PRELIMINARY PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL EFFECT TOWARD *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM WHITE LEG SHRIMP (*PENAEUS VANNAMEI*) OF THE AQUEOUS HERBAL EXTRACT OF *WEDELIA CHINENSIS*

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Abstract

The trend of using natural compounds or herbs containing antibacterial characteristics for disease prevention and treatment is being common, because of its environmentally friendly, and ensure food safety and animal harm reduction. There are numerous Vietnamese herbs that contain antibacterial and environmentally friendly features, however, the use of herbs in aquaculture is still limited. This study aims to determine the antibacterial components of *Wedelia chinensis* extract for the prevention of diseases caused by *Vibrio parahaemolyticus* in white-leg shrimp (*Penaeus vannamei*). There were 36 samples of grass (*W. chinensis*), which were collected in Central Vietnam and were extracted in 3 different solvents (distilled water, methanol 99.8%, and ethanol 96%) for phytochemical screening by the standard phytochemical assays. The results of the preliminary phytochemical screening in this study showed the extract in 3 different solvents containing various chemical compounds such as alkaloids, saponin, tannin, flavonoids, carbohydrates, organic acids, fixed oil, and fats. The total polyphenols and flavonoids of *W. chinensis* extract were analyzed by folin-ciocalteau regent (for polyphenols) and by $AlCl_3$ (for flavonoids). The highest amount of total polyphenols and flavonoids were recorded in the *W. chinensis* extract in the solvent of methanol 99.8% (74.33 ± 4.49 mg GAE/g and 24.59 ± 2.19 mg RE/g dry weight, respectively), followed by in the solvent of ethanol 96% (73.65± 5.44 mg GAE/g and 20.63 ± 4.30 RE/g of dry weight respectively). The lowest of total polyphenols and flavonoids was observed in the solvent of distilled water (53.07 ± 1.48 mg GAE/g and 3.20 ± 0.07 mg RE/g, respectively). The efficiency of aqueous herbal extraction is highest in the solvent of distilled water (up to 18.2%), followed by in the solvent of ethanol 96% (11.3%), and in the solvent of methanol 99.8%, (11%). The results of antagonistic to *V. parahaemolyticus* testing of *W. chinensis* extract in 3 solvents showed that all *W. chinensis* extract inhibited the growth of *V. parahaemolyticus*, in which the diameter of the antibacterial zone of *W. chinensis* extracts in methanol solvent was highest up to 14.7 ± 0.91 mm. The minimum inhibitory concentrations (MIC) of *W. chinensis* extract in the solvents: distilled water, methanol 99.8%, and ethanol 96% against *V. parahaemolyticus* were 2500 mg/L, 15.5 mg/L to 15.5 mg/L, respectively. The minimum bactericidal concentration (MBC) of *W. chinensis* extracts was 31.25 mg/L in the solvent of methanol 99.8%, and 62.5 mg/L in the solvent of distilled water. Toxicity test of *W. chinensis* extract in methanol solvent 99.8% in white leg shrimp (*P. vannamei*) showed the concentration of 31.25 mg/L (MBC) was safe for shrimp.

Keywords: *W. chinensis, V. parahaemolyticus,* solvents, quantification, and qualification.

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Introduction

The trend of using natural compounds or herbs containing antibacterial biological activities for disease prevention and treatment in aquaculture, is being common because of its environmental friendly, and ensuring food safety (Cos et al 2006) [22]. Natural compounds such as herbs are becoming increasingly common alternative antibiotics to prevent bacterial disease in aquaculture and ensure food safety. There are numerous Vietnamese herbs which contain antibacterial and environmentally friendly features. Several studies have shown the important role of herbs in the competitive exclusion of pathogenic bacteria, providing nutrient addition, and enzymes that contribute to fish digestion, enhancement of the fish's immune response, and disease resistant [26]. The extract of gallic could be used as an antibacterial compound for shrimp [11]. Nguyen Ngoc Phuoc et al (2007) successfully tested the antifungal ability of betel leaf extract in five species of Saprolegnia and Aphanomyces infected in fish [10]. The studies of Nguyen Quang Linh (2010) and Pham Thi Hai Yen et al (2019) [6],[13] showed the antibacterial effect of betel leaf extract and *Phyllanthus amarus* extract on pathogenic *Vibrio* strains isolated in shrimp and red drum fish in Vietnam. However, the information on phytochemical and antimicrobial compound in herbs are still limited. This study aimed to preliminary screen the phytochemical compounds and to determine the antimicrobial effect of the aqueous herbal extract of *W. chinensis* toward *V. parahaemolyticus* isolated from white leg shrimp (*P. vannamei*) and to identify the toxicity of this extract in the shrimp (*in vivo* test) to providing the scientific information for using this herbal extraction in the prevention of bacterial disease in shrimp.

2. Materials and methods

2.1. Materials, Microorganisms and animals

W. chinensis (root, stem, and leaf) were collected from Thua Thien Hue province, Vietnam. The grass parts were shade dried in an oven (FSV 280, China) at 60°C for 8 hours and ground to obtain moderately coarse powder in the machine (Phillips HR 3573/90, China) and were extracted in 3 different solvents: distilled water, methanol 99.8% and ethanol 96% according to the method of Trieu Thi Thanh Hang et al (2018) [2]

Bacterial strain *V. parahaemolyticus* (NNP 001) (Figure 1) was isolated from in white leg shrimp that was infected Acute hepatopancreas Necrosis Disease (AHPND cultured in Thua Thien Hue, was storage in Glycerol 15% at -20 ^{0}C and supplied from the Laboratory of Fish Pathology, the Faculty of Fishery, University of Agriculture and Forestry, Hue University, Hue city, Vietnam [9].

V. parahaemolyticus NNP 001 was firstly recovered on Tryptic Soy Agar (TSA, Himedia, India) supplemented with 2% NaCl, then sub-cultured in Tryptic Soy Broth (TSB, Himedia, India) supplemented with 2% NaCl and incubated at 30 ^{0}C and 180 rpm for 24 h in the GFL 3032 incubator (GFL, Germany).

Bacterial density after culture was determined by optical density (OD) = 1 at 600 nm on UV-VIS spectrophotometer (U2900, Hitachi, Japan) (equivalent to a bacterial density of 10^9 CFU/mL, based on the standard curve for *V. parahaemolyticus* strain that provided by Laboratory of Fish Pathology, Faculty of Fisheries, University of Agriculture and Forestry, Hue University), then diluted to 10^6 CFU/mL to test the antibacterial ability of the *W. chinensis* extract in different solvents toward *V. parahaemolyticus.*

Juvenile *P. vannamei* weighing 2- 2.5 g were purchased from Phan Toan hatchery at Phu Thuan Commune, Phu Vang district, Thua Thien Hue province, Vietnam. Juvenile *P. vannamei* was transferred by air-conditioned car to the wet lab of the Faculty of Fishery, University of Agriculture and Forestry, Hue University. The shrimp were maintained in 2 000 L fibre glass tanks at 28 ^{0}C ± 2 ^{0}C and four times a day fed the basal diet for 14 days in the aquaria, before use. Shrimp used in this study were quarantined for free Acute Hepatopancreatic Necrosis Disease (AHPND), White spot disease (WSD), and Yellow head disease (YHD) at the Veterinary Clinic, Sub-Department of Animal Husbandry and Veterinary Medicine in Thua Thien Hue (Figure 2).

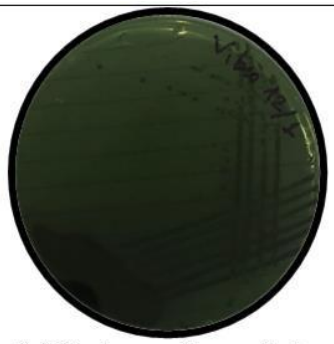


***Figure 1**. Vibrio parahaemolyticus*

***Figure 2.** Experimental white leg shrimp*

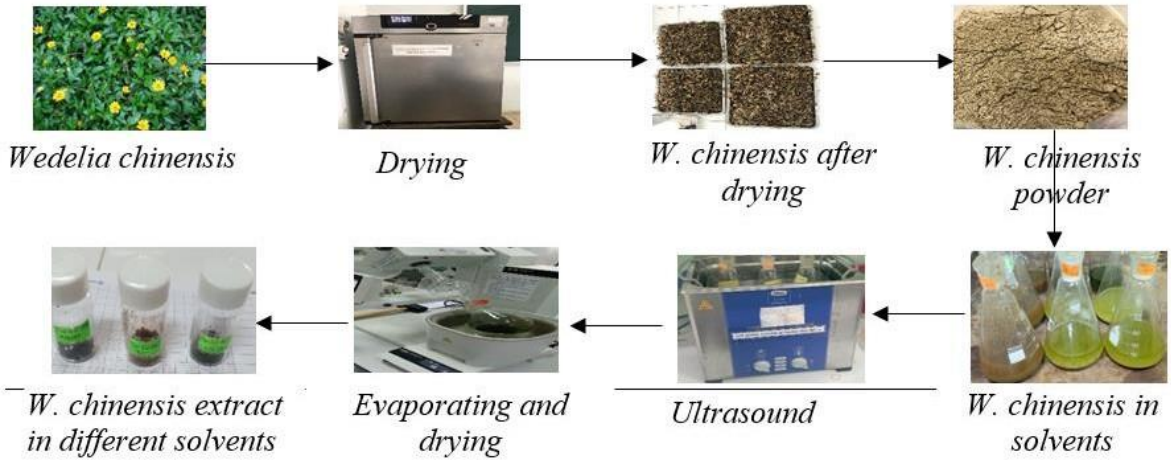


***Figure 3**. The extraction process of W. chinensis extract*

2.2. Variables

- The phytochemical compounds and antimicrobial effect of the aqueous herbal extract of *W. chinensis* toward *V. parahaemolyticus*
- The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the *W. chinensis* extract toward *V. parahaemolyticus.*
- The toxicity of the *W. chinensis* extract in white leg shrimp *P. vannamei*

2.3. Methods

- Herbal extraction in different solvent was carried out according to Trieu Thi Thanh Hang et al (2018) [2], briefly 10g of *W. chinensis* powder were extracted separately with 100 mL distilled water, or 100 mL methanol 99.8%, or 100-mL ethanol 96% in a water bath 100 ^{0}C for 20-30 minutes and leave at room temperature for 24 h. The extracts were processed by ultrasound for 1 h then filtered and concentrated to dry in a rotary evaporator for eliminating the solvents. The semi-solid extract of leaf, stem, and root thus obtained, and was re-dissolved in water and stored in a refrigerator for phytochemical screening and antimicrobial test (Figure 3) [2].
- The method of phytochemical screening of *W. chinensis* extract in different solvents (methanol 99.8%, ethanol 96%, or distilled water) was conducted according to the method of Tran Hung and Nguyen Viet Kinh (2015) [3]
- The total polyphenol of the initial extract was determined according to the Folin-Ciocalteau (a mixture of sodium volframate and sodium molybdate) method. If phenol was presented in the extract, an oxidation-reduction reaction will have occurred. The – OH groups in phenolic will be converted into quinol groups to a complex form of blue color with a wide spectrum of light absorption. The sample was tested with a standard for reaction with Folin-Ciocalteau reagent; then, the optical density was measured at the maximum absorption wavelength. The reaction proceeded in condition avoiding light. Formulate the standard curve of gallic acid in the appropriate concentration range. The total polyphenol content was calculated using the standard curve of gallic acid [16], [29].
- Defined the standard curve of gallic acid Preparation of the base standard solution and diluted gallic acid titration solutions: Weigh exactly 10.0 mg of gallic acid into rated vessels

with a capacity of 100 mL, dissolve, and add to the mark of 10mL with methanol. A standard solution of gallic acid at a concentration of 1 mg/mL was obtained. From this solution, dilute with methanol to standard gallic acid with a concentration of 50; 75; 100; 125; 150; 175 µg/mL (marked C1 to C6 respectively) [16], [29].

- Calibration step: Conduct a reaction between 0.2 mL of each diluted solution (C1 to C6), 0.8 mL of distilled water, and 1 mL of 10% Folin-Ciocalteau reagent, shake well. Leave the reaction for 5 minutes. After that added 2.5 mL of Na_2CO_3 7.5%. Measure the absorption at OD_{760nm} after 30 minutes at room temperature. From the measured optical density value, build a gallic acid benchmark.
- Sample preparation: Weight 20.0 mg of extracts and dissolved these extracts with methanol in a 10 mL rated vessel for suitable test concentration. Conduct a reaction with 10% Folin-Ciocalteau reagent similar to the above. From the obtained optical density value and based on the results of the linearity between the concentration and absorption of gallic acid standard solution, the total polyphenol content in each herbal extract was determined.
- Control sample included 0.2 mL methanol, and 1.8 mL distilled water, and 2.5 mL Na_2CO_3. The total polyphenol content (TPC) was calculated in mg of gallic acid that equivalent to 1g of herbal extract (mg GAE/g) according to the formula:

$$\text{TPC (mg GAE/g extract)} = \frac{C_x \text{ x } V \text{ x } K}{m_0}$$

Where:

TPC was the total polyphenols present in 1 g extract (mg GAE/g extract)

C_x was the concentration of total polyphenols in the photogrammetric solution in terms of gallic acid (mg/mL)

V was the initial volume of the extraction solution (mL)

K was the dilution factor

m_0 was the initial weight of extract (g)

Determine the total flavonoid in the *W. chinensis* extract was followed the colorimetric method using $AlCl_3$ [28], [33] based on the standard curve of rutin

- Accurately weigh 10.0 mg of rutin into a rated vessel with a capacity of 100 mL, dissolve with methanol solvent, and rate to the mark of 10 mL to get a standard rutin solution with concentration C_0=1 mg/mL. Took exactly 1 mL of this solution with a micropipette, put it in a 10 mL rated vessel, and diluted it to the mark with methanol, obtaining a standard rutin solution with a concentration of C_x=100 µg/mL. Diluted this solution with methanol into a series of diluted solutions C1-C6, whose concentration was 20.0; 27.5; 35.0; 42.5; 50.0; 57.5 µg/mL [28], [33].
- Calibration: Put 2 mL of each diluted standard solution (C1-C6) in test tubes and 2 mL $AlCl_3$ 2% shake well. For the reaction to take place for 10 minutes at room temperature, measure absorption at wavelength 430 nm, using a white sample of 2 mL of rutin standard at the corresponding concentration and 2 mL of methanol. Construct a rutin calibration based on the obtained value of optical density.
- Test sample preparation: Accurately weigh 10.0 mg extract in methanol or ethanol and 70.0 mg extract in distilled water, dissolved in methanol solvent in a 10 mL rated vessel for appropriate concentration. The reaction with the 2% $AlCl_3$ reagent as above, using 2 mL control sample, and 2 mL methanol. From the obtained optical density value and the results of the linear concentration and absorption of the rutin standard, the total flavonoid content of each extract was determined [28], [33]. The total flavonoid content (TFC) was calculated in mg of rutin that equivalent to 1g of herb (mg RE/g extract) according to the formula:

$$\text{TFC (mg RE/g extract)} = \frac{C_x \text{ x } V \text{ x } K}{m_o}$$

Where: TFC was the total amount of flavonoids present in 1 g of dry herb (mg RE/g extract) and C_x was the total flavonoid in rutin solution measured by colorimetric (mg /mL)

- The efficiency of extraction in different solvents was calculated as followed:

$$H = \frac{m_{cao}}{m_{tuoi}} \times 100$$

Where: H: extract yield (%); m_{cao}: Total weight of extract (g); m_{tuoi}: total weight of fresh herb (g)

- Evaluating the antimicrobial effect of *W. chinensis* extract toward *V. parahaemolyticus* which caused the acute hepatopancreatic necrosis disease in white leg shrimp

The antimicrobial effect of *W. chinensis* extract in different solvents was followed the method of

Tucker et al (2009) [35] including the following steps:

- Spread the bacteria evenly over the prepared medium of TSA+ 2% NaCl, allowed to dry for 3–5 minutes at room temperature before making 6 mm diameter well at appropriate spacing on the bacteria-spread agar plate.
- Accurately put 50 µl of distilled water into a well (1) for negative control.
- Put 50 µL of *W. chinensis* extract in 99.8% methanol into a well (2) (concentration equivalent to 2500 µg/well).
- Put 50 µL of *W. chinensis* extract in 96% ethanol into a well (3) (concentration equivalent to 2500 µg/well).
- Put 50 µL of *W. chinensis* extract in distilled water into a well (4) (concentration equivalent to 2500 µg/well).
- Placed an antibiotics disc of amoxicillin (125 µg) (positive control) on the plate.

Then allow 15 minutes for the extract diffusing into the agar layer. Check the diameter of the antimicrobial zones after 24 h at 28–30 ^{0}C. The degree of resistance to *V. parahaemolyticus* bacteria of the *W. chinensis* extract was determined according to Faikoh et al (2014) [25] (Table 1).

Table 1. *Antimicrobial activity of W. chinensis extract (Faikoh et al.,2014)*

Diameter of an antimicrobial zone (mm)	**Inference**
≥ 15	Strong
7.5 – 15	Medium
< 7.5	Weak
0	Resistance

- The minimum inhibitory concentration (MIC) of each extract was performed on the 96-well dish according to the dilution method of CLSI (2012) [21]. The minimum bactericidal concentration (MBC) was the lowest concentration in the range of extracts that can kill all bacteria in the well (bacteria that do not grow when re-subculture the solution in these wells on TSA plates) (Figure 8) [20].

- Toxicity test

The experiment was designed in plastic tanks (V = 100L) sterilized with chlorine 100 ppm and dried. Then supply seawater (salinity 30 ‰) to about 2/3 of the tank volume and continuous aeration. White leg shrimp was distributed with a density of 30 shrimp per tank. Before conducting the experiments, shrimp were tested for free *Vibrio* by taking hepatopancreatic samples of 5 shrimp and spread directly on TCBS agar plates, observing bacterial growth after 24 hours at 28 °C (Figure 4). The experimental design included:

Figure 4*. Experimental tanks*

Experimental trials with 3 different extraction concentrations (treatment) and 1 control test. Each treatment was performed in triplicates. Where:

Treatment (NT) 1: Shrimp were fed commercial feed added *W. chinensis* extract at MBC concentration.

Treatment (NT) 2: Shrimp were fed commercial feed added *W. chinensis* extract at 20x MBC concentration.
Treatment (NT) 3: Shrimp were fed commercial feed added *W. chinensis* extract at 40x MBC concentration.
Shrimp were fed commercial feed with or without *W. chinensis* extract 3 times a day at 6h, 12h, and 18h. The feed was provided at 2.0 % of the body weight of the total shrimp in each tank. Monitoring and evaluation of survival for 7 days. Based on the cumulative mortality, the LD_{50} (Lethal dose) was calculated according to the formula of Reed & Muench (1938) [34].

2.4. Data analysis

The data was processed using Excel 2016 and SPSS 16.0 software, a one-factor ANOVA variance to compare differences in antimicrobial ring diameters. Statistical testing was done at a significance level of $P \leq 0.05$, by LSD test.

Table 2. *Biological substances present in the extracts (following the method of Deepa and Padmaja, 2014 with slightly modified)*

Test for	Reagents	Reaction	Inference		
			Methanol	Ethanol	Distilled Water
Fats	Extract pressed between filter paper	Oil stain develops	+	+	-
Fixed oils	Extract pressed between filter paper	Oil stain develops	+	+	-
Carotenoids	H_2SO_4	Blue color	+	+	+
Alkaloids	Iodine in water	Reddish-brown precipitate	+	+	+
Flavonoid-pyron	HCl	Pink or red color	+	+	+
Tanins	10% alcoholic ferric chloride	Dark blue or greenish grey	-	+	+
Saponin	Distilled water	A 1cm layer of foam	-	-	+
Organic acids	Na_2CO_3	Gas released.	+	+	+
Carbohydrates	Copper sulphate+ potassium sodium tartarate+NaOH	Reddish-brown precipitate	+	+	+
Flavonoid-Proanthocyanidin	KOH 10%	Dark blue	+	+	+

Note: (+): present; (−): absent

Table 3. *Composition of polyphenol and flavonoid in W. chinensis extract in 3 dsolvents*

***W. chinensis* extract in solvents**	**Polyphenol (mg GAE/g)**	**Flavonoid (mg RE/g)**
Methanol	74.33 ± 4.49^{b}	24.59 ± 2.19^{b}
Ethanol	73.65 ± 5.44^{b}	20.63 ± 4.30^{b}
Distilled water	53.07 ± 1.48^{a}	3.20 ± 0.07^{a}

Note: a, b the differences same column significantly, $P < 0.05$

Table 4. *The efficiency of W. chinensis extract*

***W. chinensis* extract in solvents**	**Sample weight (g)**	**The weight of extract (g)**	**Efficiency (%)**
Methanol	10	1.1	11
Ethanol	10	1.13	11.3
Distilled water	10	1.82	18.2

Table 5. *Diameter of antimicrobial zone of W. chinensis extract (mm) in different solvents toward V. parahaemolyticus*

***W. chinensis* extract in solvents**	**Diameter of the antibacterial zone (mm)**
Methanol 99.8%	$14.7^{a} \pm 0.91$
Ethanol 96%	$12.7^{b} \pm 1.92$
Distilled water	$12.1^{b} \pm 1.59$
Positive Control (Amoxicillin)	$9.2^{a,b} \pm 5.3$
Negative Control (Distilled water only)	0^{d}

Note: a, b the differences same column significantly, $P < 0.05$

Table 6. *Determination of the minimum inhibitory concentration (MIC) of the extract of the W. chinensis extract toward V. parahaemolyticus.*

***W. chinensis* extract Dilution concentration (mg/L)**	**Bacterial concentration (CFU/mL)**	**Minimum Inhibitory Concentration (MIC)**		
		Methanol	Distilled Water	Ethanol
5000		-	-	-
2500		-	-	-
1000		-	+	-
500		-	+	-
250	10^6 CFU/mL	-	+	-
125		-	+	-
62.5		-	+	-
31.25		-	+	-
15.5		-	+	-

Note: (+): bacteria growth, (-): no bacteria growth

Table 7. *Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of W. chinensis extract toward V. parahaemolyticus.*

***W. chinensis* extract in solvents**	**Minimum Inhibitory Concentration (MIC) (mg/L)**	**Minimum Bactericidal Concentration (MBC) (mg/L)**
Distilled water	2500	5000
Methanol	15.5	31.25
Ethanol	15.5	62.5

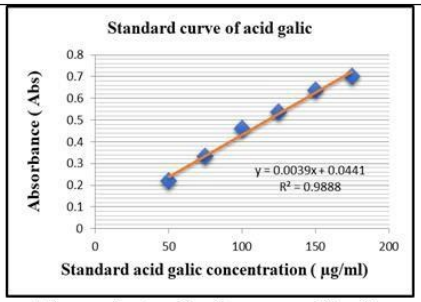


Figure 5. *standard curve acid galic*

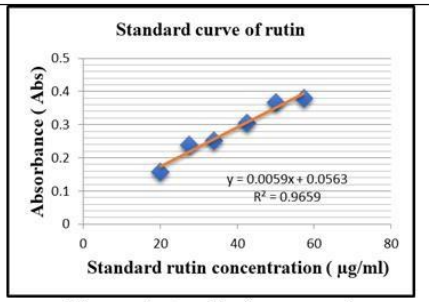


Figure 6*. standard curve ruin*

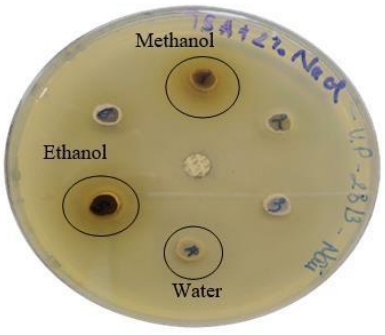


Figure 7. *Antimicrobial activities of Wedelia chinensis extract in different solvents (metanol, ethanol, Distilled water) toward Vibrio parahaemolyticus*

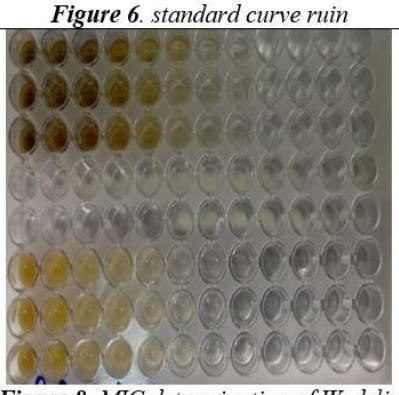


Figure 8. *MIC determination of Wedelia chinensis toward Vibrio parahaemolyticus*

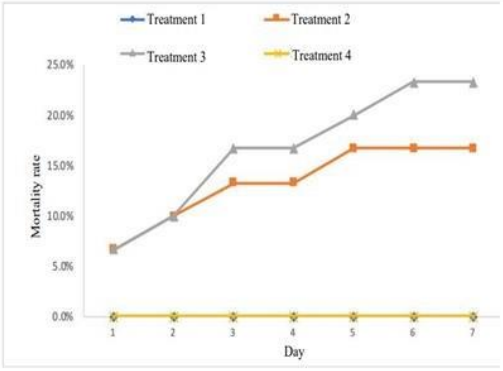
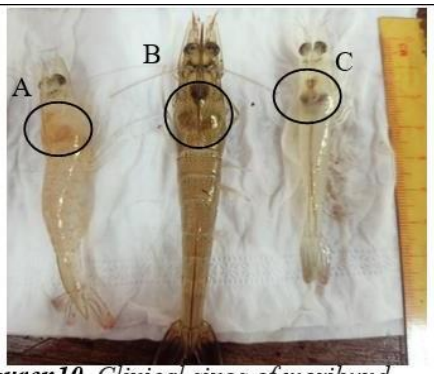


***Figure 9**. Mortality percentage of shrimp after 7 days of feeding with Wedelia chinensis extract*



***Figuren10.** Clinical sings of moribund shrimp when fed with high dose of W. chinensis extract, (A Hepatopancreas of shrimp was pale when fed with W. chinensis extract at a dose of 1250 mg/L (treatment 3), (B,C) healthy shrimp with dark hepatopancreas when the fed at a dose of 31.25 mg/L of W. chinensis (treatment control and treatment 1*

3. Results and discussions

3.1. Results of the biological compound composition of *W. chinensis*

- Qualitative determination of biological component of *W. chinensis* extract

The qualitative determination of biological component screening of *W. chinensis* extract in the solvent of methanol, ethanol, or distilled water showed the presence of biochemical compounds had good antibacterial effects and helped to enhance the immune system's response and increased the survival rate for white leg shrimp when infected *V. parahaemolyticus*. The results also indicated that the *W. chinensis* extracts in all three solvents contained components such as alkaloid, flavonoid, carotenoid, fat, oil, tannin, organic acid,s and reducing agents (Table 2). Especially, the flavonoid content was most abundant in the *W. chinensis* extracts in all three solvents. Saponin, meanwhile, was only present in aqueous solvents. The results of Govindappa et al (2011) on biological compounds in the stems and leaves of *W. trilobata* (L.) indicated the presence of phenolic, flavonoid, and alkaloid in these parts [26]. According to Tran Thanh Men et al (2019), when studying the ability to inhibit seed germination of the *W. trilobata* (L.) extract showed the presence of phenolic, tanin, flavonoid, and alkaloid were in all three parts of stem, leaf, and flower [8]. The presence of tanin in the extract of the *W. trilobata* leaves in ethanol solvent had an antibacterial effect. The antimicrobial activity of tanin was indicated by its ability of inhibiting extracellular enzymes of microorganism (Muhammad and Mudi (2011); Akiyama et al (2001); Doss et al (2009) [30] [15], [24]. Tanin also inhibited the growth of microorganism by forming metal ion complexes or inhibiting phosphorylation. Ahn (2017) identified that alkaloids, glycosides, polyphenols, and terpene from plant extracts have antimicrobial abilities [14]. Therefore, the result of the qualitative determination of some biochemical compounds present in the *W. chinensis* extract helps to explain the antibacterial properties of the *W. chinensis* extracts toward *Vibrio* infected in white-leg shrimp. Many studies determined biological compounds from plants that were able to kill *Vibrio*. The study of Rahman and Rashid (2008) showed the compounds of eclalbasaponin I and eclalbasaponin II from ginseng (*Eclipta prostrata*) extract inhibited the growth of *V. parahaemolyticus* and *V. mimicus* at concentrations of 100 μg.

In addition, the study of Munaeni et al (2019) noted 7-hydroxy-1,2-dimethoxyxanthoneisoquinolinein, naphthalen, and phenol from the extract of *Eleutherine bulbosa* in ethanol solvent could inhibit the growth of *V. parahaemolyticus* by damaging structure of bacterial cell wall and reducing bacterial cells attached [31]. Furthermore, the herb extract also contained biochemical compounds such as flavonoid, alkaloid, tanin, phenol, oil, terpenoid, glycoside, and saponin, which provided many benefits to cultured species such as appetite stimulation, stress reduction, growth promotion, immune stimulation, anti-pathogen activity and promotion of its maturity [18], [19]. Thus, the results of this study showed the biological compound in the *W. chinensis* extracts in three solvents (methanol, or ethanol, or distilled water)

they have great potential applications in aquaculture.

3.2. The total of polyphenol and flavonoid in the extract of *W. chinensis* in different solvents

3.2.1. Defined the standard curve of gallic acid and rutin

To determine the polyphenol content in the *W. chinensis*, the standard curve of gallic acid was formulated with concentration levels of 50; 75; 100, 125; 150, and 175 µg/mL. The gallic acid calibration equation in methanol (Figure 5) showed that the optical density values obtained from different gallic acid concentrations exhibit a strong correlation (R^2 = 0.9888) and the linear equation obtained from gallic acid concentrations was y = 0.0039x + 0.0441 (Figure 5).

The standard curve of rutin was formulated with concentration levels of 20; 27.5; 35; 42.5; 50 and 57.5 µg/mL to determine the flavonoid content in the *W. chinensis* extract (Figure 6). The study results showed the optical density values obtained from different rutin concentrations exhibit a strong correlation (R^2 = 0.9659) and the linear equation obtained from these rutin concentrations was y = 0.0059x + 0.0563 (Figure 6).

3.2.2. Total polyphenol and flavonoid in the W. chinensis extract

Flavonoid and polyphenol are two important groups of biochemical compounds that are highly bioactive and especially antimicrobial (Kumar et al (2011); Courts-Williamson, (2015) [27], [23]. Therefore, the quantity of total flavonoid and total polyphenol are two important indicators to evaluate the antimicrobial ability of herbal extract. The results of total flavonoid and polyphenol are presented in Table 3. The total polyphenol and flavonoid of *W. chinensis* extract were analyzed by folin-ciocalteau regent (for polyphenol) and by $AlCl_3$ (for flavonoid). The highest amount of total polyphenol and flavonoid were recorded in the *W. chinensis* extract in the solvent of methanol 99.8% (74.33 ± 4.49 mg GAE/g and 24.59 ± 2.19 mg RE/g of extract respectively), followed by those in the solvent of ethanol 96% (73.65± 5.44 mg GAE/g and 20.63 ± 4.30 RE/g of extract respectively). The lowest of total polyphenol and flavonoid was observed in the extract of *W. chinensis* in the solvent of distilled water (53.07 ± 1.48 mg GAE/g and 3.20 ± 0.07 mg RE/g, respectively). This result was similar to the previous study of Neelam et al (2012), when studying the biochemical compounds in the extract of *W. trilobata*. The total polyphenol content of 74.38 ± 1.03 mg GAE/g and a total flavonoid of 16.67 mg RE/g determined in the extract of this grass in the ethanol solvent [32]. In addition, study of Tran Thanh Men et al (2019) [8], showed that the flavonoid content in the extract of stem, leaves, and flower of *W. trilobata* was 22.70%, 28.84%, 55.81% and the total polyphenol content was 30.04%, 30.66% and 50.62%, respectively. Thus, the polyphenol in the extract of *W. chinensis* in methanol or ethanol was similar with the extract of *W. trilobata* in ethanol, but the flavonoid content in in the extract of *W. chinensis* in methanol or ethanol was higher than the extract of *W. trilobata* in ethanol, by contrast, the polyphenol and flavonoid in the extract of *W. chinensis* in distilled water were lower than those in the extract of *W. trilobata* in ethanol. According to the study of Trinh Thi Trang and Nguyen Thanh Hai (2016), when using different types of solvents for herbal extraction gave different dissolved biochemical compounds [12]. For example, using ethanol solvents for betel leaf extraction gave more biochemical compounds than using methanol and distilled water solvents. Thus, our research results show that highly polar solvents (ethanol and methanol) gave the highest ability to extract biochemical ingredients in the *W. chinensis* extract.

3.2.3. The efficacy of W. chinensis extraction in different solvents

The efficiency of herbal extraction was highest in the solvent of distilled water (up to 18.2%), followed by those in the solvent of ethanol 96% (11.3%), or in the solvent of methanol 99.8%, (11%) (Table 4).

These results showed that different solvents used for extraction gave a difference in the efficacy of the extraction of herbs. According to the study of Turker et al (2009) [35], the efficiency of herbal extraction and the active ingredient obtained in the extract were different depending on the type of solvents used. In particular, using methanol or ethanol as a solvent for herbal extraction gave higher antibacterial capacity in both Gram-positive and Gram-negative bacteria than those in distilled water. In addition, these authors also believed that extracts in all three solvents have great potential in disease control in aquaculture. Methanol was a higher polar solvent than ethanol or distilled water, then herbal extraction in methanol gave a higher capability of natural compounds found than others. These biochemical compounds in herbs can protect aquatic animals from pathogenic microorganisms in water.

3.3. Antimicrobial effect of *W. chinensis* extract toward *V. parahaemolyticus*

The extract of *W. chinensis* in all 3 solvents showed strong antibacterial effects toward *V. parahaemolyticus* that caused acute hepatopancreatic necrosis on white-leg shrimp cultured in Thua Thien Hue (Figure 7). The diameter of the antimicrobial zone of *W. chinensis* extracts in methanol solvent was highest and higher than those in the solvent of ethanol or distilled water (Table 5). The diameter of the antimicrobial zone of the extract in three solvents of methanol, ethanol, or distilled water was 14.7 ± 0.91 mm; 12.7 ± 1.92 mm; 12.1 ± 1.59 mm, respectively, which was statistically higher than amoxicillin antibiotics (9.2 ± 5.3 mm) (Table 5). This demonstrated that the antimicrobial ability of *W. chinensis* extracts could be replaced by antibiotics in the prevention and treatment of diseases caused by *V. parahaemolyticus* in white-leg shrimp.

Based on the microbial resistance assessment table (Table 1), it is found that *W. chinensis* extracts in all three solvents (methanol, ethanol, or distilled water) were moderate antimicrobial effect toward *V. parahaemolyticus* (the diameter of the antimicrobial zone was 10–14 mm). Methanol is a highly polar solvent with a high capability of dissolving natural compounds in herbs, therefore, the extract of herbs in methanol showed the highest antimicrobial effect. The crude extract from the stem, and leaves of *Polygonum chinense* L. at doses of 10 μg/mL showed the diameter of antibacterial zone against *V. parahaemolyticus* causing AHPND in brackish water shrimp reached to 20.6 mm [1]. Meanwhile, the extract of seed of *Rhodomyrtus tomentosa* at a concentration of 30 μg/μL had the diameter of antibacterial zone toward *V. parahaemolyticus* KC13.14.2; *V. parahaemolyticus* KC12.02.0 and *Vibrio* sp. KC13.17.5 were 17.67, 18.00, and 19.33 mm, respectively. In addition, among seven types of herbal extracts, the extract of *Ricinus communis* L showed the highest efficacy antimicrobial on 2 strains of *V. harveyi* which caused luminous disease, and *V. parahaemolyticus* causing AHPND in shrimp, and its diameter of antimicrobial zone were 18.0 ± 1.4 mm and 17.5 ± 0.7 mm, respectively [4], [5]. Thus, the antagonistic of *W. chinensis* extract in methanol solvent obtained in this study to *V. parahaemolyticus* has moderate and strong antimicrobial capacity [5] reaching 14.7 ± 0.91 mm, however, this was smaller than crude extracts of herbs that used in the study of Hong Mong Huyen et al (2020), but crude extracts are often more difficult to preserve than high extracts [4].

Results of determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of *W. chinensis* extract for *V. parahaemolyticus* was presented in Table 6 and 7. In the extraction of *W. chinensis* in distilled water, bacteria still grow at the concentration of 1000 mg of extract/ L (OD = 0.2 after 24 hours of incubated bacteria with herb extract at 28-30 ^{o}C). The *W. chinensis* extract in methanol, or ethanol solvents inhibit the growth of *V. parahaemolyticus* at very low concentrations (15.5 mg/L).

However, bacteria re-grow on TSA plates after 24 hours of culture at concentrations of 15.5 mg/L for extract in methanol solvent and 31.25 mg/L for extract in ethanol solvent. From the results of screening and determination of minimum inhibitory concentration (MIC) on *V. parahaemolyticus* (Table 6), the minimum bactericidal concentration (MBC) of the extract on *V. parahaemolyticus* was presented in Table 7.

The MBC of *W. chinensis* extract on *V. parahaemolyticus* in distilled water or ethanol or methanol was 5000 mg/L, 62.5 mg/L, and 31.25 mg/L, respectively. According to Cadillac and Mourey (2001), if the MBC/MIC ratio is ≤ 4, the herbal extract has been considered as a bacterial effect. If this ratio was greater than 4, they only inhibit the growth of bacteria [17]. From the results of the study, it is shown that the extract of *W. chinensis* in methanol, or ethanol solvents has a bacterial effect on *V. parahaemolyticus* causing acute hepatopancreatic necrosis AHPND on white leg shrimp with an MBC/MIC ratio was 2 and 4, respectively.

3.4. Results of the toxicity test

Based on the above experiment, the highest value of MBC of *W. chinensis* extract toward *V. parahaemolyticus* was used in this experiment for evaluating the toxicity of this extract in white-leg shrimp.

The result of the toxicity test showed the mortality occurred on the first day and finished on day 6 after feeding with feed mix with *W. chinensis* extract in treatments 2 and 3. After 7 days of feeding with *W. chinensis* extract at a concentration of 20 x MBC (625 mg/L) (treatment 2), the cumulative mortality was 16.7% and was lower than the mortality of shrimp in the treatment 3 (23.3%) which shrimp was fed with *W. chinensis* extract at a concentration of 40x MBC (1250 mg/L).

The highest cumulative mortality was observed in treatment 3 compared to other treatments. By contrast, no mortality was recorded during the experiment in treatment 4 (control) where shrimp fed with commercial feed without *W. chinensis* extract was added, and in treatment 1 which the shrimp fed with *W. chinensis* extract was added at a concentration of 1x MBC (31.25 mg/L) (Figure 9).

The cumulative mortality in all treatments was less than 50%, therefore the LD_{50} has not been determined. No bacteria or none *V. parahaemolyticus* was recovered from the hepatopancreas of dead or moribund shrimp. However, dead and moribund shrimp showed pale liver (Figure 10). The *W. chinensis* contains biochemical compounds that have a high antibacterial and antifungal effect such as phenolic, saponin, alkaloid, and flavonoid, however, if the concentration of *W. chinensis* in feed was too high, this extract can hurt the liver of animal [7]. In this study, the dose of 625 mg/L or 1250 mg/L seemed too high for shrimp used, and the dose of 31.25 mg/L of *W. chinensis* was safe for shrimp.

4. Conclusions

- The extract of *W. chinensis* contains biochemical compounds such as fat, oil, alkaloid, carotenoid, flavonoid, organic acid, tannin, reduce agents
- The highest amount of polyphenol and flavonoid (74.33 mg GAE/ g extract and 24.59 mg RE/g extract, respectively), was identified in the *W. chinensis* extract in the methanol solvent followed by the 3.65 mg GAE/g extract (polyphenol) and 20.63 mg RE/g extract (flavonoid) were determined in the *W. chinensis* extract in the solvent of ethanol. The lowest total polyphenol and flavonoid (high 53.07 mg GAE/g and 3.20 mg RE/g extract, respectively) was observed in the *W. chinensis* extract in the distilled water solvent.
- The efficiency extraction of *W. chinensis* was 18.2% in a distilled water solvent, 11.3% in ethanol solvent, and 11% in methanol solvent.
- The extract of *W. chinensis* in all three solvents (distilled water, 99.8% methanol, and 96% ethanol) showed good antimicribial effects toward *V. parahaemolyticus*, in which tract of *W. chinensis* in methanol solvent gave the best results with a diameter of antibacterial zone was 14.7 ± 0.91 mm.

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