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Isolation and Evaluation of Antimicrobial Activity of Endophytic Actinobacteria from horsetail Plant (*Equisetum diffusum* D. Don) against Bacterial Disease in Aquatic Animals

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ABSTRACT: Aquaculture is rapidly expanding in area andin intensity, however, there are serious problems caused by bacterial infection. The use of antibiotics is not sufficient to mitigate the outbreaks due to increase in antibiotic resistance. Therefore, to overcome the challenges of antibiotic resistance, antimicrobial compounds with a new mechanistic approach should be urgently sought. The aim of this study is to isolate and evaluate antimicrobial activity of endophytic actinobacteria from Horsetail plant (May Chang, *Equisetum diffusum* D. Don) against two pathogenic bacterial species *Aeromonas hydrophila* GL14, *A. veronii HY15* causing severe disease on common carp and catfish. The results showed that 9/32 (28.2%) endophytic actinobacteria isolates could inhibit at least one target pathogenic bacteria. Three isolates TB13, TB21 and TB17 showed the highest antibacterial response with minimium inhibitory concentration (MIC) ranging from 93.3 to 300 µl/mL. Amongst these, the lowest value is for TB21 and MTR622 without significant difference. When combining three individual actinobacteria mentioned above for fractional inhibitory concentration (FIC) test, the synergistic effect was found for the pair of TB13-TB17 against two tested pathogenic bacteria chosen with ∑FIC ≤0.5. The combination of two actinobacteria TB13 and TB17 improved bacterial inhibitory effect at least 4 times compared to individual treatment. The results are motivating enough to conduct further studies on use of endophytic actinobacteria for treating pathogenic bacteria in aquatic animals.

Keywords: May Chang, actinobacteria, *Aeromonas*, common carp, catfish

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INTRODUCTION

In recent 20 years, aquaculture in Vietnam has been expansing in the fishing area and enhancing in the level of intensification. However, the aquaculture industry is facing serious problems from environmental pollution and disease outbreaks. The problem of misuse or overdose of antibiotics was found to be the main cause ofthe phenomenon "antibiotic resistance" (Naylor *et al.*, 2000; Cabello *et al.*, 2006; Chouitad and Meddah, 2018). Many authors have reported that hundreds of antibiotics such as oxytetracycline, tetracycycline, ampicillinm florfenicolfaced high resistance against a range of pathogenic microorganisms (Goldburg *et al.* 2001; Miranda and Rojas, 2007; Su, *et al.*, 2011). Therefore, many countries around the world have regulated the use of antibiotic in aquaculture (Markestad and Grave, 1997; Cabello *et al.*, 2013).

Endophytic actinobacteria are known as producers of antibiotics and other biologically active substances with high commercial value for both humans and animals. A lot of herbal plants contain antibacterial compounds such as tannin, phenol, citral, quinone (Tanaka and Omura, 1993; Reverter *et al.*, 2014; Abdalla and Abdelgadir, 2016; Chouitad *et al.*, 2017). Numerous studies have shown that antimicrobial activity of the herbal plants are related to the beneficial actinobacteria as endophytic symbionts. They synthesize biological compounds which inhibit the bacteria and are safe for human. Therefore the selection of potential actinobacteria from herbal plants is a promising solution (Wang and Liu, 2010).

Horsetail plant (*Equisetum diffusum* D. Don) is an herbal plant containing many antimicrobial components that grows in Asian countries including Vietnam (Vo Van Chi, 2012).

Although Horsetail plant oil is in use in daily life, but there are no studies reporting the existence of the endophytic actinobacteria in Horsetail plant. Also, their antimicrobial activity against pathogenic bacteria which cause diseases on fish in particular and on other aquatic animals in general is yet to be understood. This is the reason that our research has focused on isolation and evalution of endophytic actinobacteria on microbial resistance against *Aeromonas hydrophila*, *Aeromonas veronii* causing severe diseases in common carp and catfish in Vietnam.

MATERIALS AND METHODS

A. Materials

Pathogenic bacteria. Tested isolates *Aeromonas hydrophila* GL14, *A. veronii HY15* which causered spot disease in common carp and epizootic ulcerative syndrome in channel catfish were provided from Environmental and Fish Pathology Department, Faculty of Fisheries, Vietnam National University of Agriculture, Vietnam.

Medium. Nutrient Agar (NA) and Nutrient Broth (NB) (Merck) were prepared at 121°C in15 minutes. The composition of medium Gause I includes starch powder - 20; K_2HPO_4 – 0.5; $MgSO_4.H_2O$ - 0.5; NaCl - 0.5; KNO_3 - 0.5; $FeSO_4$ - 0.01 (g/l); pH = 7-7.4. The composition of the antibiotic producing medium A4-H includes Glucoza – 15; Soybean powder – 15; NaCl – 5; $CaCO_3$– 1 (g/l); pH = 7 -7.4.

B. Methods

Endophytic actinobacteria (EA) isolation. Roots, stems and leaves of Horsetail plant were collected fromSon La, Yen Bai, Bac Ninh province, Vietnam. After collection, the surface of samples were disinfected following the process of Justin and Christopher (2003) and then cultured on Gause I with complementary nalidixic acid (25 mg/l), nystatin (50 mg/l) and $K_2Cr_2O_7$ (50 mg/l) to inhibit the growth of negative bacteria and fungi. After incubation of 4 days at 30°C, EAs were sub-cultured 3 times before screening antibacterial activity against tested pathogenic bacteria. Classification of EAs were based on the system of color wheels of Tresner and Buckus (1963).

Screening of EAs antibacterial activity. After isolation from Horsetail plant, EAs were determined for antimicrobial activity against pathogenic bacteria *A. hydrophila* GL14, *A. Veronii HY15* by agar diffusion method (Dhanasekaran *et al.,* 2012). Briefly, EAs were inoculated in medium Gause I and incubated by shakingat 200 rpm, 28°C for7 days and there after centrifuged at 6000 rpm for 10 minutes to get crude supernatant of each isolated EA strain. Tested bacteria were cultured on NB at 28°C for 24h and then adjusted to 10^8 CFU/mL by measurement using a spectrophotometer with a 600nm wavelength light and confirmed by colony counting method on NA medium (Putman *et al.,* 2005). Bacteria were spread and inoculated on sterile NA medium in separate plates using sterile glass stick. Sterile paper discs (6mm) were placed on agar where bacteria was placed. Crude supernatant of each EA strain(50 µl) was added separately into each disc and incubated at room temperature for 24 h, bacterial growth was observed and the zone of inhibition was measured (Kafur *et al.,* 2011).

Determination of minimium inhibitory concentration (MIC) of EAs supernatant. Isolated EAs showing antimicrobial activity were selected for determination of MIC **(**Dore *et al.*, 1999). EAs were inoculated by mixing in antimicrobial producing medium A4-H at 200 rpm and 30°C. After 7 days of incubation, crude supernatant was separated by centrifuging at 6000 rpm for 10 minutes and then serially diluted twice. Briefly, mixed NB was obtained by adding each type of tested bacteria at 10^8 CFU/ml. 100 µl of EAs crude supernatantat dilluted concentrations was separately added to 900 µl mixed NB and incubated at 28°C, 24h before placing inoculumon NA plate and examined after 24h. The MIC was defined as the lowest concentration of EAs crude supernatnant preventing visible growth. All tests were performed in duplicate and analysed by software SPSS 20 and the differences are assessed by Turkey test.

Evaluation of interaction between endophytic actinobacteria (∑FIC). After identification of MICs of EAs supernatant, the interaction between EA metabolites was evaluated by determining the fractional inhibitory concentration (∑FIC) based on the method of Gutierrez *et al*. (2008). The test was carried out on 96 plates with 270 µL of each tested bacteria suspension containing 10^8 CFU/mL and15µL crude supernatants of each EA. After that, the plates were incubated at 30°C for 24h before placing on NA to check the growth of bacteria. A combination of crude supernatant of two EA sat different concentration was presented in Table 1.

Table 1: Combination of EAs crude supernatant at different concentration of MICs.

∑FIC		EA 1						
		2 MIC	**1.5 MIC**	**1 MIC**	**1/2 MIC**	**1/4 MIC**	**1/8 MIC**	**1/16 MIC**
EA 2	**2 MIC**	4.00	3.50	3.00	2.50	2.25	2.13	2.06
	1,5 MIC	3.5	3.00	2.50	2.00	1.75	1.63	1.56
	1 MIC	3.00	2.50	2.00	1.50	1.25	1.13	1.06
	1/2 MIC	2.50	2.00	1.50	1.00	0.75	0.63	0.56
	1/4 MIC	2.25	1.75	1.25	0.75	0.50	0.38	0.31
	1/8 MIC	2.13	1.63	1.13	0.63	0.38	0.25	0.19
	1/16 MIC	2.06	1.56	1.06	0.56	0.31	0.19	0.13

∑FIC was determined as a minimum combination of two EAs crude supernatant which can inhibit the growth of bacteria. So, ∑FIC was calculated as FIC_{EA1} + FIC_{EA2}; whereas FIC_{EA1}= $MIC_{EA1\ in\ combination}/MIC_{EA1\ in\ single}$ and FIC_{EA2} = $MIC_{EA2\ in\ combination}/MIC_{EA2\ in\ single}$. The result was interpreted the combination of EA1 and EA2 as: synergy with ΣFIC≤0.5, addition with 0.5 < ΣFIC≤ 1, indifference with 1 < ΣFIC≤ 4, antagonism with ΣFIC > 4. The test was carried out in triplicate.

RESULT AND DISCUSSION

A. Isolation of endophytic actinobacteria (EA)

Twenty-six (26) of EA strains were isolated from Horsetail plant (Table 2). Based on the system of color wheels of Tresner and Buckus (1963) and the color of sporulating aerial mycelium, EAs were classified into 5 color groups as White, Grey, Pink and Brown and Blue. In total of 26 EA strains, Grey group accounts for the biggest portion with 12 strains (46.2%), followed by White group (26.9%) and Brown group (11.5%). This result was in disagreement with the study of Le Thi Hien *et al.* (2014) which showed 37.1% of total 43 EA strains from soil belonging to White group. Apart from that, the isolation of EA was carried out on different kind of herbs such as *Aloe vera*, *Mentha* and *Ocimum sanctum* (Gangwar *et al.*, 2011).

B. Screening of antimicrobial activity of EA strains in Horsetail plant

The total of 26 EA strains were tested for antimicrobial activity against 2 isolates of pathogenic bacteria *A. hydrophila* GL14, *A. Veronii* HY15 which cause diseases in common carp and catfish. From Table 2, the results show that eight in the 26 strains (30.7%) exhibited inhibitory activity against at least one of the pathogenic microorganisms tested. Whereas, six out of the eight strains exhibited antimicrobial activity with the bacteria tested at different levels (Table 3). The results show that two strains TB13 and TB21 have large inhibitory zone ranging from 22.6 to 26.2 mm with the pathogenic bacteria tested. In addition, the value of TB17 fluctuated from 16.4 to 17.5 mm.

Table 2: Color classification and antimicrobial activity of endophytic actinobacteria.

S. No.	Color group of EAs	Number of EAs	Percentage of EAs (%)	Antimicrobial activity	
				Number of EAs	*Percentage of EAs (%)*
1.	White	7	26.9	3	11.5
2.	Grey	12	46.2	4	15.4
3.	Brown	3	11.5	0	0
4.	Pink	2	7.7	1	3.8
5.	Blue	2	7.7	0	0
	Total	**26**	**100**	**8**	**30.7**

Table 3: Antimicrobial activity of endophytic actinobacteria (EA) in Horsetail plant.

S. No	EA strains	Inhibitory zone (mm)	
		A. hydrophila GL14	*A. veronii HY15*
1	TB21	22.6 ± 1.5	23.6 ± 1.2
2	TB13	25.5 ± 1.3	26.2 ± 1.6
3	TB12	-	4.4 ± 0.6
4	TB411	-	5.6 ± 1.2
5	TB32	5.4 ± 0.7	6.8 ± 0.8
6	TB17	16.4 ± 0.7	17.5 ± 0.9
7	TB31	12.2 ± 1.8	1.8 ± 1.3
8	TB32	6.7 ± 2.4	8.4 ± 2.2

(-) None of antimicrobial activity

The inhibitory activities of these strains against a variety of pathogens suggested that these endophytic actinobacteria may be potential candidates for the production of bioactive compounds.

Although remaining five EA strains showed antimicrobial capacity, their inhibitory zone was small and unstable. Therefore, only 3 strains TB21, TB13 and TB17 were selected for further tests.

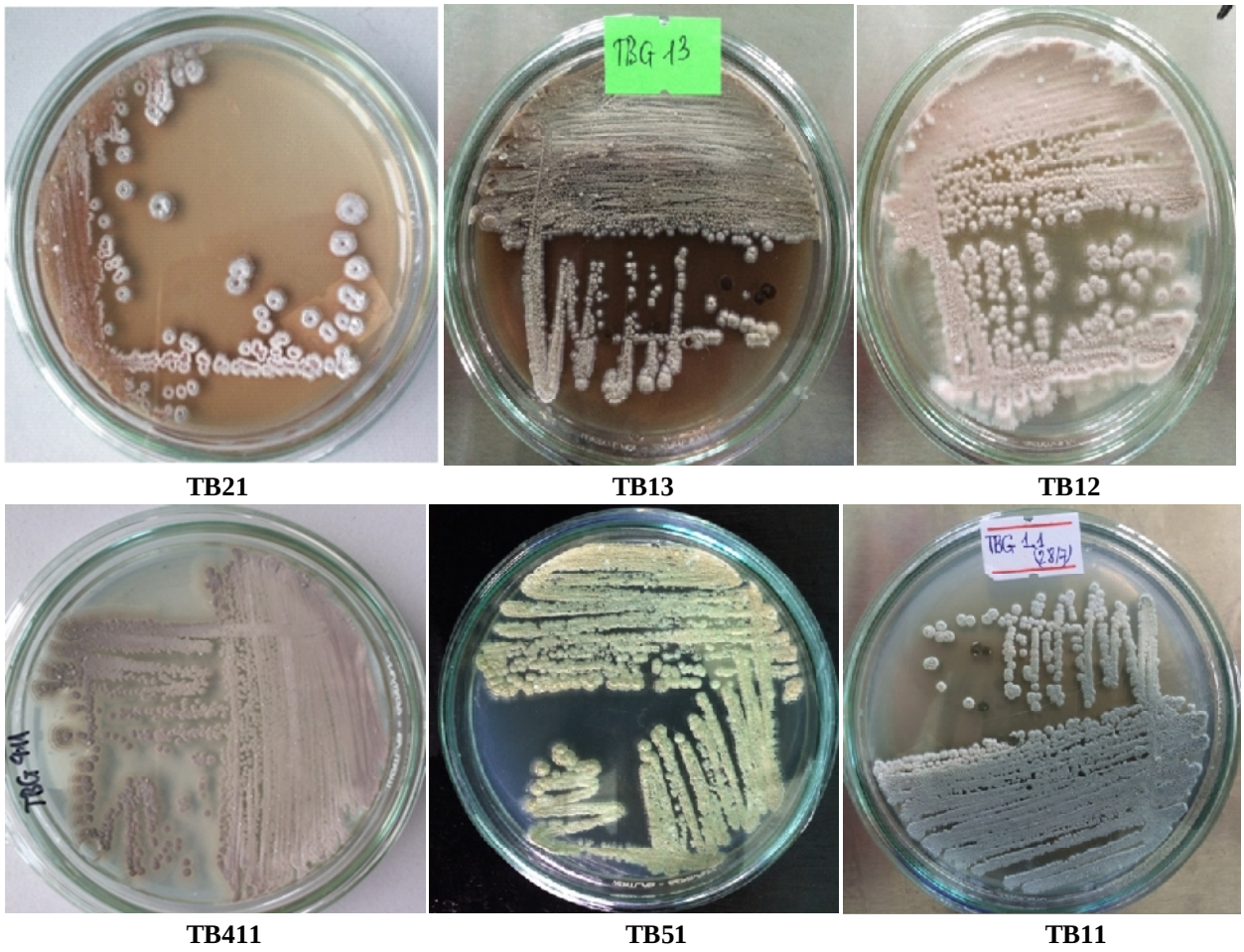

Fig. 1. Endophytic actinobacteria strains in medium Gause I.

Many endophytic actinobacteria have been approved for production of bioactive compounds against pathogenic micro organisms such as fungi, bacteria. Therefore, many of them are used as materials for extraction, synthesis of drugs and chemicals to mitigate diseases in humans and animals. Many studies have confirmed antimicrobial activity of EAs. Zhao *et al.* (2012) reported that there were 26 out of total 560 EA strains isolated from 26 medical plants in Panxi, China exhibiting inhibitory activity with at least 10.7%. Similarly, Li *et al.* (2008) isolated 41 EAs belonging to *Streptomyces*, which includes 65.9% and 24.4% of total EAs against *E. coli* and *Staphylococcus aureus*, respectively. Radulović *et al.* (2006) carried out the study on the volatile constituents of the sterile stems of *Equisetum arvense* L. (Equisetaceae) which were potential in inhibiting seven pathogenic micro-organisms by a disk diffusion method. The findings showed that the 1:10 dilution of the essential oil of *Equisetum arvense* L. possesses a broad spectrum of a very strong antimicrobial activity against all tested strains. In spite of many investigations on antimicrobial activity of EAs on human pathogenic microorganisms, there is a lack of investigation on *Equisetum diffusum* D. Don carried out in aquatic animals.

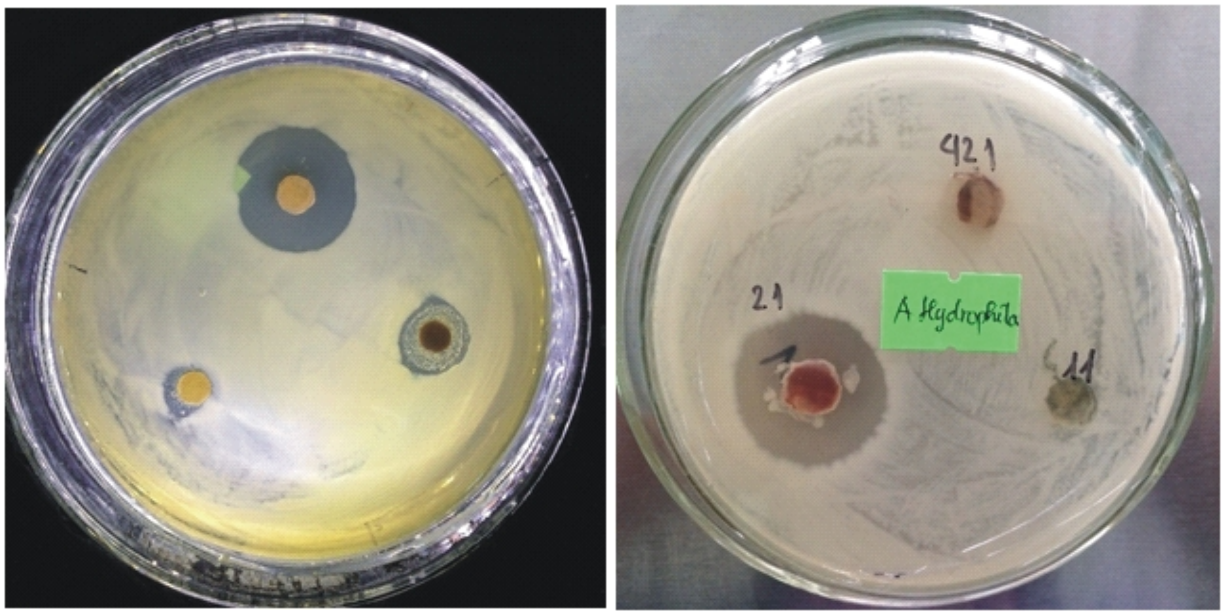

Fig. 2. Inhibitory zones of TB21 against pathogenic bacteria: (A) *A. veronii* HY15 (B) *A. hydrophila* GL14.

C. Minimium inhibitory concentration (MIC) of EA strains in Horsetail plant

From the results above, three EA strains TB13, TB21 and TB17 presenting the biggest inhibitory zone were chosen for MIC determination. The results from Table 4 show that TB21 has the lowest MIC ranging from 102.4 to 114.7 µl/mL against *A. veronii HY15*, *A. hydrophila* GL14 and was significantly different from two TB13 and TB17 ($p \leq 0.05$). The MIC of strain TB17 showed the highest MIC value with the range of 250.0 – 267.3µl/mL. These results proved that the antimicrobial effect of the strains TB13 were higher than that of TB21 and TB17. Our results are in agreement with Nguyen Hai Van *et al.* (2016) which report MIC of endophytic actinobacteria named MPT28 in May chang leaf ranged from 50–333 µl/mL against human pathogenic bacteria.

Table 4: Minimium inhibitory concentration of endophytic actinobacteria (EA) in Horsetail plant.

S. No	EA strains	Minimium inhibitory concentration (MIC) (µl/mL)	
		A. hydrophila GL14	*A. veronii HY15*
1	TB13	$114.7^{a} \pm 3.6$	$102.4^{a} \pm 7.8$
2	TB21	$123.3^{b} \pm 2.4$	$110.6^{b} \pm 5.7$
3	TB17	$250.0^{c} \pm 6.4$	$267.3^{c} \pm 8.3$

Note: Values followed by different letters within a column are significantly different ($p \leq 0.05$)

D. Interaction effect of EA strains (∑FIC) on antimicrobial activity

The interaction effect of 3 EA strains in pair combination is presented in Table 5. The results indicate that the combination of TB13 and TB17 has synergy effect of antimicrobial activity against all two tested bacteria ($\sum FIC \leq 0.5$). The combination of TB21 and TB17 has resulted in addition effect with ∑FIC in range of 0.5 – 1.0. In difference effect of TB13and TB21 was observed with ∑FIC >1.0. Therefore, the combination of TB13 and TB17 could decrease the concentration at least 4 times compared to the single treatment. The interaction effect of antimicrobial compounds were conducted by some studies. Cai *et al.* (2007) reported that MIC of allicin alone was 512 µg/mL, but it facilitated antibacterial activity of all three β-lactams tested at subinhibitory concentrations. In particular, ∑FIC of cefazolin was 0.5 ($1/4MIC_{allicin\ alone}$ and $1/4MIC_{cefazolin}$), ∑FIC of oxacillin was 0.375 ($1/8MIC_{allicin\ alone}$ and $1/4MIC_{oxacillin}$).

Table 5: Interaction effect of EA strains on antimicrobial activity.

Combination of EA strains	Pathogenic bacteria	∑FIC	Interaction*
TB13-TB21	*A. hydrophila*	1.4	Indifference
	A. caviae	1.7	Indifference
TB13- TB17	*A. hydrophila*	0.45	Synergy
	A. caviae	0.3	Synergy
TB21-TB17	*A. hydrophila*	0.8	Addition
	A. caviae	0.7	Addition

**Synergy (ΣFIC ≤0.5); Addition (0.5 < ΣFIC≤ 1); Indifference 1 < ΣFIC≤ 4; Antagonism (ΣFIC > 4)*

The study of Zafar Ahmed *et al*. (2013) showed that Amoxicllin and Cefadroxil have synergy effect against 47 isolates *Staphylococcus aureus* with value∑FIC in the range of 0.14 – 0.5.Whereas, Streptomycin and Cefadroxil showed synergestic antimicrobial activity against 44 isolates *S. aureus* ($\sum FIC_{min}$ 0.03 – 0.5). The study of Nguyen Hai Van *et al.* (2016) on interaction between EAs and May Chang oil indicated synergestic effect of the oil and EA strain named MPT28 against 4 isolates of human pathogenic bacteria.

CONCLUSION

1. There were 8 out of total 26 EA strains in Horsetail plant exhibiting antimicrobial effect on the two pathogenic bacteria *A. hydrophila* GL14, *A. Veronii HY15* which cause diseases in common carp and catfish. Three EA strains TB21, TB13 and TB17 showed wide inhibitory zones ranging from 26.2 to 17.5 mm.
2. MICs of 3 strains TB21, TB13 and TB17 displayed no significant difference in a range of 102.4 to 267.3 µl/mL against both tested bacteria.
3. The combination of TB13 andTB17 showed synergistic effect against two tested bacteria to enhance antimicrobial activity at least 4 times compared with single strain. This result could be of potential and promising application for sustainable therapy in aquaculture.

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