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In vitro antibacterial activity of several plant extracts against fish bacterial pathogens

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ABSTRACT

Crude methanol extract of 9 Vietnamese plants were in vitro screened for their antibacterial activity against three common freshwater fish pathogens including Aeromonas hydrophila, Edwardsiella ictaluri, and Streptococcus agalactiae. Agar disc diffusion method was used to evaluate the antibacterial activity, then solvent extract was performed for the extracts which exhibited the strongest and a broad-spectrum antibacterial activity. Minimal inhibitory concentration (MIC) was conducted for effective plant extracts using broth dilution method. The results indicated that most of the plant extracts exhibited antibacterial propeties to at least one tested bacterium. Headache tree (Premna corymbosa), bushwillows (Combretum quadrangulare) and Celandine spider flower (Cleome chelidonii) showed a broad-spectrum antibacterial activity. The largest inhibitory zones of 35 mm and 21 mm were observed for the extract of Premna corymbosa against E. ictaluri and S. agalactiae, respectively. E. ictaluri was found to be the most susceptible for all of the extracts while A. hydrophila was the most resistant. The MIC of effective plant extracts against tested bacteria ranged between 0.39 mg/mL and 3.125 mg/mL. The result can be considered for further investigation of the development of an alternative therapy against bacterial infection in aquaculture.

1. INTRODUCTION

Bacterial diseases continue to be a severe constraint to sustainable aquaculture industry due to its high mortality level and heavy economic losses (Mishra et al., 2018). Amongst the common freshwater fish pathogens, *Edwardsiella ictaluri* and *Aeromonas hydrophila* were documented as the main cause of high mortality in striped catfish (*Pangasianodon hypophthalmus*) (Tu, Nguyen, et al., 2008). Moreover, *Streptococcus agalactiae* infection in tilapia outbreaks have been reported in many Asian countries, leading to 90% of mortality rate (Ha et al., 2011). Control and treatment of bacterial infection commonly relies on the use of chemical agents, particularly antibiotics to aquaculture ponds (Rico et al., 2013). However, the improper use of antibiotics is the main reason leading to the emerge and selection of antibiotic resistant-bacteria (Quach et al., 2014; Tu, Haesebrouck, et al., 2008). These resistant-bacteria or its genes, can be easily transferred to human via food consumption, via direct contact or via environment (Evans et al., 2009; Sreedharan et al., 2012). Thus, development of reliable alternative therapies against bacterial pathogens is crucial for improving both quality and quantity in aquaculture production.

Plant extracts have been chosen as a promising alternative to antibiotics due to its antibacterial properties and ability to promote growth, stimulate the immune system against bacterial infection (Bulfon et al., 2015; Ngo, 2015). Researchers have reported the antibacterial activity of many plants from different regions in the world. Turker *et al.* (2009) investigated antibacterial activity of aqueous, ethanol and methanol extracts obtained from 22 Turkish medicinal plants against fish pathogens and found that various solvent of *Nuphar lutea*, *Nymphaea alba*, *Stachys annua*, *Genista lydia*, *Vinca minor*, *Fragaria vesca*, *Filipendula ulmaria*, *Helichrysum plicatum* extracts revealed the highest inhibitory activity. In addition, the methanol extract of *V. minor* and the ethanol and aqueous extract of *N. lutea* showed a broad-spectrum antibacterial activity which against all of the tested bacteria including *A. hydrophila*, *Yersinia ruckeri*, *Lactococcus garvieae*, *S. agalactiae* and *Enterococcus faecalis*. In another study, various solvents extracts of 9 edible herbs as black pepper, clove, curry leaf, onion and Vietnamese coriander exhibited antibacterial activity against 9 common pathogenic bacteria in fish (Najiah *et al.*, 2011). Furthermore, a number of studies indicate that some plants with antibacterial activity can be used as an alternative agent against bacterial infections in aquaculture (AftabUddin et al., 2017; Mohammed & Arias, 2016; Zilberg et al., 2010).

Considering the huge potential of diversity plants as a source for antibacterial drugs, the present study aims to investigate the *in vitro* antibacterial activity of nine Vietnamese plant extracts (Table 1) against three common pathogenic bacteria including *A. hydrophila, E. ictaluri* and *S. agalactiae*, with the view of providing preliminary information about the antibacterial activity of local plants and its potential application in freshwater aquaculture.

Table 1. Plants used in the study

No.	Plant name	Family	Common name	Local name	Collection place
1	*Azadirachta indica*	Meliaceae	Neem	Sầu đâu	An Giang
2	*Cayratia trifolia*	Vitaceae	Fox grape	Dây vác	Hau Giang
3	*Cleome chelidonii*	Cleomaceae	Celandine spider flower	Mần ri	Hau Giang
4	*Combretum quadrangulare*	Combretaceae	Bushwilows	Trâm bầu	Hau Giang
5	*Cynara scolymus*	Asteraceae	Artichoke	Atiso	Da Lat
6	*Kalanchoe pinnata*	Crassulaceae	Air plant	Sống đời	Can Tho
7	*Premna corymbosa*	Lamiaceae	Headache tree	Lá cách	Can Tho
8	*Wedelia chinensis*	Asteraceae	Chinese wedelia	Cúc sài đất	Can Tho
9	*Xanthium strumarium*	Asteraceae	Cocklebur	Ké đầu ngựa	Hau Giang

2. METHODOLOGY

2.1. Preparation of plant extracts

Fresh plants were collected from some areas in the Mekong Delta, Vietnam. All collected plants were washed through tap water and oven dried at 45ºC. After drying, dried plants were finely powdered. Subsequently, 100 g of plant powder were macerated 5 times in 1 L of methanol, each time for 24 hours at room temperature. The extracts were then filtered through Whatman filter paper and were evaporated in a rotary evaporator for obtaining crude methanol extracts. Based on the screening result of crude methanol extract, the extracts which demonstrate strong and broad spectrum inhibitory effect were selected for solvent extraction. The crude methanol extract was well-mixed with water followed by shaking with hexane and ethyl acetate to give a methanol-water extract, hexane extract and ethyl acetate extract, respectively (Nguyen Kim Phi Phung, 2007).

2.2. Preparation of paper discs

The plant extracts were dissolved in DMSO (VWR Prolabo, USA), then impregnated onto a paper disc (8 mm, Advantec, Tokyo, Japan) and air-dried in a sterilized flow cabinet for 30 min. The positive controls were Doxycycline (30 µg), Flofenicol (30 µg) and Ampicillin (10 µg) for *A. hydrophila*, *E. ictaluri* and *S. agalactiae*, respectively. Paper discs impregnated with DMSO were used as negative control.

2.3. Preparation of bacterial inoculum

Three isolates of fish pathogenic bacteria including *A. hydrophila, E. ictaluri* and *S. agalactiae*, provided by the Department of Aquatic Pathology, Can Tho University, were recovered on Tryptone Soya Agar (Himedia, India) plates and incubated at 28ºC for 16-36 hours. Direct suspension method was used by picking some colonies then suspended in sterilized sodium solution (0.85% NaCl). Afterward, the bacterial inoculum was adjusted to a concentration of 10^8 CFU/mL by measuring the optical density through a spectrophotometer (OD_{610}=1 for *E.*

ictaluri and OD_{610}=0.8-0.9 for *A. hydrophila* and *S. agalactiae*).

2.4. Determination of antibacterial activity

Agar disc diffusion method was used to screen antibacterial activity of the plant extracts (Oonmetta-aree et al., 2006). The bacterial inocula were spread on Mueller Hinton Agar (Himedia, India) and kept for about 15 min in a flow cabinet to allow the surface of agar plate to dry. The prepared paper discs were placed onto MHA plates inoculated with respected bacteria, followed by incubation at 28ºC for 16-48 hours. The antibacterial activity of plant extracts was determined by measuring the diameter of inhibitory zone forming around the paper discs. All of experiments were performed in triplicate and the inhibitory zone's diameter of each plant extract is calculated as mean ± standard deviation (SD). Describe the positive and negative controls.

2.5. Determination of minimum inhibitory concentration (MIC)

The plant extracts which showed inhibition ≥15 mm were selected to determine the minimum inhibitory concentration (MIC) using the broth dilution method (Oonmetta-aree et al., 2006). Briefly, a series of concentrations of plant extract was prepared by two-fold serial dilution, ranged from 0.024 to 25 mg/mL. Tubes containing 1 mL broth were inoculated with broth medium in the absence of bacteria (blank control sample), bacteria with medium (positive control sample), and bacteria with respective antibiotic (negative control samples). *A. hydrophila* and *E. ictaluri* were grown in Nutrient Broth (Difco, USA) while *S. agalactiae* was grown in Brain Heart Infusion Broth (Merck, Germany). The test tubes were incubated at 28ºC for 16-48 hours with gentle shaking. The MIC of the plant extracts was determined as the lowest concentration of plant extract where no bacterial growth by eyes. The MIC experiment was performed in triplicate.

2.6. Data analysis

Mean and standard deviation values of inhibitory zones were calculated using Microsoft Excel version 2010.

3. RESULTS AND DISCUSSION

3.1. Antibacterial activity of crude methanol extracts

The screening assay of nine crude extracts against three common fish antibacterial pathogens were performed by agar disc diffusion method and the result was summarized in Table 2. There were no inhibitory zone of negative controls (DMSO) while the positive controls including Doxycycline, Florfenicol and Ampicillin showed strong antibacterial activity against *A. hydrophila* (20±0.58 mm), *E. ictaluri* (48.67±2.08 mm) and *S. agalactiae* (31.7±0.83 mm), respectively. The diameters of inhibitory zones for the tested plant extracts were varied, ranging from 9.2 to 35 mm. Most of the plant extracts showed the inhibitory activity against at least one tested bacterium. Among these extracts, *Cleome chelidonii* (Celandine spider flower), *Combretum quadrangulare* (bushwillows) and *Premna corymbosa* (headache tree) revealed a broad spectrum of inhibitory effect, although *C. chelidonii* extract showed a moderate activity against all tested bacteria. Additionally, the strongest antibacterial activity against *E. ictaluri* and *S. agalactiae* was demonstrated by the extract of *P. corymbosa* with the mean of inhibitory zone being 32.2±2.9 mm and 18.2±1.79 mm, respectively. Limited information existed on the antibacterial activity against 3 tested bacteria of *P. corymbosa* and *C. chelidonii* extracts. However, their inhibitory effect against other bacteria was documented in literature (Immaculate & Rani, 2015; Rahman et al., 2016). Sridhar *et al.* (2014) revealed the inhibitory activity of *C. chelidonii* methanol extract against both Gram-positive and Gram-negative bacteria including *Bacillus subtilis, Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa.* The leaves extract of *P. corymbosa* was found to contain some bioactive compounds as alkaloids, tannins, flavonoids and glycosides with refer to antibacterial activity (Uppin & Naik, 2017). Likewise, most isolated chemical constituents from *C. quadrangulare* belonging to the class of triterpenoids and flavonoids (Roy et al., 2014). Furthermore, the antibacterial activity of *C. quadrangulare* extract in this study is correlation with Trieu Thi Thanh Hang *et al.* (2018) who reported high antibacterial potential against *A. hydrophila* and *E. ictaluri.*

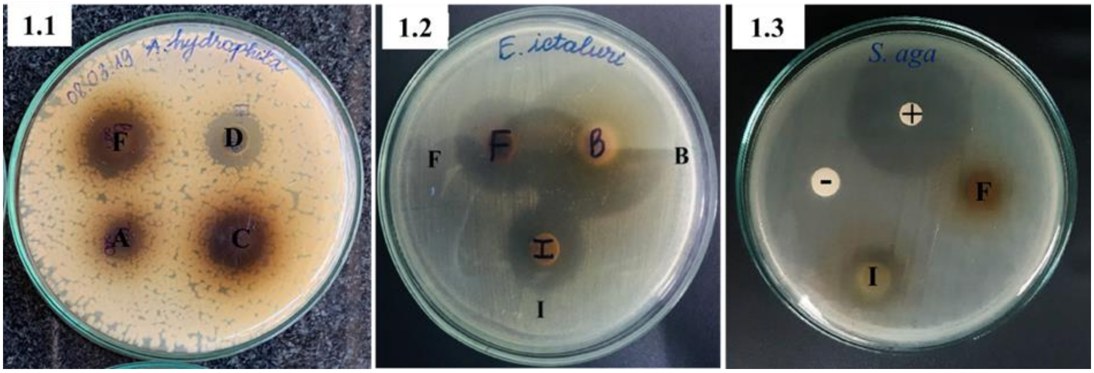

Figure 1. Antibacterial activity of crude methanol extracts against aquatic pathogenic bacteria

(1.1) A. hydrophila, (1.2) E. ictaluri, (1.3) S. agalactiae, (A) A. indica, (B) P. corymbosa, (C) C. quadrangulare, (D) C. scolymus, (F) K. pinnata, (I) C. trifolia, (+) Positive control, (-) Negative control

Among the tested bacteria, *E. ictaluri* was found to be the most sensitive to all tested plant extracts (Table 2), in which *P. corymbosa*, *C. quadrangulare* and *Kalanchoe pinnata* extracts showed strong inhibition, while the extracts of *C. trifolia* (fox grape), *W. chinensis* (Chinese wedelia), *Azadirachta indica* (neem), *Xanthium strumarium* (cocklebur), *C. chelidonii* (Celandine spider flower) and *Cynara scolymus* (artichoke) all showed a moderate activity (11-14 mm of inhibitory zone). As opposed to *E. ictaluri*, *A. hydrophila* was the most resistant bacterium, with 5/9 plant extracts including *A. indica*, *C. trifolia*, *W. chinensis*, *X. strumarium* and *C. scolymus* showed no inhibition or inhibition at weak level. The other extracts showed inhibitory effect at intermediate level with the largest zone of inhibition was recorded for *C. trifolia* extract (15.5±0.61 mm). Previous study also revealed the sensitivity of these two bacteria to various plant extracts (Huynh Kim Dieu, 2010), in which *E. ictaluri* and *A. hydrophila* were sensitive to 28/30 and 15/30 plant extracts, respectively. The results of present study were in contrast to those of Dao *et al.* (2020) who reported the ethanol extracts of *C. trifolia*, *A. indica* and *W. chinensis* were inactive to *E. ictaluri*. However, the three mentioned plant extracts did not exhibit inhibitory effect to *A. hydrophila* which was in agreement to the observation of Dao *et al.* (2020). Variations in the inhibitory effect of plant extracts against tested bacteria might be because of the differences in the plant part used, the age of plants and the local environmental conditions that affected the potency of plants (Ref). Furthermore, the extraction method and also solvent used could affect the amount of extracted bioactive compounds (Eloff, 1998; Azwanida, 2015).

Table 2. Antibacterial activity of crude methanol extracts

No.	Plant extracts	Zone of inhibition (mm ± SD)		
		A. hydrophila	***E. ictaluri***	***S. agalactiae***
1	*Azadirachta indica*	0	13.20±1.30	10.60±1.81
2	*Cayratia trifolia*	0	13.90±1.58	10.50±1.10
3	*Cleome chelidonii*	12.00±0.00	12.66±1.53	12.00±1.00
4	*Combretum quadrangulare*	13.67±0.58	23.00±1.00	16.67±0.58
5	*Cynara scolymus*	10.00±0.00	11.30±0.58	10.00±1.73
6	*Kalanchoe pinnata*	14.00±2.65	19.66±0.58	0
7	*Premna corymbosa*	13.30±1.60	32.20±2.90	18.20±1.79
8	*Wedelia chinensis* (Osbeck) Merr	0	13.66±0.58	19.70±1.53
9	*Xanthium strumarium*	9.20±1.30	12.80±2.60	12.20±1.09
10	Doxycycline (30 µg per disc)	20.00±0.58	-	-
11	Florfenicol	-	48.67±2.08	-
12	Ampicillin (10 µg per disc)	-	-	31.70±0.83
13	DMSO	0	0	0

Notes: (-) means not be tested.

Susceptible (S) ≥15 mm, Intermediate resistant (I) 11 – 14 mm and Resistant (R) ≤10 mm (Okoth et al., 2013)

According to previous antibacterial assay for screening purpose, the plant extracts were generally more effective to Gram-positive than Gram-negative bacteria (Dahiya & Purkayastha, 2012), due to the cell wall structure complexity in Gram-negative bacteria (Silhavy et al., 2010). In this study, *S. agalactiae* was sensitive to almost tested plant extracts, except *K. pinnata* which showed no inhibition. Although no information on the antibacterial potential of *K. pinnata* against *S. agalactiae*, previous study demonstrated that various solvent extract of *K. pinnata* did not revealed any inhibitory effect against various bacteira such as *B. subtilis, B. cereus, Staphylococcus epidermidis*, and *E. coli* (Kamal et al., 2014). However, Castro *et al.* (2008) found that *S. agalactiae* was the most resistant bacteria when screened 46 methanol plant extracts against 3 fish pathogenic bacteria including *A. hydrophila*, *F. columnare* and *S. agalactiae*. Only 5 methanol plant extracts including *Calyptranthes clusiifolia* (Miq.), *Croton floribundus*, *Heisteria silvianii*, *Merremia tomentosa*, *Zanthoxylum riedelianum* Engl. exhibited the inhibitory effect to *S. agalactiae* (Castro et al., 2008).

3.2. Antibacterial activity of solvent plant extracts

P. corymbosa was selected for solvent extraction due to its strong and broad-spectrum antibacterial activity (Table 3). The result showed that crude methanol and methanol-water extract of *P. corymbosa* exhibited higher antibacterial activity than hexane and ethyl acetate extract. The crude methanol and methanol-water extract revealed strong inhibitory against all tested bacteria, while hexane and ethyl acetate extract was ineffective against *A. hydrophila* and *S. agalactiae*. However, the results showed an intermediate inhibition against *E. ictaluri*, with the diameters of the zone of inhibition being 10.8±1.5 and 15.8±1.5 mm for hexane and ethyl acetate extract, respectively. Extraction is the important process because it is necessary to extract the desired bioactive compounds from the plant materials (Abdullahi R. & Haque, 2020). The composition of bioactive compounds of the resulting extract is affected by various factors including extraction time, temperature, solvents extraction method and solvent type. The objective of extraction process is to maximize the amount of target compounds and to obtain the highest biological activity of these extracts.

Table 3. Antibacterial activity of *P. corymbosa* solvent extracts

Plant extracts	Solvent fraction	Zone of inhibition (mm)		
		A. hydrophila	***E. ictaluri***	***S. agalactiae***
Premna corymbosa	Methanol	13.3±1.5	32.2±2.9	18.2±1.8
	Methanol-Water	14.3±0.7	35.0±1.6	21.0±1.4
	Hexane	0	10.8±1.5	0
	Ethyl acetate	0	15.8±1.5	0

3.3. Minimum inhibitory concentration (MIC) of plant extracts

The MIC value of each plant extract was in agreement with its zone of inhibition diameter in the case of *E. ictaluri*. For *S. agalactiae*, it was slightly on the contrary with the preliminary zone of inhibition which means some extracts having larger inhibitory zone but their MIC value was equal. As indicated in Table 4, the maximum MIC of 3.125 mg/mL against *S. agalactiae* was recorded in methanol extract of *C. quadrangulare, W. chinensis* and methanol-water extract of *P. corymbosa*. However, the methanol-water extract of *P. corymbosa* showed the minimum MIC of 0.39 mg/mL against *E. ictaluri*. Rajendran & Basha (2010) documented the MIC values of various solvent fractions (hexance, chloroform, ethyl acetate and ethanol) of *P. corymbosa* against *S. aureus, E. coli, Pseudomonas aeuroginosa, Salmonella typhi, S. typhi typhi A, S. typhi B, Vibrio chlorea, Entero cocci*, ranging from 33 to 133 µg/mL, which was much lower than the MIC found in current study. The differences in bacterial strains and solvents used in the research could be explained in this case.

Table 4. The MIC of plant extracts against respected bacteria

Plants	Extract	Bacteria	MIC (mg/mL)
Combretum quadrangulare	Methanol	*E. ictaluri*	1.56
		S. agalactiae	3.125
Kalanchoe pinnata	Methanol	*E. ictaluri*	0.78
Premna corymbosa	Methanol	*E. ictaluri*	1.56
		S. agalactiae	1.56
	Methanol-Water	*E. ictaluri*	0.39
		S. agalactiae	3.125
Wedelia chinensis	Methanol	*S. agalactiae*	3.125

Although future study of *in vivo* antimicrobial activity and the phytochemical analysis of solvent fractions are required, our study confirmed the antibacterial activity against several aquatic pathogens was demonstrated by various plant extract materials, that can be considered as an alternative to synthetic antibiotics currently used in aquaculture practice.

4. CONCLUSION

The present study indicates that *P. corymbosa* and *C. quadrangulare* methanol extracts possess a significant strong and broad-spectrum of antimicrobial activity against the three bacterial pathogens such as *E. ictaluri, A. hydrophila* and *S. agalactiae*. It is assumed that these plants can be potentially used in combating fish bacterial diseases.

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