Malaysian Journal of Microbiology, Vol 17(5) 2021, pp. 539-547
DOI: http://dx.doi.org/10.21161/mjm.211189

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Antibiotic resistant *Vibrio harveyi* isolated from shrimp pond and anti-*Vibrio* activity of *Combretum quadrangulare* Kurz. and *Mimosa pudica* extracts

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Received 1 June 2021; Received in revised form 8 July 2021; Accepted 23 August 2021

ABSTRACT

Aims: The occurrence of bacterial disease in shrimp ponds is a major problem faced in shrimp farming. Thus, the aims of this study were to isolate and evaluate antibiotic resistant profile of *Vibrio harveyi* strain isolated from shrimp pond water, as well as to study the potential anti-*Vibrio* activity of *Combretum quadrangulare* Kurz. (CQ) and *Mimosa pudica* (MP) leaves extracts.
Methodology and results: *Vibrio harveyi* WSC103 was isolated from water in white shrimp (*Litopenaeus vannamei*) culture pond and identified using 16S rRNA gene sequencing analysis. This strain showed characteristics of multidrug-resistant (7 antibiotics). It had become more sensitive to antibiotics (9 out of 10 antibiotics) after plasmid curing. It is showed CQ and MP leaves extracts contain potent bioactive compounds (tannins, flavonoids, steroids, cardiac glycosides and alkaloids) against *V. harveyi* WSC103. The aqueous, 95% ethanolic and 75% acetone extracts of CQ (MIC value of 3.13-12.50 mg/mL) and MP (MIC value of 3.13-25.00 mg/mL) leaves revealed strong vibriostatic activity, but aqueous and 95% ethanolic extracts in both plants showed vibriocidal activity. The 95% ethanolic extract of both CQ and MP leaves displayed the excellent vibriocidal property with MBC value of 100 mg/mL with zone of inhibition at 11.44 ± 1.01 and 11.78 ± 1.01 mm by agar disc diffusion.
Conclusion, significance and impact of study: The isolated *Vibrio harveyi* WSC103 was successfully characterized as a novel multidrug-resistant strain. The ethanolic *C. quadrangulare* Kurz. and *M. pudica* extracts exhibited prominent vibriostatic and vibriocidal capacities. These finding is proven that *C. quadrangulare* Kurz. and *M. pudica* extracts would be an alternative anti-*Vibrio* agent for aquaculture infectious treatment.

Keywords: Vibrio harveyi, antibiotic resistance, plasmid curing, plant extracts**,** anti-*Vibrio* activity

INTRODUCTION

Vibrio harveyi strain was a serious pathogen for many marine vertebrates and invertebrates (Austin and Zhang 2006; Tinrat and Singhapol, 2020). Many *Vibrio* sp. such as *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus* are the major species that cause the vibriosis in fish and shrimps (Austin and Austin, 1999), especially luminous disease has caused serious loss in shrimp farming. Shrimp larvae are particularly susceptible to *Vibrio harveyi* strain causing luminescent bacterial disease that may lead to high mortalities of larval stages in shrimps. There are many studies reported that *V. harveyi* was isolated from various sources such as sea bass (*Dicentrarchus labrax*) (Uzun and Ogut, 2015), sea bream (*Sparus aurata*) (Deane *et al*., 2012; Firmino *et al*., 2019), yellowfin sea bream (*Acanthopagrus latus*) (Esmaeili *et al*., 2019), tiger shrimp (*Penaeus monodon*) (Alavandi *et al*., 2006; Kannappan *et al*., 2018), white shrimp (*Litopenaeus vannemei*) (Aguilera-Rivera *et al*., 2017; Muthukrishnan *et al*., 2019). In the past, antibiotics have played an important role in the inhibiting or killing of *Vibrio* spp. in the aquaculture industry such as shrimp and fish. However, antibiotic properties have affected both pathogenic and beneficial bacteria. As a result, the normal flora strains in the aquatic animals and aquacultures are also eliminated which may affect the immunity of the aquatic animals themselves. *V. harveyi* strains have been recognized as major pathogenic bacteria in aquatic animals and non-pathogenic bacteria in humans (Zhou *et al*., 2012). Unfortunately, *V. harveyi* have been recently diagnosed in human infection (Del Gigia-Aguirre *et al*., 2017; Brehm *et al*., 2020). Therefore, *V. harveyi* infection would affect human health. It is normal norm for using antibiotics as first-line treatment for bacterial infection but, drug resistant has caused treatment abolishment (Cabello *et al*., 2013). Taken together, seeking a novel natural compound against the

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(5) 2021, pp. 539-547
DOI: http://dx.doi.org/10.21161/mjm.211189

virulence effect of *V. harveyi* is urgently needed.

Nowadays, there is an increasing demand of therapeutic drugs from diverse natural resources. Plant is a source of valuable bioactive compounds which has been well established and exploited (Tinrat, 2021). *Combretum quadrangulare* Kurz. and *Mimosa pudica* are the medicinal plants that commonly found in Thailand, Vietnam, Cambodia, Laos and Myanmar (Ahmad *et al*., 2012; Joseph *et al*., 2013). Both plants contain numerous bioactive constituents that have potential antioxidant (Nopsiri *et al*., 2014; Lakshmibai and Amirtham, 2018), antimicrobial (Thoa *et al*., 2015; Vijayalakshmi and Udayakumar, 2018), anticancer (Nopsiri *et al*., 2014), anti-inflammatory (Azam *et al*., 2015; Park *et al*., 2020), anti-diabetic activity (Sundaresan and Radhiga, 2015; Sutrisna *et al*., 2015) and hepatoprotective activity (Adnyana *et al*., 2001; Kumaresan *et al*., 2015). Previously, *C. quadrangulare* Kurz and *M. pudica* extracts demonstrated an antibacterial effect against various pathogens. Yet, the extracts have not been declared against *Vibrio* sp. Thus, the objectives of this study were to (i) isolate and identify the *V. harveyi* from water in *Litopenaeus vannamei* culture pond using biochemical and molecular techniques, (ii) determine the antimicrobial resistance profiles of *V. harveyi* isolated, (iii) screen the phytochemical constituents and (iv) assess the anti-*Vibrio harveyi* activities of *C. quadrangulare* Kurz. and *M. pudica* leaves extracts by agar disc diffusion and broth micro-dilution methods.

MATERIALS AND METHODS

Isolation and identification of *Vibrio harveyi* strain

Vibrio harveyi (VH) strains were isolated from water in *Litopenaeus vannamei* (white shrimp) culture ponds from Samut Sakhon Coastal Fisheries Research and Development Center, Samut Sakhon, Thailand by culturing on tryptic soy broth (TSB) supplement with 3% NaCl at 37 °C for 18-24 h and re-streaked on Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar containing 3% NaCl agar for purity checking. The morphology and biochemical tests (including casein utilization, lipid utilization, oxidase and fermentation test, blood hemolysis test and phenylalanine deaminase test) were studied. All isolates were identified as *V. harveyi* based on the Baumann and Schubert scheme (Baumann and Schubert, 1984). Haemolytic activity was performed on Columbia agar base (Oxoid) containing 3% (w/v) NaCl and 5% (v/v) sheep's blood and was then incubated at 30 °C for 24 and 48 h.

16S rRNA gene sequencing determination

DNA extraction

The overnight culture of isolated *Vibrio harveyi* was inoculated into TSB medium containing 3% (w/v) NaCl, following incubation at 37 °C for 24-48 h. Nucleic Acid Extraction Kit (QIAGEN) was used for extracting the bacterial genomic DNA of isolated strain, according to the manufacturer's protocols.

Primers, PCR condition and agarose gel electrophoresis

The VHARF-VHARR primer set (VHARF 5'-CCGCATAATACCTACGGGTC-3' and VHARR 5'-ACCCGAAGTGCTGGCAAACA-3') were used for identification of *V. harveyi*. PCR condition was fixed as initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 53 °C for 30 sec, 72 °C for 40 sec and final extension at 72 °C for 7 min. (Fukui and Sawabe, 2007). Gel electrophoresis (1.2% agarose) was used to observe the amplified PCR products. The PCR negative control were *Vibrio* ssp. (*V. parahaemolyticus* WS001) and the PCR master mix. BLAST analysis was carried out using the programs at NCBI's GenBank database. The sequences were also analyzed and aligned using the ClustalW program (MEGA X bioinformatics package). Phylogenetic tree was constructed using MEGA X with a level of 1000× bootstrap (Kumar *et al*., 2018).

Antibiotic susceptibility test

A total of the ten antibiotics, including amikacin, ampicillin, bacitracin, chloramphenicol, erythromycin, gentamycin, nalidixic acid, norfloxacin, penicillin and streptomycin (OXOID®, England) were performed antimicrobial susceptibility testing by using Kirby-Bauer disc diffusion assay (NCCLS, 2002; Hindler and Stelling, 2007). The zone of inhibition (diameter in mm) for each antibiotic was measured and expressed as resistance, R (0 mm $\leq$ IZs $\leq$ 15 mm), intermediate, I (16 mm $\leq$ IZs $\leq$ 20 mm) and susceptible, S (IZs $\geq$ 21 mm) (Mulaw *et al.*, 2019).

Plasmid curing

The plasmid of isolated VH was cured using protoplast formation following modifications of the methods by Tinrat *et al*. (2011). The overnight culture of VH with a concentration of 10^8-10^9 CFU/mL (OD_{600} = 0.2 by spectrophotometer) was used as the inoculum in 10 mL of TSB culture. After incubation at 37 °C for 24 h, the bacterial cells were collected by centrifugation at 3,000× *g* for 10-15 min at 4 °C and washed with ultrafiltration water. The re-suspension cell in a protoplast buffer (0.2 M sodium phosphate + 0.5 M sucrose + 20 mM $MgCl_2$, pH 7.0) was re-centrifuged and mixed with protoplast buffer supplemented with 10 mg/mL lysozyme (Sigma). Protoplast cells were then harvested by centrifugation after incubation at 37 °C for 2-3 h. Next, the protoplast cell was re-suspended in protoplast buffer and re-cultured on tryptic soy broth (TSA) supplemented with 0.5 M sucrose at 37 °C for 24 h for regenerating cell. Simultaneously, the viability measurement of protoplasts was then performed by plate counting method. The protoplast *V. harveyi* WSC103 was also regrown in the culture medium for antibiotic susceptibility testing.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(5) 2021, pp. 539-547
DOI: http://dx.doi.org/10.21161/mjm.211189

Preparation of crude extract of *C. quadrangulare* Kurz. and *M. pudica*

The dried leaves of *C. quadrangulare* Kurz. and *M. pudica* were obtained from the Si Prachan District, Suphan Buri, Thailand. The collected plant samples were carefully cleaned with distilled water and shade dried for 7-10 days at 35-37 °C. Dried plants were cut into small pieces (1-1.5 cm in diameter). Then, all plant samples were separately extracted by maceration with 95% ethanol and 75% acetone with a ratio of 1:5 for 7 days and decoction (ratio of 1:4) with distilled water at 60 °C for 2 h. The crude extracts were filtered (Whatman no.1 filter paper) and centrifuged (8,000 rpm or 6,720× *g* for 20 min). Afterwards, the filtrates were concentrated in a vacuum rotary evaporator and subsequently stored at –20 °C with the dark condition until further use. The percentage yield of crude extracts was calculated as follows:

Yield (%) = [(Weight of crude extract)/(Total weight of sample)] × 100

Phytochemical analysis

The qualitative phytochemical screening was carried out by chemical tests. The major natural chemical categories such as saponins, flavonoids, tannins, anthraquinone, steroids, cardiac glycosides, alkaloids and terpenoids were identified for presence and/or absence following prescribed methods (Harborne, 1998). The results were reported as follows: - = absent present (nil); + = trace/mildly present (1–30%); ++ = moderately present (31–50%); +++ = highly present (>50–70%); ++++ = very highly present (>70%) (Tinrat, 2021). If the colors or precipitates appearing after the testing is not more than one, two and three-sixth of the total volume in the tested tube, the amount of each phytochemical compounds will be reported as not more than 30%, 50% and 70%, respectively. If the colors or precipitates was shown more than half of the total volume in the tested tube, the amount of phytochemical compounds will be reported as >70%. The percentage of phytochemical compounds was determined by the amount of precipitate and the change in apparent color intensity compared to the initial control tube.

Antimicrobial activities

Agar disc diffusion method

Anti-*Vibrio harveyi* activity was assessed by agar disc diffusion method with some modification (Tinrat and Singhapol, 2020). *Vibrio harveyi* (VH) WSC103 was cultured in TSB (Difco, USA) containing 3% NaCl and incubated at 37 °C for 18-24 h. The stock solutions of crude extracts (400 mg/mL) were prepared and serially diluted with sterile distilled water to give concentrations of 200, 100, 50 and 25 mg/mL. An overnight-cultures of VH WSC103 strain was adjusted to OD_{600} of 0.2 (approximately 10^8-10^9 CFU/mL) by spectrophotometer. Briefly, the tested bacterial strain was swab onto the TSA (containing 3% NaCl) plates and sterile 6 mm paper discs was placed on the surface of TSA plate. Afterward, approximately 20 μL of the crude extracts were added in each paper discs. The bacterial growth was monitored on day 1, 3 and 5 after incubation at 37 °C in term of inhibition zones (IZ) in millimeters (mm), comparing with antibiotics as positive controls (ampicillin; Amp, 5 μg/mL and norfloxacin; Nor, 10 μg/mL).

Broth micro-dilution assay

Minimum inhibitory concentration (MIC) values of leaves extracts were determined by the broth micro-dilution assay with some modification (Tinrat and Singhapol, 2020). The concentration ranging from 0.195 to 200 mg/mL of the crude extracts were evaluated. Briefly, 200 μL of starter extract concentration were added into 96-wells microplate containing 200 μL of TSB supplement with 3% NaCl using 2-fold dilution method. About 5 μL of the VH culture (10^8-10^9 CFU/mL; OD_{600} = 0.2) was then dropped to each well. The well without VH strain was served as a negative control. The lowest crude extract concentration was determined as MIC values for inhibiting the VH-growth after 18-24 h of incubation at 37 °C. After that, the visually clear broth dilution wells were subculture on TSA plates containing 3% NaCl and incubated 37 °C for 24 h for examination of minimum bactericidal concentration (MBC) values.

Statistical analysis

All data were presented as a mean ± SD (n=9). One way analysis of variance (ANOVA) and paired t-test were used analyzed the significant difference (p<0.05).

RESULTS AND DISCUSSION

Isolation and identification of *Vibrio harveyi*

The bacterial consortia were screen from culture ponds of white shrimp (*L. vannamei*). The individual 13 isolates were grown on the agar plates that exhibited distinct morphology. All of them were labeled as WSC101 to 113 for tracking. Among 13 isolates, WSC103 strain was exhibited a promising character as *Vibrio* strains under molecular technique with specific primer set. The growing colony appeared as green colony on TCBS medium and emitted light in dark on TSA containing 3% NaCl after 24 h incubation. The morphology and biochemical profiles of WSC103 strain is shown in Table 1. This isolate was a Gram-negative, short-rod shape, motile, oxidase and catalase-positive. Furthermore, this strain could utilize glucose, lactose and mannitol as the carbon sources to produce acid under fermentation process. It was β-hemolytic strains because of appearing the clear zone on blood agar and also are able to survive at 0.5-6.0% NaCl and temperature range from 30 to 45 °C.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(5) 2021, pp. 539-547
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Table 1: Biochemical and physiological characteristics of *V. harveyi* WSC103.

Characteristic	Observation
Colony morphology / Shape	Circular, white (on TSA) / Short rod
Gram reaction	Negative
Cell arrangement	Single and Chain
Colony color on TCBS medium	Green
Hemolytic activity	β-hemolysis (clear zone)
Motility test	+
Catalase	+
Oxidase	+
Acid and gas from glucose (Triple sugar iron; TSI)	+/-
Fermentation	
Glucose	+
Fructose	-
Sucrose	-
Lactose	w/+
Mannitol	w/+
Sorbitol	-
Growth in NaCl (%) at	
0.5	+
1.0	+
3.0	+
6.0	+
9.0	-
Growth at	
30 °C	+
37 °C	+
45 °C	+
Casein utilization	+
Gelatin liquefaction	-
Arginine dihydrolase	-
Phenylalanine deaminase	-

+ = positive; - = negative; w = weak.

Based on molecular technique, WSC103 strain was identified by using 16S rRNA gene. The result showed that WSC103 strain displayed the PCR product at VHARF-VHARR region that was specifically detected *V. harveyi* (Figure 1A) comparing with *V. parahaemolyticus* WS001 as a negative control (Lane 2 in Figure 1A) in this study. The size of the amplicon was approximately 967 bp.

After blasting the WSC103 sequences at NCBI's GenBank database, the Mega X program was used for making multiple sequence alignments (MSAs) to obtain the NJ phylogenetic tree with a level of 1000× bootstrap. The result showed that the WSC103 strain had the similarity of 99% (Accession no. MT605241.1) with *V. harveyi* strain. The phylogeny distance of *V. harveyi* WSC103 nucleotide sequence was presented in Figure 1B.

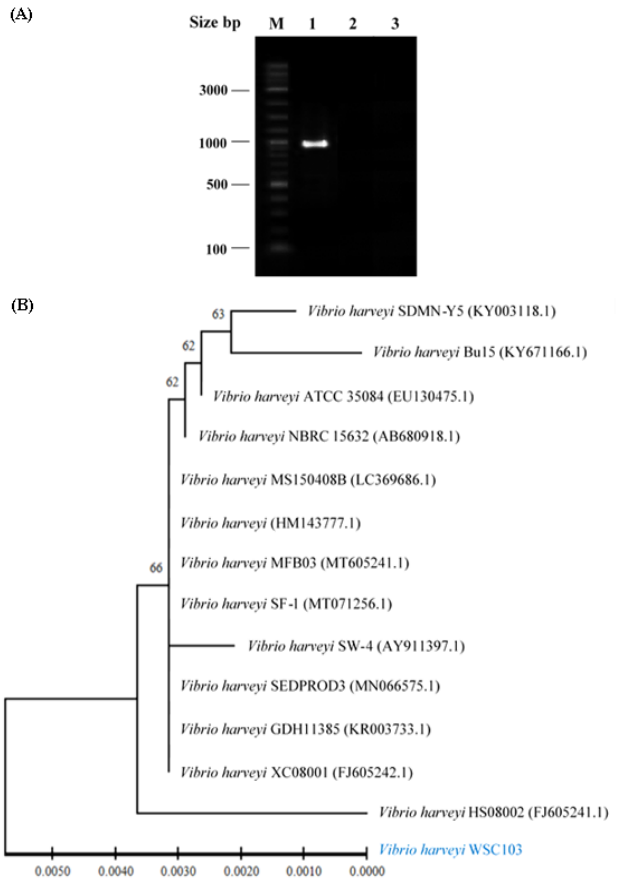

Figure 1: (A) PCR product of *Vibrio harveyi* WSC103 using VHARF-VHARR primer on 1.2% agarose gel electrophoresis. Lane M: 1 kb DNA ladder, Lane 1: WSC103 strain (~967 bp), Lane 2: Negative control (*V. parahaemolyticus* WS001), Lane 3: Negative control (PCR master mix). (B) Phylogenetic tree of *V. harveyi* WSC103 constructed by neighbor joining tree method using Mega X program.

Susceptibility of antibiotics testing

The results of antibiotic sensitivity test revealed that the *V. harveyi* WSC103 was sensitive to chloramphenicol, nalidixic acid and norfloxacin but it was resistant to ampicillin, penicillin, bacitracin, gentamycin, streptomycin, erythromycin and amikacin (0 mm ≤ IZs ≤ 15 mm) (Table 2). It is known that different antibiotics have distinct mechanisms against microorganism. For example, penicillin, ampicillin and bacitracin are classified as a cell wall synthesis inhibition antibiotic. Whilst gentamicin, streptomycin, erythromycin and amikacin abolished protein synthesis by interrupting translation process (Tinrat *et al*., 2011). Hence, based on the result of antibiotic sensitivity test, it is indicated that the isolated strain was resistant to multiple antibiotics through both mechanisms, including inhibition of cell wall synthesis and protein synthesis.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(5) 2021, pp. 539-547
DOI: http://dx.doi.org/10.21161/mjm.211189

Table 2: The susceptibility of antibiotics to the isolated *V. harveyi* WSC103.

Antibiotic agents	Disc content (μg; ∅ = 6 mm)	Inhibition zones (IZs; mm)	
		Normal cell	Protoplast cell
Group I: Inhibiting cell wall synthesis			
Ampicillin (Amp)	10	13.63 ± 0.26^{d}	18.50 ± 0.50^{d}
Penicillin (P)	10	0.00^{g}	8.00 ± 0.00^{h}
Bacitracin (B)	10	0.00^{g}	0.00^{i}
Group II: Inhibiting protein synthesis			
Gentamycin (CN)	10	10.48 ± 0.17^{e}	16.93 ± 0.53^{e}
Streptomycin (S)	10	7.50 ± 0.50^{f}	10.50 ± 0.50^{g}
Erythromycin (E)	15	7.00 ± 0.00^{f}	11.00 ± 0.00^{g}
Amikacin (AK)	30	9.93 ± 0.53^{e}	15.50 ± 0.50^{f}
Chloramphenicol (C)	30	27.59 ± 1.56^{b}	31.50 ± 0.50^{b}
Group III: Inhibiting nucleic acid synthesis			
Nalidixic acid (NA)	30	22.56 ± 0.91^{c}	26.93 ± 0.53^{c}
Norfloxacin (NOR)	10	30.48 ± 0.57^{a}	36.50 ± 0.50^{a}

R = Resistant (0 mm ≤ IZs ≤ 15 mm); I = Intermediate (16 mm ≤ IZs ≤ 20 mm); S = Sensitive (IZs ≥ 21 mm).
Different superscript alphabet in the same columns represents significantly difference (p<0.05).

Vibrio harveyi WSC103 was as a shrimp pathogen that demonstrated the multidrug resistance (ampicillin, penicillin, bacitracin, gentamycin, streptomycin, erythromycin and amikacin) in this study which presented the similar finding from Kumar *et al.* (2009). It was reported that *V. harveyi* isolated strains from seafood products (coastal India) were resistance to streptomycin. Moreover, *V. harveyi* WSC103 was also highly resistant to penicillin and bacitracin. The findings were also in agreement with studies of Parvathi *et al.* (2011) which presented various antibiotic especially penicillin and bacitracin. In contrast to previous studies, it is reported that *Vibrio* isolates (56%) were susceptible against bacitracin (Parvathi *et al*., 2011; Amalina *et al*., 2019).

Plasmid curing was performed to investigate whether the isolates contain plasmid harboring drug resistant genes. After the plasmid curing, the protoplast cell of *V. harveyi* WSC103 were performed to monitor changes in antibiotic resistance profile and results were shown in Table 2. The antibiotic susceptibility profiles of regenerated protoplast cell obtained in current study clearly indicated that the protoplast cell of *V. harveyi* WSC103 displayed decreasing antibiotic resistance (DAR) of range from 0.00-61.55% when compared with normal cell (Figure 2). *Vibrio harveyi* WSC103 was less resistant to penicillin but remained unchanged to bacitracin when compared with non-regenerated protoplasts (normal cells). The drug-resistant phenotype of *V. harveyi* WSC103 was altered due to the loss of plasmid DNA after the plasmid curing. *V. harveyi* WS103 showed an increased sensitivity to other antibiotics including ampicillin, gentamycin, streptomycin, erythromycin, amikacin, chloramphenicol, nalidixic acid and norfloxacin. The susceptibility of ampicillin and gentamycin was moderately increased (16 mm ≤ IZs ≤ 20 mm; DAR of 37.73-61.00%) while that of chloramphenicol, nalidixic acid and norfloxacin was strongly raised (IZs ≥ 20 mm; DAR of 14.17-19.75%) after plasmid curing. Antibiotic resistant of *V. harveyi* might be the putative negative impact for aquaculture industry in the event of epidemics such as luminous disease in shrimp. The antibiotic resistances of most bacterial strains are caused by the presence of a plasmid as an extra-chromosomal DNA within the bacterial cell that can also transferred drug resistance gene to other bacteria via plasmid (Manjusha and Sarita, 2013). The isolated *V. harveyi* WSC103 was declared to be a multidrug resistant strain by plasmid curing techniques. The finding indicated that *V. harveyi* WSC103 contains plasmids harboring drug-resistant genes.

Extraction yield and phytochemical screening

Many medicinal plants are the source of bioactive compounds that had potent antimicrobial activity to replace the antibiotic when antibiotic-resistant of bacteria occurs. *Combretum quadrangulare* Kurz. (CQ) and *M. pudica* (MP) leaves were choose for assessment of antimicrobial activity in the present study. The different secondary metabolites in *C. quadrangulare* Kurz. and *M. pudica* leaves were extracted by maceration in different solvents and decoction. The results showed that the highest yield extracts were obtained by aqueous extracts for MP (25.70 ± 0.06%) and CQ (13.90 ± 0.06%) leaves, followed by 95% ethanolic (5.80 ± 0.05-7.80 ± 0.05%) and 75% acetonic (5.50 ± 0.04-6.40 ± 0.05%) extracts in both plants, respectively.

High diversity of bioactive compounds was detected from preliminary phytochemical screening in ethanolic and 75% acetonic extracts. The preliminary phytochemical screening results of CQ and MP leaves extracts are shown in Table 3. The crude 95% ethanolic extracts of both plants were found to contain the rich secondary metabolites of tannins, flavonoids, steroids, cardiac glycosides and alkaloids in present study which are consistent with this previous observation of Kaur *et al*.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(5) 2021, pp. 539-547
DOI: http://dx.doi.org/10.21161/mjm.211189

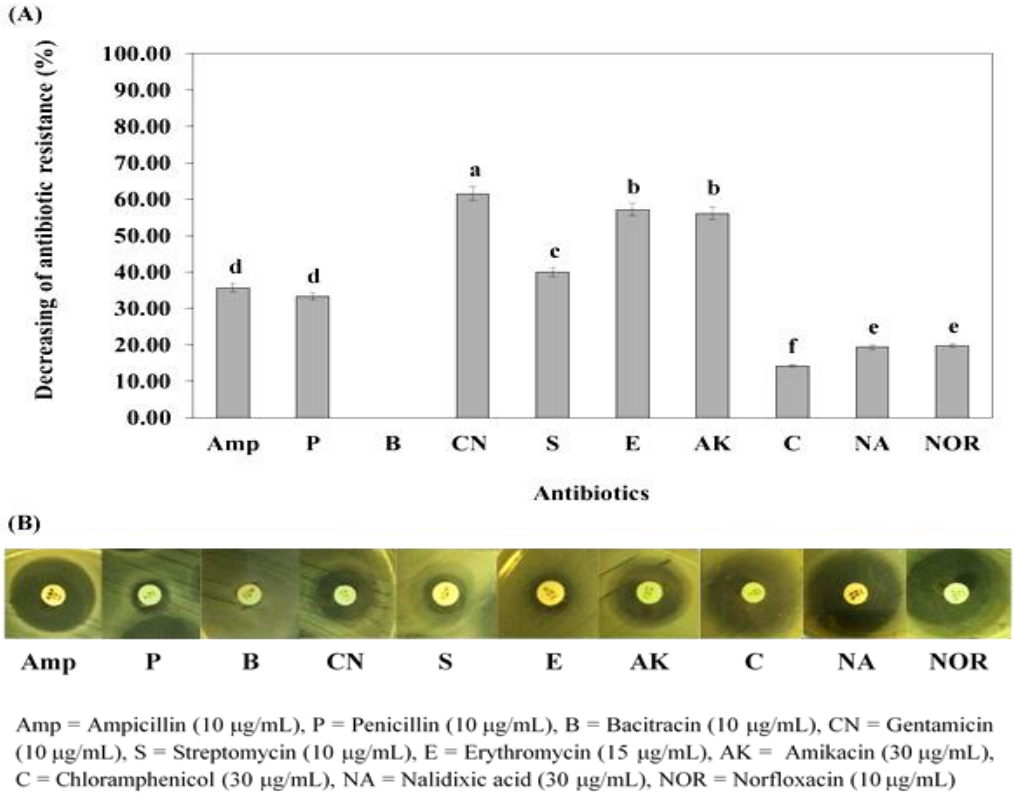

Figure 2: The decreasing of antibiotic resistance pattern of protoplast cell (*V. harveyi* WSC103) (A) and antimicrobial susceptibility test (B) after plasmid curing. Values with different alphabet represent significantly difference at $p<0.05$.

Table 3: Preliminary phytochemical profiles of *Combretum quadrangulare* Kurz. and *Mimosa pudica* plant leaves extracts.

Plant extracts	*C. quadrangulare* Kurz. leaves			*M. pudica* leaves		
	Distilled water	95% Ethanol	75% Acetone	Distilled water	95% Ethanol	75% Acetone
Saponins	++	-	++	-	-	-
Tannins	-	++++	-	-	++	++
Flavonoids						
Shinoda test	+	+	+	+	+	+
10% Lead (IV) acetate test	++	++++	+++	+	+	+
Anthraquinones	-	-	-	-	-	-
Steroids						
Liebermann test	-	++	+	-	+	+
Keller-Kiliani test	-	+++	++++	-	+	+
Terpenoids	-	-	+	-	-	-
Cardiac glycosides						
Keddy's test	-	+	-	-	+	+
Keller-Kiliani test + 10% $FeCl_3$ test	-	++	-	-	+	++
Alkaloids						
28% NH_4OH test	-	+++	+++	-	++++	++++
Wagner's test	-	+	++++	-	++++	++++
Dragendoff's test	-	++	+	-	++	++

- = absent present (nil); + = trace/mildly present (1–30%); ++ = moderately present (31–50%); +++ = highly present (>50–70%); ++++ = very highly present (>70%).

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Table 4: The anti-*Vibrio* activity of *Combretum quadrangulare* Kurz. and *Mimosa pudica* plant leaves extracts.

Anti-VH activities	*Combretum quadrangulare* Kurz.			*Mimosa pudica*		
	Distilled water	95% Ethanol	75% Acetone	Distilled water	95% Ethanol	75% Acetone
Agar disc diffusion (∅ =6.0 mm; mg/mL)						
50	8.00 ± 0.50^{ab}	9.22 ± 0.62^{b}	7.83 ± 0.56^{a}	7.28 ± 0.62^{a}	8.56 ± 0.88^{c}	10.22 ± 0.56^{a}
100	8.56 ± 0.83^{a}	11.44 ± 1.01^{a}	6.50 ± 0.00^{b}	6.67 ± 0.25^{a}	11.78 ± 1.01^{a}	8.32 ± 0.43^{c}
200	7.33 ± 0.50^{bc}	11.78 ± 0.88^{a}	7.39 ± 0.70^{ab}	6.55 ± 0.25^{a}	11.78 ± 0.93^{a}	8.67 ± 0.50^{bc}
300	7.17 ± 0.25^{c}	12.00 ± 1.01^{a}	7.50 ± 0.75^{a}	6.67 ± 0.50^{a}	12.11 ± 1.01^{a}	9.33 ± 0.50^{ab}
400	7.33 ± 0.50^{bc}	11.56 ± 1.07^{a}	6.67 ± 0.25^{b}	6.55 ± 0.33^{a}	10.56 ± 1.01^{b}	10.00 ± 1.01^{a}
Broth micro-dilution (mg/mL)						
MICs	6.25	3.13*	12.50	6.25	3.13*	25.00
MBCs	200	100*	>200	200	100*	>200

^{ab} = values in the same columns with different superscript differed significantly (p<0.05).
^{ABC} = values in the same rows with different superscript differed significantly (p<0.05).
* = significant (p<0.05).

(2011) and Chittasupho and Athikomkulchai (2018). On the other hand, 75% acetonic extracts of CQ leaves exhibited the presence of terpenoids while other solvents extraction did not. Among solvent extraction, the aqueous extracts of CQ (saponins and flavonoids) and MP (flavonoids) leaves were exhibited the minimal secondary metabolites categories (Table 3). The phytochemical compounds of the extracts seemed to contain both hydrophilic and hydrophobic compounds. It would be promising to apply in various applications. Solvent polarity and extraction techniques had affected the quantities and phytochemical types. In addition, plant parts, plant species and seasonal growth are common factors that affect the differentiation of the detected biochemical composition in plants (Tinrat, 2021).

Anti-*Vibrio* activities

Plant extract can be an alternative to antimicrobial agents to reduce the usage of antibiotic. *Mimosa pudica* and *C. quadrangulare* Kurz. leaves extracts were thus evaluated the potential of anti-*Vibrio* activity (normal cell) after extraction and phytochemical screening. The results of anti-*Vibrio* activities of crude extracts by agar disc diffusion assay are shown in Table 4. All CQ and MP leaves extracts exhibited anti-*Vibrio* activities with inhibition zone (IZs) of 6.55 ± 0.25-12.11 ± 1.01 mm. *Vibrio harveyi* WSC103 showed the most sensitive to 95% ethanolic extracts of CQ (IZs of 9.22 ± 0.62–12.00 ± 1.01 mm) and MP (IZs of 8.56 ± 0.88-12.11 ± 1.01 mm) leaves and followed by 75% acetone and aqueous extracts of both plants, respectively. Various concentrations (50-400 mg/mL) of the plant extracts were performed for agar diffusion method. The result showed that the maximum of anti-*Vibrio* activity in both leaves extracts in 95% ethanol was at 300 mg/mL with IZs of about 12.00 ± 1.01-12.11 ± 1.01 mm. Whereas, the optimal concentration of 95% ethanolic extracts was 100 mg/mL in both extracts (IZs of 11.44 ± 1.01-11.78 ± 1.01 mm). The aqueous extracts of *C. quadrangulare* Kurz. leaves had higher antibacterial potential than that of *M. pudica* leaves but it showed the contrary result on the acetone extract by agar disc diffusion method. MIC and MBC value have been widely used to determine the effect of antimicrobial activity of plant extracts (Omar *et al*., 2010).

Based on micro-dilution assay, the 95% ethanolic extracts in both plant samples were exhibited the highest anti-*Vibrio* capacity with MIC/MBC values of 3.13/100 mg/mL, following by aqueous and 75% acetone extracts, respectively (Table 4). Intriguingly, the 95% ethanolic and aqueous extracts in both plants significantly showed the vibriocidal activity with MBC values of 100 and 200 mg/ml, respectively (p<0.05). The 75% acetone crude extracts of *C. quadrangulare* Kurz. (MIC value of 12.50 mg/mL) had higher antagonistic activity against *V. harveyi* WSC103 than that of *M. pudica* (MIC value of 25 mg/mL), but both extracts had no bactericidal effect to *V. harveyi* WSC103 (> 200 mg/mL).

The MIC/MBC values of standard amoxicillin and ciproflaxacin against *V. harveyi* WSC103 in this study were 0.313/0.313 μg/mL and 0.039/0.625 μg/mL, respectively. However, there was hardly to find any data on *M. pudica* and *C. quadrangulare* Kurz. leaf extracts to inhibit the growth of *V. harveyi*. It is thus interesting novel information that both plant extracts represented the potent inhibit the *V. harveyi* WSC103, especially 95% ethanolic extracts at 100 mg/mL of concentration by agar disc diffusion and broth micro-dilution assay (MICs and MBCs value).

CONCLUSION

Vibrio sp. is a major pathogen in marine agriculture that causes pathogenesis in various marine livestock. In this work, *Vibrio harveyi* WSC103 was successfully isolated from shrimp farming ponds. The isolate was identified by 16S rRNA sequencing to be a pathogenic strain causing luminous disease in shrimp. Awareness of zoonotic pathogen transmission to human and drug resistant occurrence have been concerned. Therefore, antibiotic susceptibility profile of isolated *V. harveyi* WSC103 was

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

also evaluated. *Vibrio harveyi* WSC103 was shown to be a multidrug resistance strain. Seeking an alternative strategy has been developed for treatment. Natural compounds are still a potential molecule for infectious treatment. Surprisingly, ethanolic *C. quadrangulare* Kurz. and *M. pudica* extracts not only revealed the highest anti-bacterial activities against *Vibrio harveyi* WSC103 by agar disc diffusion (IZs of 8.56 ± 0.88-12.11 ± 1.01 mm) and micro-dilution (MIC of 3.13 mg/mL) but also exhibited the vibriocidal capacity with MBCs at 100 mg/mL. Taken together, the findings indicated that *C. quadrangulare* Kurz. and *M. pudica* leaves contain several potential bioactive compounds against *V. harveyi* WSC103. The extracts would be an alternative strategy to being used instead of antibiotics in the aquaculture industry for consumer safety.

ACKNOWLEDGEMENTS

The authors would like to thank the Department of Biotechnology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Thailand, for provision of all laboratory facilities.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538