *P. demselae* 4137 and *V. harveyi* strains demonstrated the diameter of the inhibition zone ranging from 11 to 21 mm (Table 6). Besides, the guide of interpretation in Table 3 suggests resistance for these bacteria (WHO, 2003). This family of drug that inhibits protein synthesis of cells and included in the 50S class, often lose its bactericidal potency after the elongation has passed critical length (Tenson, 2003).

The *E. tarda* strains, *P. damselae* FP 2137, *P. damselae* FP 2261 and *V. harveyi* RFHW 1KA exhibited resistance to the treatment by OTC and issued the diameter of the inhibition zone ranging from 9.6 to 20.3 mm (Table 6). It was affirmed by Grossman (2016) that mutations in rRNA of bacteria result in resistance against OTC group. This is under the statement that 30S ribosome inhibitor such as OTC cannot block the access of aminoacyl-tRNAs to the ribosome of resistant strains (Chopra and Robert, 2001). It has shown full inability to combat satisfactorily against the current situation emergence in resistant bacteria related to aquaculture. Moreover, the drug is directly associated with incapacity to treat

microorganisms affected by mutations on the expression or functionality of their repressor (Afolayan, 2003). Therefore, *E. tarda* EET 34 was intermediate susceptible to the therapy.

The result is a demonstration of obsolescence and requests an urgent alternative. Many aspects can influence the difference in susceptibility or drug affectivity against certain bacteria and resistance of others (Lee, 2014). The main comes from the mechanism of resistance (Afolayan 2003), depending on dissimilarity in their cell wall structure and exterior membrane (McDonnell and Russell, 1999). Additionally, antibiotics may be a better solution against some pathogens and worst against others. The group of ribosome inhibitor therapies is broadly bactericidal when applied as medicine for specific species but not for another (Kohanski et al., 2008). Even so, ERY and OTC have demonstrated obsolescence and requested for an urgent effective alternative against fish pathogenic bacteria.

Table 6. Antibacterial activity of erythromycin (ERY) and oxytetracycline (OTC) against fish pathogenic bacteria

Bacterial strain	**Zone of inhibition (mm)**	
	ERY (5 mg/disc)	**OTC (30 mg/disc)**
Edwardsiella tarda EET 34	22.6	9.6
Edwardsiella tarda EET53	29	10.3
Edwardsiella. tarda EET 54	29	12.6
Photobacterium damselae FP 2137	29.3	10.6
Photobacterium damselae FP 2261	30	12
Photobacterium damselae FP 4137	11	29.6
Vibrio harveyi RFHW 9L	21.6	36.6
Vibrio harveyi RFHW 1KA	19.6	20.3
Vibrio harveyi RFHW 3KA	27.3	41.6

Inhibition zone diameter interpretation: susceptible, ≥26; Intermediate, 23–25; and, resistant, ≤22 mm. Data are the averages of triplicate experiments.

4. Synergistic between EtOAc with antibiotics

The EtOAc exhibited strongest and broad-spectrum antibacterial activity between the MeOH extract, *n*-Hexane, H_2O and DCM soluble fractions of *E. bicyclis* against, *E. tarda, P. demselae,* and *V. harveyi* strains. For this reason, it was chosen for further studies in combination with well-known old-fashionable antibiotics, ERY and OTC. The synergistic test was performed alluding to the fact of those bactericidal have failed to show any effective antibiotic propriety.

4.1 Synergistic effect of EtOAc in combination with Ery against fish pathogens bacteria

Erythromycin is ranked in the 6th position as the top used medicine for the treatment of fisheries diseases (Lavilla-Pitogo, 2017). It is believed to be effective in opposition to a wide range of gram-negative and gram-positive bacteria (Kohanski et al., 2010). This reason makes it an important drug to verify the concentration needed to inhibit the growth of fish pathogenic bacteria. The carried out MIC assay found 64 to 256 µg/mL as the concentration that inhibited 90% of bacterial growth (Table 7). Meaning that *E. tarda* EET 34, *P. damselae* FP 4137, *V. harveyi* RFHW 9L, and *V. harveyi* FRHW 1KA developed own sophisticated system to fight against the effect of phagocyte and to generate reactive oxygen species (ROS) on the immunologic response (Kohanski et al., 2008).

The conjugation between EtOAc and ERY resulted in the FIC index ranged from 0.75-1.5 as shown in Table 7. It is linked to the fact that 50S ribosome inhibitor as ERY is highly likely to interact with other drug molecules that behave like antibiotics (Kohanski et al., 2008). Thus, the application of this conjugation would impact positively in aquaculture and adjacent areas such as pharmacology, immunology, and environment (Hasan et al., 2018). These findings report synergism and interaction between bioactive molecules of ERY and EtOAc (Yang et al., 2017). Therefore, the action of both drugs together is better than one alone.

Table 7. FIC index of erythromycin (ERY) in combination with EtOAc soluble fraction from methanolic extraction of *Eisenia bicyclis* against ERY-resistant fish pathogenic bacteria

Bacterial strain	MIC[c] alone µg/mL	EtOAc[a] fraction MIC	Combined MIC	FIC[b] index	Minimum synergistic concentration	Interpretation
E. tarda EET 34*	64	128	32	0.75	32	SYN
P. damselae FP 4137	256	128	64	0.75	64	SYN
V. harveyi RFHW 9L	256	128	64	0.75	16	SYN
V. harveyi FRHW 1KA	256	128	128	1.5	128	S-AD

FIC index = MIC$_{combined}$ / MIC $_{alone\ \mu g/mL}$) + (MIC $_{Combined}$ / MIC $_{EtOAc\ fraction}$).

E. tarda, Edwardsiella tarda; P. damselae, Photobacterium demsielae; V. harveyi, and, *Vibrio harveyi.* [a] EtOAc, ethyl acetate soluble extract; [b] FIC fractionary inhibitory concentration; and, [c] MIC, minimum inhibitory concentration; *, a strain that shows intermediate inhibition zone diameter interpretation. Interpretation: SYN, synergistic, FIC index was <1; and, S-ADD, sub-additive, FIC index was between 1.0 and 2.0.

4.2 Synergistic effect of EtOAc in combination with OTC against fish pathogens bacteria

OTC is the world's most used antibiotic in aquaculture together with other tetracycline derivates (Lavilla-Pitogo, 2017) and known for a wide spectrum of effectivity against bacterial infection in farmed fish (Kohanski et al., 2010). The concentration of this drug needed to kill 90% of fish pathogenic bacteria was ranged from 0.5 to 512 µg/mL as reported in Table 8. This MIC variation depended on the target strain and the observed value represents a resistant breakpoint (IDEXX, 2019). It meaning that most of the tested strains are resistant and it would need an application of high dosage of this therapy to kill referred bacteria (Kohanski et al., 2010).

The combination of EtOAc and OTC resulted in FIC index >2.0 as shown in Table 8. It means the conjugation mainly resulted in antagonism (Yang et al., 2017) and the bioactive compounds of the used therapies are intrinsically divergent and enable to improve potency, whether *in-vitro* or *in-vivo* (Borisy et al., 2003; Grossman, 2016). It would be needed a high concentrated dose to cause damage to the bacteria cell pathway (Kohansky et al., 2008). Moreover, this combination resulted in an additive effect against *E. tarda* EET 34, meaning the fusion of active molecules of combined drugs occur in a less significant proportion (Borisy et al., 2003).

Table 8. FIC index of oxytetracycline (OTC) in combination with EtOAc soluble fraction from methanolic extraction of *Eisenia bicyclis* against OTC-resistant fish pathogenic bacteria

Bacterial strain	MIC [c] alone µg/mL	EtOA[a] fraction MIC	Combined MIC	FIC[b] index	Minimum synergistic concentration	Interpretation
E. tarda EET 34	0.5	128	0.5	1.00	0.5	ADD
E. tarda EET 53	512	128	512	>2	512	ANT
E. tarda EET 54	512	128	1024	>2	128	ANT
P. damselae FP 2137	256	256	1024	>2	256	ANT
P. damselae FP2261	512	256	1024	>2	128	ANT
V. harveyi FRHW 1KA	64	128	128	>2	64	ANT

FIC index = $MIC_{combined}$ / $MIC_{alone\ \mu g/mL)}$ + ($MIC_{Combined}$ / $MIC_{EtOAc\ fraction}$).

E. tarda, Edwardsiella tarda; P. damselae, Photobacterium demsielae; V. harveyi, Vibrio harveyi.[a] EtOAc, ethyl acetate soluble extract; [b] FIC fractionary inhibitory concentration; [c] MIC, minimum inhibitory concentration. Interpretation: ADD, additive, FIC index was 1.0; and, ANT, antagonistic, FIC index >2.0.

Conclusion

The world demand for alternatives and therapeutic approaches from a natural source to solve the problem of multidrug resistance properties developed by bacteria in aquaculture. In this sense, the present study aims to evaluate the antibiotic potential of brown seaweed *Eisenia bicyclis,* and its synergistic effect in combination with ERY or OTC against fish pathogenic bacteria. It was performed to access the therapeutic activity of MeOH soluble extract and its organic fractions by disc diffusion and MIC.

E. bicyclis contain biologically active substances with a broad spectrum of antibacterial activity qualified to inhibit the growth of fish pathogenic bacteria at the MIC of 128 to 1,024 µg/mL. Additionally, the largest diameter of the inhibition zone was 12 mm at 1 mg/disc and 20 mm at the concentration of 5 mg/disc. However, the fraction potency depends on the organic solvent used and the EtOAc fraction was found to be significantly effective, followed by BuOH, MeOH, *n*-Hexane, DCM and DW. Moreover, *P. demselae* 4137 and *V. harveyi* strains are resistant to the application of conventional ERY. Auspiciously, a combination of EtOAc fraction and ERY issued FIC index ranging from 0.0474 to 1.5, alluding interaction between their bioactive molecules. In contrast, *E. tarda* strains, *P. damselae* FP 2137, *P. damselae* FP 2261 and *V. harveyi* RFHW 1KA are OTC resistant. Besides, the union of this drug and EtOAc fraction result in antagonism.

The utilization of *E. bicyclis* has immense potential for controlling bacterial diseases in farmed fish, alone or combined with ERY therapy. Finding the mechanism of inhibitory activity of EtOAc fraction against fish pathogenic bacteria would be a future study. Moreover, it could play a key factor in killing the multidrug-resistant bacteria which are known to causes disease in aquaculture.

Acknowledgements

I would like to express my thanks to the Korean International Cooperation Agency (KOICA) for funding the degree,

Prof. Wongyu Park for being my supervisor and encouragement for research,

Prof. Young-Mog Kim for overseeing the project, guidance and expert advice,

Prof. Sung-Yun Hong for lending me his extraordinary books about scientific writing,

Profs. Antonio Hoguana and Manecas Baloi for intellectual support from Mozambique, and,

KOICA-PKNU coordinators for the prompt guidance and support that make everything easy.

I want to extend my appreciation to my classmates and lab-mates for collaboration,

To my family and Dulce, and for those who have been on my way to push forward.

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