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IN VITRO ANTIBACTERIAL ACTIVITY OF WAX APPLE LEAF EXTRACT (*Syzygium samarangense*) AGAINST SEVERAL PATHOGENIC STRAINS OF VIBRIO

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Abstract – *The purpose of this study is to determine the antibacterial activity of wax apple leaf extract (WALE) against several pathogenic Vibrio strains. Antibacterial activity, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) of wax apple leaf extract were examined in Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio alginolyticus, and Vibrio harveyi strains. The results showed that the zones of bacterial inhibition against Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio alginolyticus, and Vibrio harveyi were 16.00±0.00 mm, 16.67±0.58 mm, 20.00±0.00 mm, 17.00±0.00 mm, respectively. The highest minimum inhibitory concentration and minimum bactericidal concentration values of wax apple leaf extract (2,500 and 5,000 µg/mL) were determined for V. harveyi while the lowest minimum inhibitory concentration (156.25 µg/mL) and minimum bactericidal concentration values (312.5 µg/mL) were found for V. vulnificus. The minimum inhibitory concentration and minimum bactericidal concentration values were observed to be 1,250 µg/mL and 2,500 µg/mL for V. parahaemolyticus; doubled that of minimum inhibitory concentration (625 µg/mL) and minimum bactericidal concentration (1,250 µg/mL) for V. alginolyticus. The wax apple leaf extract was proven to have antibacterial activity against four strains of Vibrio above, with a ratio of MBC/MIC = 2.0.*

Keywords: antibacterial activity, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), Vibrio, wax apple leaf extract (WALE).

I. INTRODUCTION

On a global scale, white shrimp *Litopenaeus vannamei* and tiger shrimp *Penaeus monodon* are two important species that continue to contribute to crustacean aquaculture production with 58.1% of marine species [1]. However, the shrimp culture faces serious economic losses annually due to disease outbreaks. Approximately 60% of disease losses in shrimp aquaculture were estimated to be caused by viral pathogens and 20% by bacterial pathogens [2]. Vibriosis is one of the major problems of disease in culture shrimp by responsible for the mortality of shrimp, especially diseases caused by *Vibrio* spp. The use of chemicals and antibiotics to prevent outbreaks of diseases in the pond. However, the excessive use and misuse of antibiotics have resulted in antibiotic residuals in food and the spread of antibiotic-resistant pathogens in the aquatic environment. Therefore, several solutions have been proposed for the effective use of plant extracts to control disease outbreaks in shrimp and stimulate non-specific immune responses in shrimp [3–5].

Wax apple leaves contain many biologically active substances such as alkaloids, tannins, saponins, flavonoids, phenols, and glycosides [6]. Many studies have indicated that extracts from wax apple flowers, seeds and leaves have activity against many bacteria and fungi, including *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Cryptococcus neoformans*, *Mycobacteria smegmatis*, *Candida albicans*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus au-*

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reus, *Salmonella typhimurium*, *Enterococcus faecalis* [7–9]. Therefore, the purpose of this study was to determine the *in vitro* antibacterial activity of wax apple leaf extract (WALE) against pathogenic Vibrio. The research results contribute scientific information about the possibility of applying medicinal herbs in aquaculture.

II. LITERATURE REVIEW

Vibriosis is considered the major disease problem causing the mortality of farmed shrimp worldwide [10]. Vibrio-related infections frequently occur in hatcheries, but epizootics also commonly occur in pond-reared shrimp species. Vibriosis is caused by gram-negative bacteria in the family Vibrionaceae. Vibriosis is caused by gram-negative bacteria which belong to the Vibrionaceae family. Vibriosis is caused by a number of Vibrio species of bacteria, namely *V. harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*, and others [11]. Effective treatment for diseases caused by bacteria is using antibiotics such as tetracycline, oxytetracycline, cephalosporin, quinolone, sulfonamide, etc. [12, 13]. Herbs containing biologically active compounds, alkaloids, terpenoids, lectins, polyphenolics, phenolics, quinones, and polypeptides can be replaced with antibiotics. Therefore, herbs are important as raw materials used to prepare drugs. The antibacterial activity of some leaf extract from *Hedyotis corymbosa*, *Gymnanthemun amygdalilinum*, *Moringa oleifera*, *Callisia fragrans*, *Acanthus iliciforlius* and *Sphagneticola calendulacea* for *V. parahaemolitycus* and *V. harveyi* was examined. The results show that the zones of inhibition were 7 mm, 9.5 mm, 9 mm, 7.5 mm, 9 mm, and 8 mm for *V. parahaemolitycus*, respectively; 7 mm, 11 mm, 11 mm, 8 mm, 10.5 mm, and 10 mm for *V. harveyi*, respectively [14]. Extracts from *Psoralea corylifolia*, *Murraya koeniji*, and *Quercus infectoria* effectively inhibited bacterial pathogens, *Pseudomonas aeruginosa*, *S. aureus*, and *V. harveyi*, in shrimp with inhibition zones ranging from 9 to 14 mm [15]. Najiah et al. [16] examined the antibacterial activity of *Syzygium aromaticum* on some pathogenic bacteria in marine fish. The results showed that the diameter of the inhibition zone and minimum inhibitory concentration (MIC) value was 15.3 mm and 1.56 mg/mL for *V. parahaemolyticus*; 7 mm and MIC = 3.13 mg/mL for *V. vunificus*; 10 mm and MIC = 1.56 mg/mL for *Streptococcus aureus*; 8 mm and MIC = 0.78 mg/mL for *Streptococcus aginosus*.

III. RESEARCH METHODOLOGY

A. Materials

Wax apple leaves were collected from Ben Tre Province. Four bacterial strains, *V. parahaemolyticus*, and *V. vulnificus* were isolated from diseased white shrimp [18], while V. alginolyticus and V. haveyi were donated from the Research Institute for Aquaculture No.2 (Ho Chi Minh City, Vietnam), were used in these experiments. The chemicals, the thiosulfate citrate bile salt sucrose (TCBS; Merck), tryptic soy broth (TSB; Merck), tryptic soy agar (TSA; Merck), Dimethyl sulfoxide (DMSO, China), NaCl (China) and ethanol, also were in this study.

Preparation of WALE

The harvested wax apple leaves were washed, dried, ground into powder, and soaked in 96% ethanol at a ratio of 1:5 (100 g of wax apple leaf powder soaked in 500 mL of ethanol) for seven days. The crude extracts were filtered using Whatman No-1 filter paper, evaporated, and concentrated into solid extracts at room temperature. The WALE obtained was stored at 4^oC for further experiments. The extraction efficiency was calculated using the following formula [17].

Yield (%) = [weight of extract (g)/weight of sample (g)] * 100.

Determination of antibacterial activity of extracts by disc diffusion method

Each bacterial strain, namely *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, and *V. haveyi*, was recovery cultured on thiosulfate citrate bile salt sucrose (TCBS; Merck) for 24 hours at 37^oC and then transferred to 10 ml of tryptic soy broth (TSB; supplemented with 1.5% NaCl, Merck) for 24 hours at 37^oC. Bacterial density was

measured by spectrophotometer at 610 nm, then it was diluted to get a density of 1×108 CFU/ml for determination of antibacterial activity. One hundred microliters (100 μl) of bacterial suspension were placed in petri dishes and dispersed tryptic soy agar (TSA +1.5% NaCl). In the following step, the sterile tip (200 μl) was used to make the three holes in the agar plate. Then, 100 μl of WALE extract was added to each of the two holes. One hole with 100 μl of Dimethyl sulfoxide (DMSO) added was used as a negative control. The plates were incubated at 37^oC for 24 hours. The results were recorded by measuring the zone of bacterial growth inhibition (mm) surrounding the holes. Each assay was repeated in triplicates. Levels of antibacterial activity of WALE extract were assessed based on the diameter of the inhibition zone, according to Lorian [19]: Resistant: $\leq$ 9 mm; Medium: $\geq$ 10–13 mm; Sensitive: $\geq$ 14 mm.

Determination of minimum inhibitory concentration (MIC)

Each bacterial strain was grown in tryptic soy broth (TSB supplemented with 1.5% NaCl) for 24 hours at 37oC. Bacterial density was measured by spectrophotometer at 610 nm, then it was diluted to get a density of 2x106 CFU/ml for this experiment. WALE was diluted in DMSO (2%) to reach concentrations of 160,000 μg/mL; 80,000 μg/mL; 40,000 μg/mL; 20,000 μg/mL; 10,000 μg/mL; 5,000 μg/mL; 2,500 μg/mL; 1,250 μg/mL; 625 μg/mL; 312.5 μg/mL; 156.25 μg/mL, and 78.125 μg/mL. Then, 2x106 CFU/ml was added into each concentration above with a ratio of 1:1. Each mixed concentration was repeated three times for each bacterial strain. The MIC of the extract was determined as the lowest concentration of the extract in a liquid medium without bacterial growth [20].

Determination of minimum bactericidal concentration (MBC)

The dilutions of WALE that inhibited the growth of each bacterial strain were used to test the MBC, using the colony count method on TCBS agar plates. 100 μL of each concentration was spread on TCBS agar plates. The MBC of WALE was determined as the lowest concentration of extract in TCBS plates that showed no bacterial growth [20].

Statistical analysis

All data were subjected to a one-way analysis of variance (ANOVA), and Duncan's multiple-comparison test was conducted to examine significant differences among treatments using IBM SPSS (Version 20.0). Significant differences were considered at $p < 0.05$.

IV. RESULTS AND DISCUSSIONS

A. Antibacterial activity

The yield of WALE using ethanol solvent was 8.4%. Different studies gave different extraction yields, with *R. communis* at 23.5%, *H. corymbosa* at 8.2%, *G. amygdalina* at 24.8%, *M. oleifera* at 15.3%, and *C. fragrans* at 10.8% [14]. The extraction yield of M. oleifera from seeds, flowers and leaves was 16.2%, 13.7%, and 15.5%, respectively [17]. It was demonstrated that the yield of WALE using water (4.21±0.03%), ethanol (4.13±0.15%), and hexane (4.07±0.03%) [21]. Different extraction methods and solvents gave different extraction yields [22].

Table 1: Antibacterial activity of WALE

Herbal extract	Diameter of inhibition zone (mm)			
	V. parahaemolyticus	*V. vulnificus*	*V. alginolyticus*	*V. harveyi*
WALE	16.00±0.00^a	16.67±0.58^b	20.00±0.00^c	17.00±0.00^b

Note: Values with different letters in the same row indicate significant differences ($p < 0.05$)

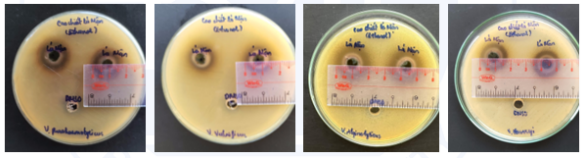

Fig. 1: Diameter of inhibition zone of WALE for pathogenic Vibrio

In this study, the antimicrobial property of WALE was determined by growing *V. alginolyticus*, *V. harveyi*, *V. vulnificus*, and *V. parahaemolyticus* on TSA plates. The zones of bacterial inhibition were 20.00±0.00 mm, 17.00±0.00 mm, 16.67±0.58, and 16.00±0.00 mm against *V. alginolyticus*, *V. harveyi*, *V. vulnificus* and *V. parahaemolyticus*, respectively. The results showed that the highest antibacterial activity of WALE was found for *V. alginolyticus* while the lowest one was found for *V. parahaemolyticus*. No significant difference in antibacterial activity was recorded between *V. vulnificus* and *V. harveyi* (Table 1).

The antibacterial activity of *Phyllanthus urinaria*, *Punica granatum*, *Camellia sinensis*, *Cleome spinose*, and *Chromlacna odorata* was examined for *V. parahaemolyticus* and *V. harveyi*. For *V. parahaemolyticus*, the zones of bacterial inhibition were 21.7±1.53 mm, 20.7±0.58 mm, 11.8±1.92 mm, 9±0.61 mm, 8.9±0.22 mm, respectively. For *V. harveyi*, the zones of bacterial inhibition were 19.7±0.58 mm, 18.3±0.58 mm, 14±2.56 mm, 12.7±0.58 mm, 9.7±1.52 mm, respectively. However, *Moringa oleifera* and *Carica papaya* extracts did not show antibacterial activity [23]. Similarly, the antibacterial activity of *M. oleifera* extracts against *V. vulnificus* and *V. parahaemolyticus* differed among seeds, flowers, and leaves. The zone of bacterial inhibition against *V. vulnificus* by seed extract showed maximum inhibition (23.33±0.58 mm), followed by leaves (18.33±0.58 mm) and the minimum one by flowers (14.67±0.58 mm). For *V. parahaemolyticus*, the maximum zone inhibition was found in seed (20.7±0.58 mm), followed by in flower (17.3±0.58 mm) and the minimum one was found in leaf extracts (15.3±0.57 mm) [18]. Therefore, the antibacterial efficacy of herbal extracts may depend on the plant species, and the parts of the plant used for the extract.

The antibacterial activity of WALE was studied on two strains of gram-positive bacteria, *B. cereus* and *S. aureus* with zones of inhibition 12±0.78 mm and 13±0.53 mm, respectively; and two strains of gram-negative bacteria, *E. coli* (10±0.93 mm) and *P. aeruginosa* (9±0.45 mm) [8]. However, Idris [9] demonstrated that the antibacterial activity depended on the concentration of WALE extract in the experiment. At a concentration of 0.2 mg/ml of WALE, the diameter of the inhibition zone against *S. aureus*, *Enterococcus faecalis*, *Salmonella typhimurium*, and *E. coli* were determined to be 8.33±0.43 mm, 13.7±1.25 mm, 10.67±1.61 mm, 15.13±1.03 mm, respectively. At a concentration of 1 mg/ml of WALE, the antibacterial activity was significantly higher than that of 0.2 mg/ml, with the diameter of the inhibition zone against *S. aureus* (18.33±0.58 mm), *E. faecalis* (19.03±0.45 mm), *S. typhimurium* (20 ± 0.5 mm), *E. coli* (21.13±0.81 mm). However, the antibacterial activity of WALE for *B. cereus* was significantly lower than that of *E. coli*, with inhibition zone diameters against *B. cereus* (9±0.5 mm) and *E. coli* (20.2±0.7 mm) [24]. The antibacterial activity of plums also depends on the plant parts, extraction method, concentration, and bacterial strains. Wax apple parts contain phenols, flavonoids, and other antioxidant components including glycosides, proanthocyanidins, anthocyanidins, ellagitannins, flavanones, flavonols, triterpenoids, and volatile terpenoids. Extracts from wax apple flowers, seeds, and leaves have been shown to be active against a wide range of bacteria and fungi [25].

B. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of WALE against Vibrio strains

The results of the MIC and MBC of WALE on V. parahaemolyticus, V. vulnificus, V. alginolyticus, and V. harveyi strains are presented in Table 2.

Table 2: MIC and MBC of WALE against *Vibrio* strains

Vibrio strains	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC
V.parahaemolyticus	1,250	2,500	2
V.vulnificus	156.25	312.5	2
V.alginolyticus	625	1,250	2
V.harveyi	2,500	5,000	2

The results showed that the lowest MIC and MBC values of WALE were determined in *V. vulnificus*, with MIC and MBC values of 156.25 μg/mL and 312.5 μg/mL, respectively. In contrast, the highest MIC = 2,500 μg/mL and MBC = 5,000 μg/mL were determined in *V. harveyi*. In addition, the MIC (1,250 μg/mL) and MBC (2,500 μg/mL) values in *V. parahaemolyticus* were twice as high as those recorded in *V. alginolyticus* (Table 2). Canillac et al. [26] demonstrated that if the MBC/MIC ratio is less than or equal to 4, the extract is considered to have bactericidal ability; conversely, if this ratio is greater than 4, it has a bacteriostatic effect. In this study, the ratio of MBC/MIC = 2.0 indicated that WALE has the bactericidal ability against all the Vibrio strains (Table 2). Tran Thi Tuyet Hoa et al. [23] reported that the values of the MIC and MBC of *P. rinaria* were determined to be 0.09 mg/mL and 6.25 mg/mL for *V. harveyi*; 0.39 mg/mL and 6.25 mg/mL for *V. parahaemolyticus*. The value of MIC (0.19 mg/mL) and MBC (6.25 mg/mL) was determined for *P. granatum* against *V. harveyi*; MIC value = 0.39 mg/mL and MBC = 12.25 mg/mL against *V. parahaemolyticus*. It was indicated that both *P. urinaria* extract and *P. granatum* extract showed bacteriostatic activity for *V. parahaemolyticus* và *V. harveyi* [22]. In this study, the MIC of WALE for *V. harveyi* and *V. parahaemolyticus* was higher than that of *P. urinaria* extract and *P. granatum* extract. However, the MBC value of WALE for *V. harveyi* and *V. parahaemolyticus* was lower than that of the two extracts above.

However, the MIC = 5 mg/mL, MBC = 10 mg/mL was determined on the *M. oleifera* seed extract; MIC = 10 mg/mL, MBC = 20 mg/mL was determined on the *M. oleifera* flower extract and MIC = 20 mg/mL, MBC = 40 mg/mL was recorded on the *M. oleifera* leaf extract against V. parahaemolyticus. In contrast, the MIC values of the *M. oleifera* seed, flower, and leaf extracts were 2.5 mg/mL, 20 mg/mL, and 10 mg/mL, respectively; the MBC values of the *M. oleifera* seed, flower, and leaf extracts against *V. vulnificus* were 5 mg/mL, 40 mg/mL and 20 mg/mL, respectively [18]. The MIC and MBC values of WALE for *V. parahaemolyticus* and *V. vulnificus* in this research were lower than those of different parts of *P. urinaria* (seed, flower, and leaf). Another study also reported the MIC and MBC values of *P. urinaria* extract were 125 mg/mL and 500 mg/mL for *V. parahaemolyticus*, 62.5 mg/mL, and 250 mg/mL for Vibrio sp [27].

The oil extracts from five species of Syzygium showed antibacterial activity against *S. aureus*, *B. cereus*, *L. monocytogenes*, *E. coli*, and *S. thypi*. MIC value ranged between 250–500 μg/mL [28]. The oil extract of *S. polyanthum* was also studied to have antibacterial activity at a concentration of 31.25 μg/mL for *B. subtilis* [29]. The antibacterial activity of *S. polyanthum* at a concentration of 250 μg/mL for *B. cereus* and *S. polycephalum* at 250 μg/mL for *L. monocytogenes* [30]. It was reported that compounds in plums are able to penetrate the double membrane surrounding the bacterial cell wall [31]. Besides, hydrophilic small molecules are lipophilic macromolecules that have the property of passing through the outer membrane of gram-negative bacteria. These compounds can accumulate in the plasma membrane, leading to the loss of cell components, changes in cell structure and function, and metabolic disorders of bacteria. In addition, herbal compounds can also inhibit bacterial cell wall synthesis, causing energy depletion, mutation, cell damage, and eventually leading to bacterial cell death [32]. Compounds, such as flavonoids, triterpenes, sterols, tannins, terpenoids, and alkaloids contribute significantly to antibacterial activity [33].

V. CONCLUSIONS AND RECOMMENDATIONS

The antibacterial activity of WALE for *V. alginolyticus* was significantly higher than that for *V. parahaemolyticus*, *V. vulnificus*, and *V. harveyi*. The ratio of MBC/MIC from WALE for *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, and *V. harveyi* is 2. It can be concluded that WALE was able to kill four types of Vibrio bacteria that cause disease in shrimp. These results indicated

that the potential of WALE can be used to control bacterial disease in aquaculture.

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