

Investigation of the inhibitory ability of *Melaleuca* spp. leaf extract on *Vibrio* spp. isolated from the intestine of white-leg shrimp infected by white feces disease

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ABSTRACT

White feces disease (WFD) is a significant problem in white-leg shrimp farming, mainly caused by gut microbiota imbalance and the overgrowth of *Vibrio* spp., especially *Vibrio parahaemolyticus*. *Vibrio* colonies were isolated and cultured on thiosulfate citrate bile salts sucrose (TCBS) medium, and had typical morphological characteristics, including a round, convex shape with yellow or green pigmentation. Using a specific primer, the research identified ten *V. parahaemolyticus* strains and 13 *Vibrio* spp. strains. Notably, the extraction yields of *Melaleuca* using methanol were 10.73% and exhibited significant antibacterial activity at concentrations ranging from 25 to 100 mg/mL. *Melaleuca* extraction shows effect inhibit *V. parahaemolyticus* A2.1 and A3, with a halo diameter of 5.67 mm at 100 mg/mL. The *Melaleuca* leaf extract was qualitatively characterized by using gas chromatography-mass spectrometry. The extract contained 13 compounds, including methirol, allyl pentanoate, 1,2,5-hexanetriol, 3,3-diethoxy-1-propyne, tert-butyl hydroperoxide, eucalyptol, cis-beta-ocimene, eugenol methyl ether, β -caryophyllene, α -caryophyllene, α -cubebene, α -farnesene, and caryophyllene oxide. These compounds indicated that *Melaleuca* leaf extract could be a natural alternative to control *Vibrio* populations in shrimp ponds, restore intestinal microbiota balance, reduce WFD incidence, and improve shrimp health. Further studies should focus on identifying compounds in the extract against *V. parahaemolyticus* and investigate shrimp growth when supplemented with the extract.

1. INTRODUCTION

Shrimp is one of the most exported products in Vietnam. In the first quarter of 2025, shrimp exports to the European Union (EU) exceeded 107 million USD. Of these, whiteleg shrimp (*Litopenaeus vannamei*) remains the key commodity, accounting for about 87 million USD, accounting for about 81% of the total shrimp export value to the EU, up 31% over the same period last year [1].

Despite advances in aquaculture development and the increasing adoption of modern shrimp farming systems, the industry continues to face serious challenges, especially in disease management. Among them, white feces disease (WFD) is one of the most common and persistent problems in shrimp farming. WFD affected approximately 719 ha of shrimp ponds in the main farming provinces, including Soc Trang, Bac Lieu, Tra Vinh, and Ca Mau in 2022 [2].

WFD is still a significant problem for white-leg shrimp farming. Although it does not cause mass mortality like acute hepatopancreatic

necrosis disease, early mortality syndrome, or white spot syndrome virus, WFD spreads rapidly in an epidemic pattern, slows shrimp growth, and negatively impacts overall productivity and economic returns. The disease is mainly associated with an imbalance in the intestinal microbiota of shrimp, in which a high abundance of curved rod-shaped bacterial cells is found in the white midgut. In the digestive tract and pond water of WFD-affected shrimp, some *Vibrio* species have been identified, including *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio mimicus*, *Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Vibrio fluvialis*, *Vibrio vulnificus*, *Vibrio damsela*, and *Vibrio cholerae* [3-5].

Traditional medicine emphasizes the potential of bioactive compounds from plants in combating pathogenic bacteria. *Melaleuca* (Myrtle family) is a tree species widely distributed in Vietnam, especially in mangrove forests in Kien Giang, Ca Mau, and Dong Thap provinces.

This tree grows vigorously on alluvial and acidic soils and contributes significantly to coastal protection (seawalls and wave barriers). In addition, *Melaleuca* leaves have significant analgesic, antibacterial, and anti-allergic properties.

The medicinal potential of *Melaleuca* extract to inhibit bacteria, fungi, and viruses has been demonstrated in several recent studies. The stem inhibits *Propionibacterium acnes* ATCC 6919, the leaves

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inhibit coronavirus, and the flowers and fruits inhibit *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231 [6-8]. More than 30 bioactive compounds have been identified in the methanol extract of *Melaleuca* leaves. The main ingredients include cyclopropa[c,d]pentalene-1,3-dione, hexahydro-4-(2-methyl-2-propenyl)-2,2,4-trimethyl, docosane, 1H-Indene, 1-ethylideneoctahydro-7a-methyl, epiglobulol, (-)-oxidoselina-1,3,7(11)-trien-8-one, globulol, (+)-spatulanol, 2-Heptanone, 6-(3-acetyl-2-methyl-1-cyclopropen-1-yl)-6-methyl, veridiflorol [9].

In some previous studies showing the antibacterial potential of *Melaleuca* extract, and considering the current prevalence of WFD in shrimp, which is mainly caused by an imbalanced intestinal microbiota. This study aimed to evaluate and expected to clarify the antibacterial activity of *Melaleuca* methanol leaf extract against *Vibrio* spp. isolated from WFD-affected shrimp. Therefore, suggest a promising biological approach for controlling *Vibrio* spp. as part of a feasible strategy for the prevention and treatment of WFD in shrimp farming.

2. MATERIALS AND METHODS

2.1. Sample Collection

White-leg shrimp showing signs of WFD have been observed in Cu Lao Dung district, Soc Trang province. Symptoms of shrimp affected by WFD include opaque white intestines with a thin yellow layer covering the hepatopancreas, as well as white or yellow feces floating on the water surface. Additionally, the shrimp exhibit signs of lethargy, slow growth, reduced feeding rates, and loose shells [10]. *Melaleuca* leaves were collected in Hamlet 6, Luong Nghia Commune, Long My District, Hau Giang Province, Vietnam. *Melaleuca* leaves were collected in the morning and transported to the laboratory.

2.2. Isolation of *Vibrio* spp.

The shrimp were sterilized with 70% alcohol to remove bacteria on the surface. About 1 g of intestines and hepatopancreas were dissected and homogenized in 9 mL of a 0.9% NaCl solution. The sample was diluted to a concentration of 10^{-3} and spread in 100 μ L of the solution on the TCBS medium. The sample was incubated at room temperature ($27^{\circ}\text{C} \pm 3$) for 24 h, selecting typical colonies with blue and yellow colors [11]. The bacterial isolates were preserved by combining them with TSB medium and glycerol in a 7:3 volume ratio and then stored at -4°C .

2.3. Observe the Colony and Biochemical Character

Colonial morphology was observed under a stereomicroscope and described in terms of color, form, elevation, margin, and colony diameter after 24 h on the TCBS medium at $27^{\circ}\text{C} \pm 3$. Cellular morphology was observed under an optical microscope with a $\times 100$ objective. Gram stain, motility, and oxidase and catalase reactions were subsequently determined.

2.4. Identification of *Vibrio* spp. Bacteria and the *toxR* Virulence Gene

Bacterial cells were extracted using lysis buffer at a ratio of 1:1 (v/v) for 15 min and centrifuged at 12,000 rpm for 20 min. The supernatant was transferred into a new microcentrifuge tube and precipitated with 1 mL of 95% ethanol (4°C) for 30 min. Then, centrifuge again at 12,000 rpm for 20 min and retain the precipitate. DNA was washed with 500 μ L of 70% ethanol and centrifuged at 12,000 rpm for 20 min (repeat this step twice). After washing, the DNA was dried in a vacuum

dryer at $45\text{--}65^{\circ}\text{C}$ for 10 min. Next, add 100 μ L of 0.1X TE buffer and freeze the DNA. *Vibrio* spp. and the *toxR* virulence gene are detected by specific primer pairs [Table 1].

2.5. Extract Preparation

The *Melaleuca* leaves were transported to the laboratory, removed from the branches, washed, and dried at 40°C until a constant mass was achieved. The dried leaves were then ground into a fine powder. A total of 10 g of leaf powder was combined with 100 mL of methanol solvent and soaked repeatedly 3 times. The extract was filtered through Whatman no. 1 filter paper and evaporated in a vacuum at 50°C until a viscous extract was obtained. This extract was freeze-dried to completely remove the solvent and stored at 4°C [14]. The chemical constituents of the extract were identified by using the gas chromatography-mass spectrometry (GC-MS) method at the Can Tho Technical Center of Standards, Metrology, and Quality. The extraction efficiency (E) was determined using the following formula:

$$E(\%) = \frac{\text{Mass of freeze-dried extract (g)}}{\text{Mass of leaf powder (g)}} \times 100\%$$

2.6. The Ability to Inhibit *Vibrio* spp. of the *Melaleuca* Extract

Antibacterial activity of the extract using the disc diffusion method as described by Akshaya *et al.*, [15]. The concentrations of the extract were investigated as 10, 25, 50, 75, and 100 mg/mL, oxytetracycline at 10 mg/mL as a positive control, and 5% dimethyl sulfoxide as a negative control. Spread 1 mL of bacteria at a density of 10^8 colony forming unit/mL on the TSA medium (Himedia, India), and create agar wells with a diameter of 6 mm. Add 50 μ L of extract to each well and observe the zones of bacterial inhibition after 24 h.

2.7. Statistical Analysis

The experiment was repeated 3 times. Data are presented as mean \pm standard deviation. The results were statistically analyzed using the analysis of variance method (Tukey's test) with Minitab 16.0 software.

3. RESULTS AND DISCUSSION

3.1. Isolation and Identification of *Vibrio* spp.

Twenty-five bacterial strains were isolated from 30 shrimp samples exhibiting signs of WFD in Cu Lao Dung District, Soc Trang Province [Figure 1]. Of these, 6 strains formed yellow colonies, while 19 strains formed green colonies. Among the isolates, 6 bacterial strains had colony sizes ≥ 2 mm, and 19 bacterial strains had colony sizes < 2 mm. All colonies floated on the medium, exhibited intact borders, and consisted of curved rod-shaped cells. All bacterial strains tested negative for Gram staining and tested positive for the presence of oxidase and catalase enzymes [Table 2].

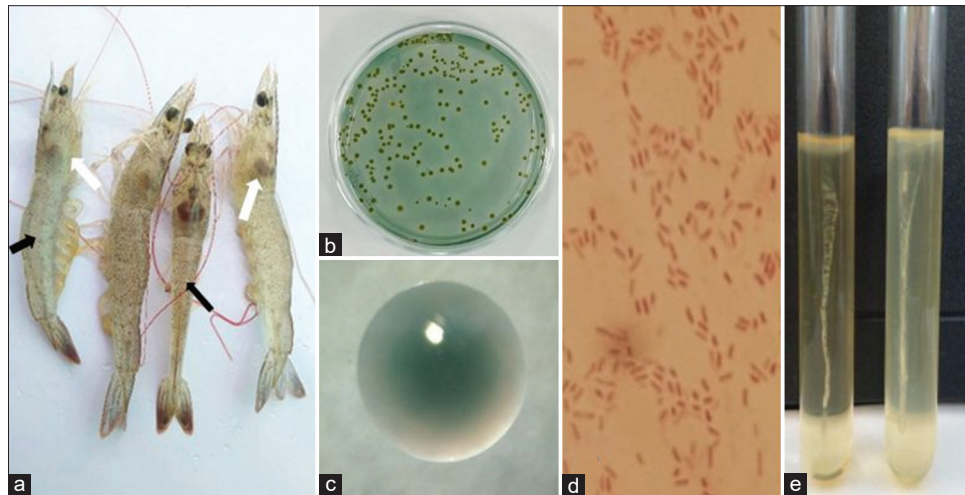
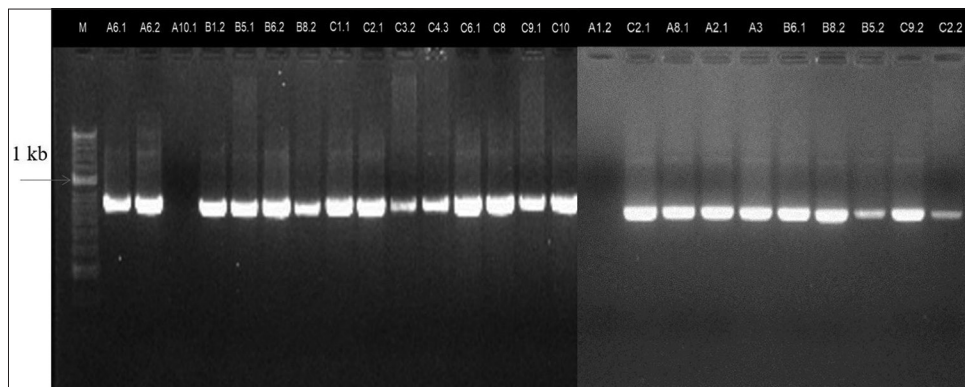
On the TCBS medium, *Vibrio* spp. typically form green or yellow colonies. for the green colonies, *V. vulnificus* ATCC27562 and *V. parahaemolyticus* ATCC27969 do not ferment sucrose. For the other, *V. alginolyticus* ATCC17749 and *V. harveyi* ATCC14126 ferment sucrose, which reduces the pH and turns bromothymol blue in the medium to yellow [16].

3.2. Identification of *Vibrio* spp. with 16S rRNA Gene Primers

PCR results using 16S rRNA-specific primers identified 24 bacterial strains as *Vibrio* spp. [Figure 2]. However, isolates A1.2 and A10.1

Table 1: Information on primer pairs used in the study.

Primers	Sequencing	Temperature cycles	Gene	bp	References
16S-F 16S-R	CAGGCCTAA-CACATGCAAGTC GCATCTGAGTGTCAGTATCTGTCC	95°C–10 min, 30 cycles of the following step 95°C–1 min, 55°C–1 min, 72–1.5 min, 72°C–5 min	16S	700	Montieri <i>et al.</i> [12]
toxR-F toxR-R	GTCTTCTGACGCAATCGTTG ATACGAGTGGTTGCTGTCATG	94°C–10 min, 20 cycles of the following step 94°C–1 min, 63°C–1 min, 72–15 min, 72°C–10 min	toxR	366	Kim <i>et al.</i> [13]

**Figure 1:** (a) Black arrow with empty intestine, white arrow with faint liver and signs of liver atrophy; shrimp with thin body (sick, loose shell), poor pigmentation on the shell (pale color); (b and c) colony on the TCBS medium; (d) *Vibrio* spp.; (e) motility test.**Figure 2:** Agarose gel electrophoresis of the 16S rRNA gene amplicons.

were not *Vibrio* spp. despite growing on TCBS medium. Isolate A1.2 had yellow colonies with a diameter of 1.8 mm, and A10.1 had blue colonies with a diameter of 1.5 mm. Some other bacterial species can also grow on TCBS medium, including *Aeromonas hydrophila* (yellow) and *Enterococcus* spp. (yellow), *Proteus mirabilis* (yellow), and *Staphylococcus aureus* (yellow) [17].

3.3. Identification of *V. parahaemolyticus* with *toxR* Gene Primers

Among the 24 *Vibrio* strains, 10 *V. parahaemolyticus* strains were identified as containing the *toxR* virulence gene [Figure 3], which is a membrane-bound regulatory protein that plays a role in virulence and fitness in *Vibrio* species [18].

In the intestines of shrimp with WFD, there is a significant diversity of pathogenic bacteria. The study isolated 23 bacterial strains, including 2 strains that were not *Vibrio* spp., 10 strains of *V. parahaemolyticus*

containing the *toxR* virulence gene, and 11 strains of *Vibrio* spp. Previous studies have demonstrated that the total number of *Vibrio* bacteria in shrimp with WFD is higher than in healthy shrimp. Additionally, there is a diversity of species, including *V. vulnificus*, *V. fluvialis*, *V. parahaemolyticus*, *V. alginolyticus*, *V. damsela*, *V. mimicus*, and *V. cholerae* non-O1 [3]. In a study conducted by Supono *et al.* [4], *V. parahaemolyticus*, known as the primary infected bacteria, is associated with the white-leg shrimp disease among *V. vulnificus*, *V. parahaemolyticus*, and *V. alginolyticus*.

Through sequence analysis of the 16S rRNA gene region, strain B5.1 was identified to exhibit a very close genetic relationship with *V. parahaemolyticus*, with a genetic distance of only 0.001 [Figure 4]. Although *V. owensii* was also identified as a closely related species in the phylogenetic tree, it did not create any amplification signal when using primers specific to the *toxR* gene - a widely used molecular marker for the identification of *V. parahaemolyticus* - as determined by Primer-basic local alignment search tool (BLAST) analysis. In this

Table 2: Characteristics of colony morphology, cells, and biochemical characteristics.

Strains	Colony morphology					Cell morphology			Biochemical characteristics	
	Color	Form	Elevation	Margin	Diameter (mm)	Motility	Shaped	Gram	Oxidase	Catalase
A1.2	Yellow	Circular	Raised	Entire	1.8	+	Curved rod	-	+	+
A2.1	Green	Circular	Raised	Entire	3.0	+	Curved rod	-	+	+
A3	Green	Circular	Raised	Entire	2.1	+	Curved rod	-	+	+
A6.1	Green	Circular	Raised	Entire	2.1	+	Curved rod	-	+	+
A6.2	Green	Circular	Raised	Entire	2.0	+	Curved rod	-	+	+
A8.1	Green	Circular	Raised	Entire	2.5	+	Curved rod	-	+	+
A10.1	Green	Circular	Raised	Entire	1.5	+	Curved rod	-	+	+
B1.2	Green	Circular	Raised	Entire	1.2	+	Curved rod	-	+	+
B5.1	Green	Circular	Raised	Entire	2.0	+	Curved rod	-	+	+
B5.2	Green	Circular	Raised	Entire	2.1	-	Curved rod	-	+	+
B6.1	Green	Circular	Raised	Entire	2.1	+	Curved rod	-	+	+
B6.2	Yellow	Circular	Raised	Entire	1.0	+	Curved rod	-	+	+
B8.1	Yellow	Circular	Raised	Entire	1.2	+	Curved rod	-	+	+
B8.2	Green	Circular	Raised	Entire	2.0	+	Curved rod	-	+	+
C1.1	Yellow	Circular	Raised	Entire	2.0	-	Curved rod	-	+	+
C1.2	Green	Circular	Raised	Entire	0.1	-	Curved rod	-	+	+
C2.1	Yellow	Circular	Raised	Entire	2.0	+	Curved rod	-	+	+
C2.2	Green	Circular	Raised	Entire	1.0	-	Curved rod	-	+	+
C3.2	Green	Circular	Raised	Entire	0.7	-	Curved rod	-	+	+
C4.3	Green	Circular	Raised	Entire	0.1	-	Curved rod	-	+	+
C6.1	Yellow	Circular	Raised	Entire	1.2	+	Curved rod	-	+	+
C8	Green	Circular	Raised	Entire	1.0	-	Curved rod	-	+	+
C9.1	Green	Circular	Raised	Entire	0.7	+	Curved rod	-	+	+
C9.2	Green	Circular	Raised	Entire	2.0	+	Curved rod	-	+	+
C10	Green	Circular	Raised	Entire	0.1	-	Curved rod	-	+	+

Observation on TCBS medium after 24 h, “-”: Negative, “+”: Positive.

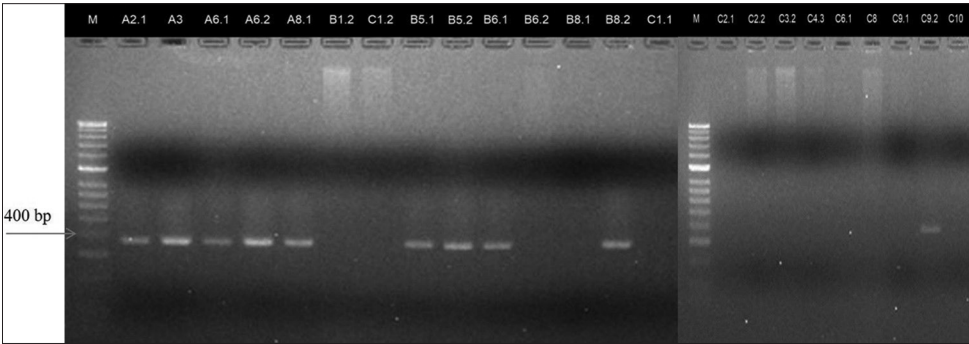


Figure 3: Agarose gel electrophoresis of the *toxR* gene amplicons.

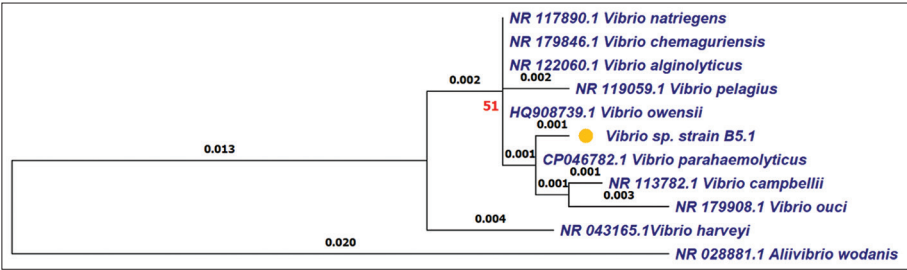


Figure 4: Maximum likelihood phylogenetic tree of the *Vibrio* spp. strain B5.1 based on *16S rRNA* gene.

study, primer sets specific to the *Vibrio* genus and *V. parahaemolyticus* species from Montieri *et al.* (2010), Kim *et al.* (1999) [12,13], respectively, were employed. Their specificity and binding affinity were evaluated by Primer-BLAST. Subsequently, the 16S rRNA sequence (isolate B5.1) was employed to confirm its accuracy. As a consequence, this strain belongs to the *V. parahaemolyticus* clade based on the result of phylogenetic analysis. Therefore, the reliability of species identification based on PCR in combination with phylogenetic analysis was enhanced.

3.4. Melaleuca Extract

Melaleuca leaf powder is light green and fluffy. The extraction yield of *Melaleuca* leaf extract is 10.73%, with a moisture content of 0%. The recovery yield of *Melaleuca* extract by Dieu [19] is 3.37% with a moisture content of 12.21%, the difference may be due to the content and compounds inside the extract. The content of compounds inside *Melaleuca* leaves can vary depending on factors such as geographical location, tree age, and nutrient content in the soil.

Table 3 and Figure 5 report 13 volatile compounds and their chemical structures achieved from GC-MS analysis of the methanolic *Melaleuca* extract. The total peak area accounted for 100%, reflecting the high extraction efficiency and purity of the sample. The majority of the detected components were eugenol methyl ether (60.08%, retention time of 13.412 min). Additionally, the others were 1,2,6-hexanetriol (4.88%), allyl pentanoate (6.63%), and α -farnesene (11.10%). Moreover, monoterpenes and sesquiterpenes, namely β -caryophyllene, α -caryophyllene, α -cubebene, and caryophyllene oxide, were minor.

3.5. Inhibitory Ability of *Vibrio* spp.

The inhibitory ability of the extract against *Vibrio* bacteria was studied using 8 isolates of *Vibrio* spp.: A2.1, A3, B1.2, B5.1, B8.1, C1.2, C4.3, and C9.1, at concentrations of 100, 75, 50, 25, and 10 mg/mL. The extract showed inhibitory ability against *Vibrio* at various concentrations [Table 4 and Figure 6].

All five concentrations inhibited *Vibrio* A2.1 and A3, with the diameter of the inhibition zone reaching 5.67 mm at a concentration of 100 mg/mL. However, *Vibrio* sp. C2.1, C4.3, and C9.1 were not inhibited at 10 and 25 mg/mL. When the concentration of extract increased, the diameter of the inhibition zone also increased; these

results showing the active compounds in the extract enhanced the resistance against *Vibrio* spp.

Only high concentrations of the *Melaleuca* leaves extract inhibited *Vibrio* spp. According to the Clinical and Laboratory Standards Institute, compounds with significant antibacterial activity usually have minimum inhibitory concentrations (MICs) below 100 μ g/mL [20]. However, the effective concentration of *Melaleuca* extract identified in this study is from 10 to 100 mg/mL, which is higher than the recommended. Antibacterial function can be decreased due to interactions among the various bioactive compounds in the extract. This observation is consistent with the findings of Semeniac *et al.* (2012). In particular, strong antibacterial activity was observed in the thyme essential oil when used alone, but this activity was reduced in combination with parsley, celery, or basil because of the antagonism [21]. In another study, 13 chemical components were identified using GC-MS-based phytochemical analysis of methanolic plant extracts. Of these, at least three compounds, 1,8-cineole (eucalyptol), eugenol methyl ether, and β -caryophyllene, showed potential antibacterial properties. According to Yoo and Jwa (2018), β -caryophyllene, a sesquiterpene commonly used in ethnopharmacology, was shown to have significant antibacterial activity; the growth of *Streptococcus mutans* was inhibited at concentrations $\geq 0.078\%$ [22]. Moreover, 1,8-cineole has been reported to inhibit biofilm formation and reduce virulence in *E. coli* O101 by downregulating the expression of the *luxS* gene, a key component of the population induction mechanism [23]. Beside that, eugenol and its derivatives were identified to have antibacterial and antifungal activities *in vitro* conditions [24]. In our research, although MIC and minimum bactericidal concentration (MBC) tests were attempted, the high concentration threshold of the crude extract precluded reliable determination. Future work will involve testing individual compounds for accurate MIC/MBC values. Therefore, to standardize and improve the accuracy of MIC and MBC assessments in antimicrobial screening, it is essential to fractionate crude extracts and evaluate the bioactivity of individual constituents, thereby clarifying the specific role and potency of each compound.

The results illustrated that the components and antimicrobial ability of the extract have potential applications in disease prevention and treatment in aquaculture, especially for shrimp diseases. In 2010, WFD in shrimp was reported to cause significant losses to the shrimp farming in the Mekong Delta (Vietnam). One of the most common symptoms is white fecal fibers floating on the water, and the white color of the intestines, which is distinct from the black color of the healthy shrimp [3]. Diseased shrimp often show signs of reduced feed intake, slow growth, discoloration of the hepatopancreas, and loose shrimp shells [25]. The causative factors have been identified as a combination of adverse environmental conditions, bacterial invasion, and microsporidia (EHP) parasites. Notably, intestinal microflora disturbances caused by *Vibrio* spp., especially *V. parahaemolyticus*, have been identified as the main agents [26]. A study identified 10 *V. parahaemolyticus* isolates carrying the *toxR* virulence gene, along with 14 other *Vibrio* strains isolated from 30 WFD-affected shrimp samples, highlighting the important role of *Vibrio* in causing intestinal microbiota dysbiosis - the main mechanism causing WFD in shrimp.

The research observed antibacterial activity from the ethanol extraction of *Melaleuca* leaves, which contributes to opening a sustainable approach to controlling pathogenic. *Melaleuca* extraction inhibits *Vibrio* sp. and *V. parahaemolyticus* at 25 mg/mL, the pathogen genus in aquaculture. The extract contains more than 50 bioactive compounds, which eucalyptol (27.512%), γ -terpinene (8.59%), terpinolene (9.047%),

Table 3: The chemical compounds in *Melaleuca* extract.

No.	Bioactive compound	Concentrations (%)
1	Methirol	0.73
2	Allyl pentanoate	6.63
3	1,2,5-hexanetriol	4.88
4	3,3-Diethoxy-1-propyne	2.91
5	Tert-Butyl hydroperoxide	0.96
6	Eucalytol	0.50
7	Cis-beta-ocimene	4.25
8	Eugenol methyl ether	60.08
9	β -Caryophyllene	3.26
10	α -Caryophyllene	1.12
11	α -Cubebene	2.55
12	α -Farnesene	11.10
13	Caryophyllene oxide	1.03

Table 4: Antibacterial ability of *Melaleuca* extract.

Strains	Inhibition halo diameter (mm)					
	Tetracycline*	100 mg/mL	75 mg/mL	50 mg/mL	25 mg/mL	10 mg/mL
<i>Vibrio</i> spp. A2.1	26.56±0.40	5.93 ^a ±0.56	4.36 ^b ±0.72	4.00 ^{bc} ±0.26	2.90 ^{cd} ±0.43	1.80 ^d ±0.34
<i>Vibrio</i> spp. A3	25.80±0.26	4.43 ^a ±0.41	4.23 ^a ±0.58	4.00 ^{ab} ±0.10	3.01 ^b ±0.08	1.30 ^a ±0.51
<i>Vibrio</i> spp. B1.2	25.30±0.96	4.10 ^a ±0.26	3.90 ^a ±0.43	2.13 ^b ±0.15	2.03 ^b ±0.15	-
<i>Vibrio</i> spp. B5.1	8.16±0.57	4.13 ^a ±0.11	4.03 ^a ±0.11	4.16 ^a ±0.05	1.96 ^b ±0.15	-
<i>Vibrio</i> spp. B8.1	22.33±0.20	3.93 ^a ±0.15	3.93 ^a ±0.05	3.96 ^a ±0.5	-	-
<i>Vibrio</i> spp. C2.1	19.23±0.75	1.96 ^a ±0.05	1.93 ^a ±0.05	-	-	-
<i>Vibrio</i> spp. C4.3	20.23±0.64	3.93 ^a ±0.05	3.96 ^a ±0.05	2.00 ^b ±0.1	-	-
<i>Vibrio</i> spp. C9.1	19.76±0.25	4.00 ^a ±0.00	4.00 ^a ±0.00	3.67 ^a ±0.057	-	-

“” Tetracycline is used at 5 mg/mL, “-” non-antibacterial, each treatment was replicated 3 times. Within each row, mean values followed by the same letters represent statistically insignificant differences at the 5% significance level according to Tukey’s test.

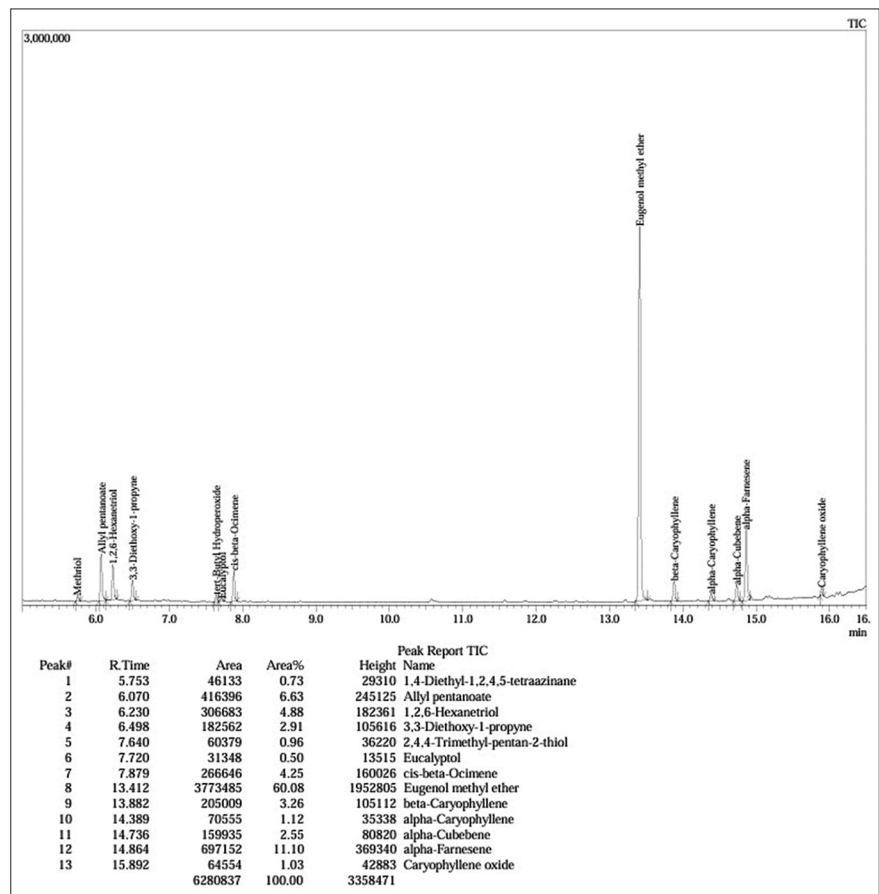


Figure 5: Gas chromatography-mass spectrometry chromatogram of the methanolic extract of the whole plant of *Melaleuca* spp.

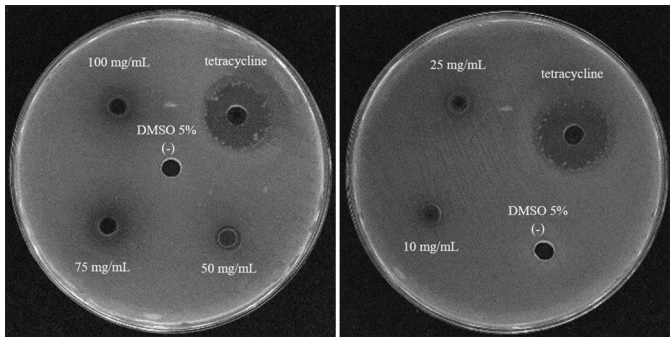


Figure 6: Anti-*Vibrio* spp. B5.1 ability of *Melaleuca* extract.

β -eudesmene (3.359%), α -selinene (3.889%), α -terpineol (4.108%), 1R- α -pinene (2.158%), caryophyllene (6.48%) and α -caryophyllene (3.522%), contributing to their strong antibacterial properties [27].

Finally, *Melaleuca* leaves extract could be a promising alternative antibiotic. Further studies should elucidate the molecular mechanism, confirm the practicality of mass production, and ensure safety for application.

4. CONCLUSION

The methanol extract of *Melaleuca* leaves demonstrated the ability to control *V. parahaemolyticus* strains carrying the virulence gene *toxR*,

as well as other *Vibrio* spp. isolated from white-leg shrimp affected by WFD. The antibacterial activity became evident at a concentration of 25 mg/mL. Chemical analysis revealed that the extract contains 13 bioactive compounds. Next studies should focus on identifying the compounds against *Vibrio* and their impact on white-leg shrimp. For industrial applications, it is urgent to optimize the extraction steps, standardize the compounds' concentration, and develop highly effective and feasible formulations in practical aquaculture.

5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

10. PUBLISHER'S NOTE

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The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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