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# Vietnamese herbal extracts exhibit potent antibacterial activity against *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease in shrimp aquaculture

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ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND), caused by *Vibrio parahaemolyticus*, poses a critical threat to global shrimp aquaculture. This study evaluated antimicrobial efficacy of ten Vietnamese herbal extracts against multidrug-resistant AHPND-causing *V. parahaemolyticus* ($Vp_{AHPND}$) isolates from diseased white-leg shrimp (*Litopenaeus vannamei*) in Vinh Long, Vietnam. Three field isolates with complete intrinsic β-lactam resistance and acquired resistance to multiple antibiotics were cultured and characterized via nested PCR and antimicrobial susceptibility testing. Among conventional antibiotics, levofloxacin showed superior efficacy (28.22 ± 2.79 mm at 50 ppm), while most alternatives demonstrated reduced activity. Remarkably, herbal extracts demonstrated comparable or superior antimicrobial efficacy: *Lagerstroemia speciosa* exhibited the strongest activity (25.67 ± 0.58 mm inhibition zone), with minimum inhibitory concentration (MIC) values of 0.19–0.39 mg/mL and bactericidal minimum bactericidal concentration/MIC ratios of 2–3. *Limnophila aromatica* showed substantial activity (18.33 ± 0.58 mm), with MIC values of 0.78–1.56 mg/mL and MBC/MIC ratios of 3–4. Qualitative phytochemical screening identified alkaloids, flavonoids, steroids, triterpenoids, tannins, saponins, and sesquiterpene lactones in both extracts. Quantitative analysis revealed *L. speciosa* contained significantly higher polyphenolic (53.1 ± 2.8 mg GAE/g) and flavonoid (195.2 ± 6.8 mg QE/g) contents compared to *L. aromatica* (48.7 ± 2.1 mg GAE/g and 182.4 ± 5.3 mg QE/g, respectively). This research establishes a crucial foundation for identifying abundant Vietnamese medicinal plants as viable plant-based alternatives for managing antibiotic-resistant $Vp_{AHPND}$ in sustainable aquaculture. These findings establish *L. speciosa* and *L. aromatica* as viable plant-based alternatives for managing antibiotic-resistant VpAHPND in sustainable aquaculture, particularly for strains with accumulated acquired resistance mechanisms.

## 1. Introduction

Shrimp aquaculture, vital for food security and economies in Southeast Asia, is severely threatened by *Vibrio parahaemolyticus* strains causing Acute Hepatopancreatic Necrosis Disease (AHPND), which can kill entire ponds in days (Choi et al., 2017; Kumar et al., 2020). Since its 2009 emergence in China, AHPND has spread across major shrimp producers, including Vietnam and Thailand, causing billions in losses (Lee et al., 2015; Tran et al., 2013; Leung and Bates, 2013). AHPND pathology involves hepatopancreatic epithelial necrosis driven by PirA/PirB toxins on the pVA1 plasmid, with hypervirulent strains carrying additional toxin genes like VHVP that worsen disease and hinder control efforts (Soto-Rodriguez et al., 2022; Zheng et al., 2021; Liu et al., 2023). Horizontal transfer of virulence plasmids and environmental stressors further complicate its epidemiology (Kumar et al., 2021).

Traditional antibiotic-based measures have failed due to rising resistance and environmental concerns (Hong et al., 2016), prompting interest in sustainable alternatives such as bacteriophages, probiotics,

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and plant-derived antimicrobials (Jun et al., 2016; Citarasu, 2010; Sivasankar et al., 2015; Soltani et al., 2019). Phytochemicals such as phenolics, flavonoids, alkaloids, tannins, and saponins act via membrane disruption, quorum sensing inhibition, and toxin suppression (Takó et al., 2020; Sun et al., 2019). Preliminary tests show - *Lagerstroemia speciosa* and *Limnophila aromatica* inhibit *Vibrio* growth and provide antioxidant protection.

The considerable biodiversity of plant species in Vietnam presents valuable opportunities for the utilization of their bioactive compounds in aquaculture, particularly in the context of shrimp farming, which holds significant economic importance. The demonstrated antimicrobial activity of these botanical extracts against *V. parahaemolyticus* isolates from shrimp culture systems underscores their potential as sustainable, plant-based alternatives to conventional antibiotics. Such species not only inhibit pathogenic bacteria but also enhance host immunity, contributing to environmentally responsible disease management practices. These approaches are congruent with current international efforts to reduce antibiotic reliance in animal production systems (Bondad-Reantaso et al., 2023; Nik Mohamad Nek Rahimi et al., 2022).

Despite the extensive cultivation of these species in the Mekong Delta, their antimicrobial potential against aquatic pathogens, particularly *V. parahaemolyticus*, remains insufficiently characterized. *Lagerstroemia speciosa* exhibits pronounced antibacterial and antioxidant activities attributed to its rich composition of saponins, flavonoids, and phenolic compounds, which confer strong bactericidal effects toward various pathogens (Nasrin et al., 2012). These bioactive constituents, especially oleanolic acid derivatives and polyacetylenes, are known to disrupt bacterial membranes effectively, showing marked efficacy against Gram-positive bacteria such as *Staphylococcus aureus* and methicillin-resistant strains (Wei et al., 2024). Similarly, *Limnophila aromatica* demonstrates notable antimicrobial and antioxidant activities, with essential oils enriched in limonene and β-*cis*-ocimene, as well as flavonoids like nevadensin, which exhibit inhibitory effects on multidrug-resistant bacteria and relevant aquatic pathogens (Nguyen et al., 2024).

This study investigated the in vitro antimicrobial effects of ten herbal extracts against prevalent *V. parahaemolyticus* strains from Vinh Long shrimp farms, determined the minimum inhibitory and bactericidal concentrations, and characterized relevant phytochemical profiles. These findings provide a scientific basis for the development of phytogenic products for shrimp disease control, supporting policy frameworks such as the European Green Deal's Farm to Fork strategy, which aims to halve antibiotic use in animal production by 2030.

## 2. Materials and methods

### 2.1. Ethical approval

The experiments were conducted at a research regulation operated by the Department of Animal Sciences at Tra Vinh University. All animal studies adhered to the recommendations of the Department of Animal Health (reference number TCVN 8400:2019), with experimental methods fully approved by the Institute of Animal Sciences for Southern Vietnam.

### 2.2. Sample collection and bacterial isolation

Shrimp samples displaying clinical signs of acute hepatopancreatic necrosis disease (AHPND) were collected from industrial shrimp farms in three districts of Vinh Long province: Cau Ngang, Duyen Hai, and Chau Thanh (three ponds/districts, 10 shrimp sample/pond, 2024). Hepatopancreatic tissues were aseptically excised and homogenized in sterile physiological saline (0.85 % NaCl). Homogenate aliquots were subsequently cultured on selective CHROMagar Vibrio medium and incubated at 37 °C for 24 h. Suspected *Vibrio parahaemolyticus* colonies, identified by their characteristic purple coloration, were selected and subcultured on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Confirmation was based on the appearance of green colonies typical of *V. parahaemolyticus*.

### 2.3. PCR detection of AHPND isolates

The isolates were further characterized by nested PCR amplification targeting the AHPND-specific virulence genes *pirA* (*toxA*) and *pirB* (*toxB*) located on the pVA1 plasmid, following the protocols described by Dangtip et al. (2015). *Vibrio parahemolyticus* 13-028/A3 strain (Tran et al., 2013) was used as a positive control, while *Vibrio vulnificus* NBRC15645 served as a negative control. The primer sequences employed were: AP4-F1 (5′-ATGAGTAACAATATAAAACATGAAAC-3′) and AP4-R1 (5′-ACGATTTCGACGTTCCCCAA-3′) for the first-round amplification; AP4-F2 (5′-TTGAGAATACGGGACGTGGG-3′) and AP4-R2 (5′-GTTAGTCATGTGAGCACCTTC-3′) for the nested PCR.

DNA templates were extracted from pure bacterial colonies using a standard boiling method. The first-round PCR was conducted in a 25 μL reaction mixture containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 U Taq DNA polymerase, and 0.5 μM of each primer (AP4-F1 and AP4-R1). Thermal cycling conditions were: initial denaturation at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 90 s; followed by a final extension at 72 °C for 2 min.

The nested PCR utilized 2 μL of the first-round product as template in a 25 μL reaction mixture with identical reagent concentrations and primers AP4-F2 and AP4-R2. Thermal cycling conditions were: initial denaturation at 94 °C for 2 min; 25 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 20 s, and extension at 72 °C for 20 s; followed by a final extension at 72 °C for 5 min and storage at 4 °C. PCR products were analyzed by electrophoresis on 1.5 % agarose gels stained with ethidium bromide and visualized under UV transillumination. Expected product sizes were 1,269 bp for the first-round PCR and 230 bp for the nested PCR product.

### 2.4. Antibiotic susceptibility test

Antibiotic susceptibility was evaluated using the disk diffusion method (Kirby-Bauer) according to CLSI standards. Eight oral antibiotics (tetracycline, doxycycline, levofloxacin, ciprofloxacin, amoxicillin, rifampicin, chloramphenicol, and cefotaxime) were prepared at concentrations of 10, 20, 30, 40, and 50 ppm in 5 % (v/v) dimethyl sulfoxide (DMSO). Additionally, two veterinary antibiotic disks (florfenicol 30 mcg and enrofloxacin 10 mcg (Oxid, UK)) were tested.

In this study, *V. parahaemolyticus* AHPND ($Vp_{AHPND}$) reference strains 13–028/A3 (Tran et al., 2013) were kindly provided by Tra Vinh University. The three $Vp_{AHPND}$ isolates ($Vp_{AHPND}$ V1, $Vp_{AHPND}$ V2, and $Vp_{AHPND}$ V3) were cultured in Nutrient Broth supplemented with 1.5 % NaCl at 28 °C for 24 h and adjusted to a bacterial inoculum of approximately $10^8$ CFU/mL. Bacterial lawns were inoculated onto Mueller-Hinton agar supplemented with 1.5 % NaCl by streaking with sterile cotton swabs. Wells (6 mm diameter) were aseptically created in the agar medium, and 50 μL of each antibiotic concentration (10, 20, 30, 40, and 50 ppm) was dispensed into the corresponding wells. The type strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were utilized as a reference for standardization. Sterile DMSO (50 μL) served as a negative control. Plates were incubated at 28 °C for 24 h, after which inhibition zone diameters were measured in millimeters and recorded. Following the CLSI breakpoints guideline M45 (CLSI, 2016), the isolates were interpreted as resistant (R), intermediate (I), or susceptible (S).

The three field isolates ($Vp_{AHPND}$ V1, $Vp_{AHPND}$ V2, and $Vp_{AHPND}$ V3), isolated from commercial shrimp farms in Vinh Long Province, were classified as non-wild-type phenotypes with evidence of acquired antibiotic resistance determinants. The reference strain 13–028/A3 served as a wild-type control for comparison. Comparative antibiotic

susceptibility testing revealed distinct phenotypic differences between reference and field isolates (Supplementary Table 1).

### 2.5. Plant material collection and extract preparation

Fresh leaves of ten herbal species (*Streblus asper*, *Triphasia trifolia*, *Leucaena leucocephala*, *Lagerstroemia speciosa, Sansevieria cylindrica*, *Sesbania grandiflora*, *Annona squamosa*, *Calophyllum inophyllum*, *Limnophila aromatica*, and *Artocarpus altilis*) were collected from Vinh Long province during the dry season (March–November 2023). Plant species were identified and authenticated by a botanist at the Faculty of Biology and Biotechnology, University of Science, Vietnam National University, Vietnam. Only mature leaves from healthy adult plants were selected for extraction.

Only adult leaves over 20 cm beyond the tip of each branch were used. Fresh leaves were thoroughly washed with distilled water, rinsed with deionized water, and oven-dried at 50 °C until constant weight was achieved. The dried material was ground into fine powder using an electric grinder and stored in airtight plastic containers until further use. The leaves extracts were obtained as follows: 500 g of powdered leaf material from each species was were soaked in 95 % ethanol at a solid-to-solvent ratio of 1:10 (w/v) for 4 days at room temperature with constant stirring. The extract supernatants were collected and filtered using Whatman paper after centrifugation for 15 min at 3000 rpm. The solvent was finally evaporated by vacuum evaporation through a rotary evaporator. The dried extracts were stored at 4 °C as previously described by El-Chaghaby et al. (2014). Working solutions of each extract were prepared at a concentration of 400 mg/mL by complete dissolving in 5 % (v/v) dimethyl sulfoxide (DMSO) for further bioactivity testing.

### 2.6. Inhibitory activity of plant extracts

The agar well diffusion method was performed to evaluate the antibacterial activity of plant extracts against four $Vp_{AHPND}$ isolates as previously described (Magaldi et al., 2004). Bacterial suspensions (approximately $1 \times 10^8$ CFU/mL) were spread evenly onto Mueller-Hinton agar supplemented with 1.5 % NaCl using sterile cotton swabs. Wells (6 mm diameter) were aseptically created in the agar, and 50 μL of plant extract at concentrations of 100 mg/mL (diluted in DMSO) was added to each well. DMSO alone served as the negative control, while doxycycline (30 μg) was used as the positive control. Plates were incubated at 28 °C for 24 h, and antibacterial activity was assessed by measuring the diameter of the inhibition zones (Oonmetta-aree et al., 2006).

MIC values were determined using the broth microdilution method. Two-fold serial dilutions of active plant extracts in Mueller-Hinton broth (MHB) supplemented with 1.5 % NaCl were prepared in test tubes, ranging from 400 to 0.195 mg/mL (400/2048). Each tube received 3 mL of diluted extract and was inoculated with standardized bacterial suspensions of the four *V. parahaemolyticus* isolates ($1 \times 10^6$ CFU/mL). Tubes were incubated at 28 °C (or 35 °C) for 24 h. The MIC was recorded as the lowest concentration of extract that completely inhibited visible bacterial growth (Oonmetta-aree et al., 2006).

To determine the MBC, 100 μL aliquots from each tube showing no visible growth in the MIC assay were subcultured onto TCBS agar plates. Plates were incubated at 28 °C for 24 h, and colonies were enumerated. The MBC was defined as the lowest concentration of extract that resulted in no bacterial growth on the TCBS agar plates (Oonmetta-aree et al., 2006).

### 2.7. Phytochemical screening

The chemical composition of the herbal extract exhibiting the highest antimicrobial activity against *Vibrio parahaemolyticus* was characterized using solvent fractionation based on differential polarity. This dissolution method sequentially partitions phytochemical constituents according to their solubility in solvents of increasing polarity. The resulting fractions were subjected to qualitative and quantitative phytochemical analysis through specific chemical reactions following standardized protocols (Ciulei et al., 1982; Sarla et al., 2012). The analytical procedures were modified and optimized by the Department of Pharmacology, Faculty of Pharmacy, University of Medicine and Pharmacy, Ho Chi Minh City (2015) to enable comprehensive detection and quantification of major bioactive compound classes, including carbohydrates, saponins, flavonoids, amino acids, anthraquinone glycosides, steroids, polyphenols, and tannins. Total polyphenolic content was determined using the Folin-Ciocalteau colorimetric method, expressing results as gallic acid equivalents (mg GAE/g dry extract). Total flavonoid content was determined using the aluminum chloride colorimetric assay, expressing results as quercetin equivalents (mg QE/g dry extract).

### 2.8. Statistical analysis

All data were analysed using one-way ANOVA and Duncan's multiple range test (SPSS v22.0, IBM, USA). Tukey-HSD was used for post-hoc mean separation. A $p$-value $<0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. Isolation and identification of Vibrio parahaemolyticus

White-leg shrimp (*Litopenaeus vannamei*) displaying clinical symptoms of AHPND were collected from three districts in Vinh Long province, Vietnam: Chau Thanh, Cau Ngang, and Duyen Hai (Fig. 1). From each district, three ponds were selected, and ten shrimp were sampled from each pond, yielding a total of 30 shrimp specimens per district. Three strains of *V. parahaemolyticus* were successfully isolated and designated as $Vp_{AHPND}$ V1 (Chau Thanh District), $Vp_{AHPND}$ V2 (Cau Ngang District), and $Vp_{AHPND}$ V3 (Duyen Hai District). All three strains exhibited characteristic morphological features on selective media, forming purple-colored colonies on CHROMagar Vibrio and green colored colonies on TCBS agar (Table 1).

Molecular identification was performed using nested PCR with AP4 primers targeting the tandem virulence genes *toxA* and *toxB*, which are specific markers for AHPND-causing *V. parahaemolyticus*. The nested PCR approach was approximately 100-fold more sensitive (100 fg total DNA template) compared to the conventional one-step AP3 method (10 pg total DNA template), enabling reliable detection of low-abundance pathogenic strains. All three isolated strains yielded positive PCR results, confirming the presence of the characteristic virulence genes associated with AHPND (Fig. 2). These results definitively confirm that all three isolates are pathogenic *V. parahaemolyticus* capable of causing AHPND ($Vp_{AHPND}$ isolates) in farmed shrimp populations.

### 3.2. Antibiotic susceptibility of V. Parahaemolyticus AHPND isolates

Antibiotic susceptibility testing of three *V. parahaemolyticus* AHPND isolates ($Vp_{AHPND}$ V1, $Vp_{AHPND}$ V2, and $Vp_{AHPND}$ V3) was performed using the disk diffusion method. Eight commercial antibiotics were tested at concentrations of 10–50 ppm, and two veterinary antibiotic disks (florfenicol 30 mcg and enrofloxacin 10 mcg) were evaluated (Fig. 2).

All three strains exhibited complete resistance to amoxicillin across all tested concentrations (inhibition zone: 0.00 ± 0.00 mm). Levofloxacin demonstrated the strongest inhibitory activity at 10 ppm (21.89 ± 2.87 mm) and 50 ppm (28.22 ± 2.79 mm). Ciprofloxacin and cefotaxime showed strong susceptibility profiles at 50 ppm (26.22 ± 0.74 mm and 25.44 ± 0.70 mm, respectively), while doxycycline, rifampicin, tetracycline, and chloramphenicol demonstrated moderate inhibitory

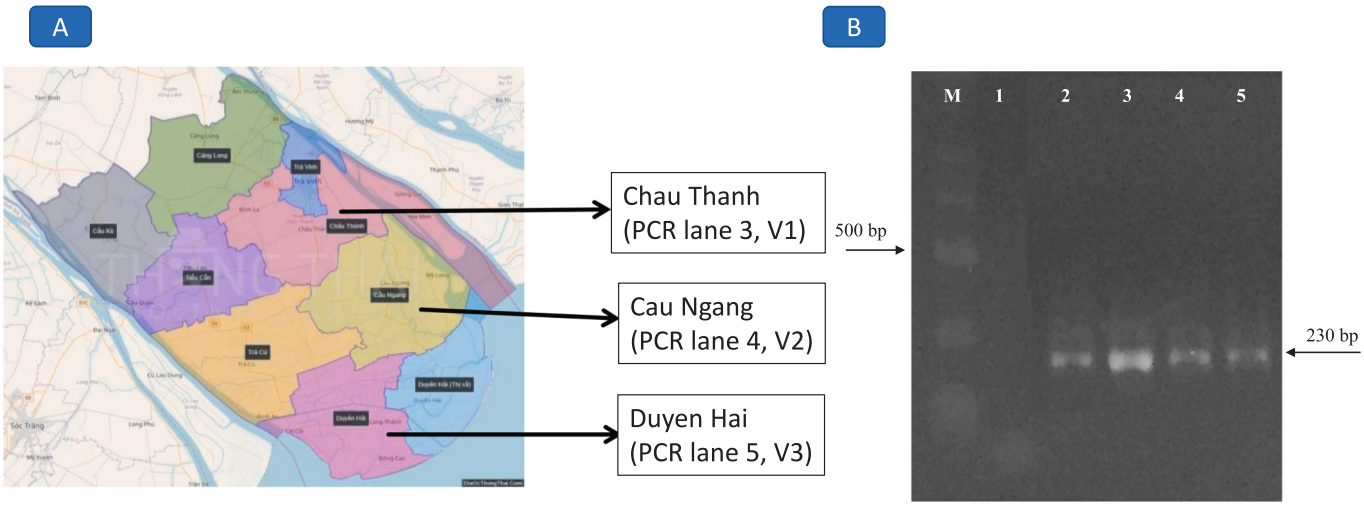


**Fig. 1.** Sampling locations in Vinh Long Province (A) and nested PCR detection of pirA/pirB virulence genes from $Vp_{AHPND}$ isolates (B). Lane M: 1 kb DNA ladder. Lane 1: Negative control *V. vulnificus* NBRC15645 (no amplification). Lane 2: Positive control *V. parahaemolyticus* strain 13–028/A3 (230 bp). Lanes 3–5: Field isolates V1, V2, V3 (230 bp), confirming virulence genes.

**Table 1**
Characteristics of *Vibrio parahaemolyticus* isolates ($Vp_{AHPND}$ V1, $Vp_{AHPND}$ V2, and $Vp_{AHPND}$ V3) from shrimp exhibiting acute hepatopancreatic necrosis disease in ponds from Tra Vinh, Vietnam.

| Isolate | Location | TCBS | Chromagar V | *pirA*/*pirB* detection *V. parahaemolyticus* |
|---|---|---|---|---|
| $Vp_{AHPND}$ V1 | Chau Thanh | Green | Purple | + |
| $Vp_{AHPND}$ V2 | Cau Ngang | Green | Purple | + |
| $Vp_{AHPND}$ V3 | Duyen Hai | Green | Purple | + |

activity at 50 ppm (18.89 ± 0.70 mm, 17.56 ± 0.95 mm, 16.78 ± 0.70 mm, and 17.00 ± 0.59 mm, respectively). Veterinary antibiotic disks produced inhibition zones of 21.67 ± 1.67 mm (florfenicol) and 21.89 ± 0.42 mm (enrofloxacin). Antibiotic susceptibility patterns were consistent across all three isolation sites.

According to CLSI interpretive criteria, $Vp_{AHPND}$ isolates were classified as susceptible to ciprofloxacin, cefotaxime, doxycycline, levofloxacin, and enrofloxacin at therapeutic concentrations. All strains were resistant to amoxicillin. Chloramphenicol, tetracycline, and rifampicin demonstrated intermediate or borderline susceptibility depending on the applied breakpoint criteria.

### 3.3. Antibiotic susceptibility phenotypes: Intrinsic vs. Acquired resistance

All three field isolates ($Vp_{AHPND}$ V1, $Vp_{AHPND}$ V2, and $Vp_{AHPND}$ V3) exhibited complete intrinsic resistance to β-lactams (amoxicillin: 0.00 ± 0.00 mm), consistent with the chromosomal β-lactamase activity characteristic of the species (Supplementary Table 1). However, these isolates also displayed intermediate resistance to tetracycline, chloramphenicol, and rifampicin, indicating acquired resistance mechanisms absent in the reference strain 13-028/A3.

Inhibition zone diameters increased progressively from 10 to 50 ppm for all antibiotics. Although dose-dependency is expected, the response to fluoroquinolones (levofloxacin, ciprofloxacin) was weaker than that of tetracycline and phenicol antibiotics. This pattern suggests differential resistance mechanisms and indicates that standard therapeutic dosing may be inadequate for achieving sufficient antimicrobial concentrations in infected tissues.

### 3.4. Antimicrobial activity of herbal extracts

Ten herbal extracts were evaluated against $Vp_{AHPND}$ isolates. All extracts exhibited inhibitory activity with varying intensities (Fig. 3). Five extracts from species (*Streblus asper*, *Triphasia trifolia*, *Leucaena leucocephala*, *Sansevieria cylindrica*, *Sesbania grandiflora*) demonstrated moderate antibacterial activity with inhibition zones ranging from 10.67 to 13.33 mm. The remaining five extracts showed sensitive antibacterial activity with inhibition zones from 14.67 to 25.67 mm. Extract of *L. speciosa* exhibited the highest activity (25.67 ± 0.58 mm), followed by *L. aromatica* (18.33 ± 0.58 mm).

Based on these results, *L. speciosa* and *L. aromatica* extracts were selected for further testing against four field isolates ($Vp_{AHPND}$ 13-028/A3, $Vp_{AHPND}$ V1, $Vp_{AHPND}$ V2, $Vp_{AHPND}$ V3) from shrimp ponds in Vinh Long province (Fig. 4). Both extracts maintained sensitive antibacterial activity against all four strains. *L. speciosa* extract exhibited inhibition zones ranging from 25.67 ± 0.58 mm to 34.33 ± 0.58 mm, while *L. aromatica* extract exhibited inhibition zones ranging from 18.33 ± 0.58 mm to 22.33 ± 0.58 mm. The $Vp_{AHPND}$ V3 isolate showed the highest susceptibility to both extracts.

### 3.5. Minimum inhibitory and minimum bactericidal concentrations

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of *L. speciosa* and *L. aromatica* extracts were determined against four $Vp_{AHPND}$ strains (Table 2). MIC values for *L. aromatica* ranged from 0.78 to 1.56 mg/mL, while MBC values ranged from 2.34 to 6.24 mg/mL. The MIC/MBC ratio was 3–4. For extract of *L. speciosa*, MIC values were lower, ranging from 0.19 to 0.39 mg/mL, with MBC values from 0.39 to 1.17 mg/mL. The MIC/MBC ratio was 2–3. Both extracts exhibited MIC/MBC ratios ≤4, indicating bactericidal activity according to Canillac and Mourey (2001) criteria. Extract of *L. speciosa* demonstrated stronger bactericidal efficacy with lower MIC and MBC values compared to extract of *L. aromatica*.

### 3.6. Phytochemical characteristics of herbal extracts

Preliminary qualitative phytochemical screening was performed on *L. speciosa* and *L. aromatica* leaf extracts to identify the presence of bioactive compounds (Table 3). Both extracts demonstrated the presence of alkaloids, flavonoids, steroids, triterpenoids, tannins, saponins, and sesquiterpene lactones. *L. speciosa* exhibited stronger reactions for

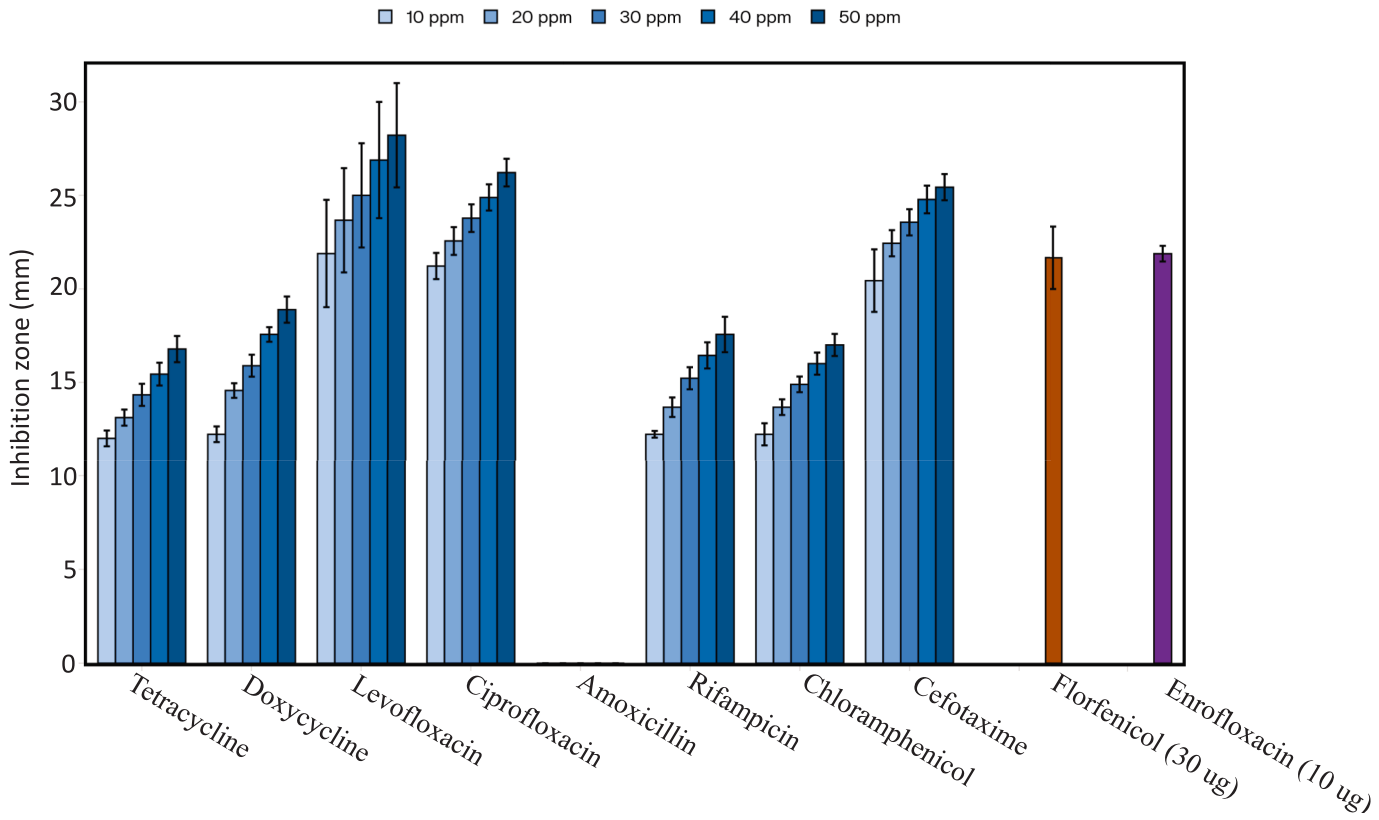


**Fig. 2.** Antibiotic susceptibility of $Vp_{AHPND}$ isolates (V1, V2, V3) from Vinh Long Province. Values represent average inhibition zone diameter (mm) ± standard deviation from well/disk diffusion assays. Amoxicillin exhibited complete resistance (0.00 mm) across all tested concentrations. Florfenicol and enrofloxacin were evaluated using fixed-dose commercial antibiotic disks only.

alkaloids (Wagner test), steroids (Liebermann-Burchard test), and saponins compared to *L. aromatica*. In contrast, *L. aromatica* showed stronger reactions for flavonoids ($H_2SO_4$ and Shinoda tests) and tannins.

Total polyphenolic and flavonoid contents were determined in both extracts using spectrophotometric methods. Extract of *L. speciosa* contained higher total polyphenolic (53.1 ± 2.8 mg GAE/g) and flavonoid (195.2 ± 6.8 mg QE/g) contents compared to *L. aromatica* (Table 4). These high concentrations of phenolic and flavonoid compounds are consistent with the potent antibacterial activities observed in the inhibition zone assays and suggest these compounds may be responsible for the antimicrobial efficacy against *V. parahaemolyticus*.

## 4. Discussion

In this study, three AHPND-causing *V. parahaemolyticus* strains representing non-wild-type, antibiotic-resistant phenotypes were successfully isolated from diseased shrimp in Vinh Long province and confirmed using combined morphological and molecular approaches. Comparative analysis against the reference wild-type strain 13-028/A3 revealed that field isolates (V1, V2, V3) carry additional acquired resistance determinants beyond the intrinsic chromosomal β-lactamase activity. Presumptive isolates recovered on CHROMagar Vibrio (purple colonies) and TCBS agar (green coloration) displayed consistent morphological characteristics across both media. CHROMagar Vibrio demonstrated superior specificity (88–95 %) compared to TCBS agar alone (51–71 %), with all three field isolates ($Vp_{AHPND}$ V1, $Vp_{AHPND}$ V2, and $Vp_{AHPND}$ V3) supporting presumptive identification as *V. parahaemolyticus*.

Our findings showed that all three isolates yielded positive nested PCR results with the characteristic 230 bp amplicon, confirming the presence of AHPND-specific virulence gene markers. In a previous study using a comprehensive panel of 104 bacterial isolates (51 AHPND-positive and 53 non-AHPND, including both *V. parahaemolyticus* and other *Vibrio* species), the AP4 method demonstrated 100 % positive and negative predictive values, establishing it as the gold standard for AHPND diagnosis (Dangtip et al., 2015). The detection of both *pirA* and *pirB* genes with the characteristic 12 bp spacer definitively distinguishes AHPND-causing *V. parahaemolyticus* from non-pathogenic strains lacking these virulence determinants.

The identical virulence gene profiles across three geographically dispersed districts in Vinh Long province (Chau Thanh, Cau Ngang, and Duyen Hai) suggest clonal relationships and potential horizontal transmission of pVA1-type plasmids among regional Vibrio parahaemolyticus populations. Multiple Vibrio species (*V. parahaemolyticus*, *V. harveyi*, *V. campbellii*, and *V. owensii*) can harbor pVA1-type plasmids and cause AHPND, complicating disease prevention and threatening sustainable aquaculture. Horizontal plasmid transfer, mediated by type IV secretion systems (T4SS), is regulated by bacterial cell density, temperature, and nutrient availability (Wang et al., 2022). The endemic distribution of AHPND virulence determinants across shrimp farming regions in Vinh Long necessitates urgent implementation of integrated management strategies to protect farming communities, aquatic ecosystems, and multiple aquatic species from this rapidly disseminating pathogen.

The three field isolates represent distinct non-wild-type phenotypes when compared to the reference strain 13-028/A3 in terms of antibiotic susceptibility patterns and resistance mechanisms. The reference strain 13-028/A3 exhibits primarily intrinsic β-lactamase-mediated resistance to β-lactam antibiotics while maintaining full susceptibility to fluoroquinolones and third-generation cephalosporins at standard testing concentrations, representing a wild-type susceptibility profile for AHPND-positive strains.

In contrast, field isolates V1, V2, and V3 demonstrate a clearly

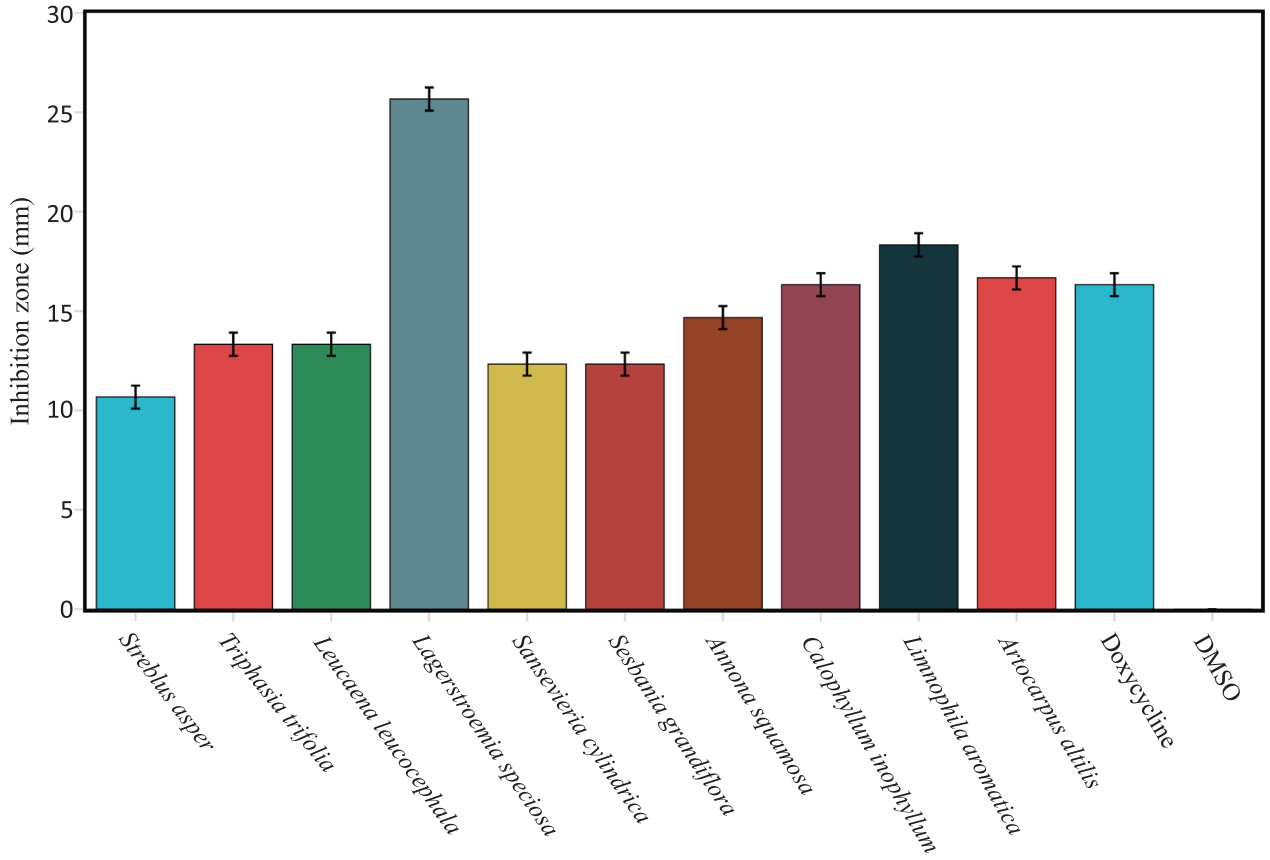


**Fig. 3.** Antibacterial activity of ten Vietnamese herbal extracts against $Vp_{AHPND}$ strain 13–028/A3. Bar chart shows average inhibition zone diameter (mm ± SD) from agar well diffusion assays at 100 mg/mL.

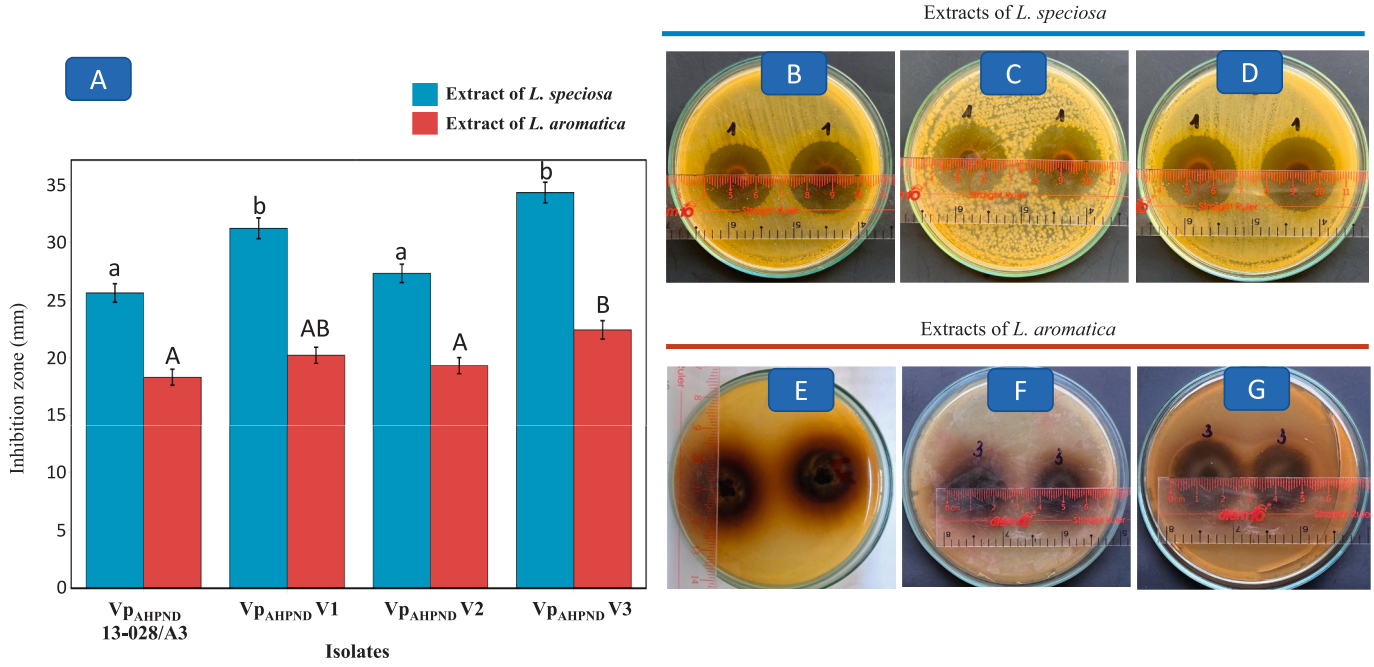


**Fig. 4.** Antibacterial activity results of *L. speciosa* and *L. aromatica* extracts. Average inhibition zone (± SD) of *L. speciosa* and *L. aromatica* extracts against $Vp_{AHPND}$ isolates (panel A). Panels B, C, and D show antibacterial activity of *L. speciosa* extract against $Vp_{AHPND}$ isolates V1, V2, and V3, respectively. Panels E, F, and G show antibacterial activity of *L. aromatica* extract against the $Vp_{AHPND}$ isolates V1, V2, and V3, respectively. Superscript letters indicate statistically significant differences between groups ($p < 0.05$).

**Table 2**
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of herbal extracts against VpAHPND isolates.

| Herbal extract | Strain | MIC (mg/mL) | MBC (mg/mL) | MIC/MBC ratio |
|---|---|---|---|---|
| *L. speciosa* | $Vp_{AHPND}$ 13–028/A3 | 0.39 | 1.17 | 3 |
| | $Vp_{AHPND}$ V1 | 0.19 | 0.57 | 3 |
| | $Vp_{AHPND}$ V2 | 0.39 | 1.17 | 3 |
| | $Vp_{AHPND}$ V3 | 0.19 | 0.39 | 2 |
| *L. aromatica* | $Vp_{AHPND}$ 13–028/A3 | 1.56 | 6.24 | 4 |
| | $Vp_{AHPND}$ V1 | 1.56 | 4.68 | 3 |
| | $Vp_{AHPND}$ V2 | 1.56 | 6.24 | 4 |
| | $Vp_{AHPND}$ V3 | 0.78 | 2.34 | 3 |

**Table 3**
Qualitative phytochemical composition of *L. speciosa* and *L. aromatica* extracts.

| Phytochemical class | Test reagent | Positive reaction | *L. aromatica* | *L. speciosa* |
|---|---|---|---|---|
| Alkaloids | Dragendorff | Brown-orange precipitate | + | + |
| | Wagner | Brown precipitate | + | ++ |
| Flavonoids | $FeCl_3$ 5 % | Brown-red or blue-black precipitate | + | + |
| | NaOH 1 % | Yellow to orange-red solution | + | + |
| | $H_2SO_4$ | Red-brown solution | ++ | + |
| | Shinoda | Solution color shift to red | ++ | ++ |
| Steroids and Triterpenoids | Liebermann-Burchard | Orange-red solution | + | ++ |
| | Salkowski | Solution color shift to brown–red | + | + |
| Glycosides | Tollens | Black Ag precipitate | – | – |
| | Fehling | Brick-red precipitate | – | – |
| | Keller-Killiani | Blue-green solution | – | – |
| Tannins | Saturated gelatin | White fibrous precipitate | ++ | + |
| | Saturated lead acetate | White fibrous precipitate | ++ | + |
| Saponins | Acid medium foam formation | Foam production | ++ | + |
| | Base medium foam formation | Foam production | ++ | + |
| | Saturated lead acetate | Multiple white precipitates | ++ | ++ |
| Sesquiterpene lactones | Legal | Pink to deep red solution | + | + |

Note: (+) Positive reaction present, (++) Strong positive reaction present, (−) Negative reaction absent.

**Table 4**
Quantitative phytochemical content of herbal extracts.

| Bioactive compound | *L. speciosa* | *L. aromatica* |
|---|---|---|
| Total polyphenolic content (mg GAE/g DW) | 53.1 ± 2.8 | 48.7 ± 2.1 |
| Total flavonoid content (mg QE/g DW) | 195.2 ± 6.8 | 182.4 ± 5.3 |

*Note: Values represent mean ± standard deviation. GAE = Gallic acid equivalent; QE = Quercetin equivalent; DW = dry weight.*

distinguishable non-wild-type phenotype characterized by three key features: (1) identical intrinsic amoxicillin resistance patterns (0.00 ± 0.00 mm inhibition zones), consistent with chromosomal β-lactamase activity; (2) intermediate to borderline susceptibility to tetracycline, chloramphenicol, and rifampicin, phenotypes absent in the wild-type reference strain; and (3) concentration-dependent susceptibility patterns with dose–response curves consistent with acquired resistance mechanisms rather than intrinsic properties.

Field isolates from Vinh Long province displayed complete intrinsic β-lactam resistance (amoxicillin: 0.00 ± 0.00 mm), consistent with 97.06 % resistance rates documented in AHPND-associated *V. parahaemolyticus* strains from the Mekong Delta (Ha et al., 2023). This intrinsic resistance phenotype results from chromosomally encoded β-lactamase, which is upregulated through a two-component regulatory system where the histidine kinase sensor *Vbr*K detects β-lactam antibiotics and triggers *Bla*A β-lactamase expression via the response regulator *Vbr*R (Li et al., 2016). Regional surveillance confirms this resistance pattern is standardized among AHPND strains, with studies from the Mekong Delta showing 100 % ceftazidime and 97.06 % amoxicillin resistance among 34 AHPND-positive isolates. Consequently, β-lactam-based treatments are therapeutically ineffective for aquaculture applications in the region (Lee et al., 2018).

The non-wild-type phenotype of field isolates suggests that they have accumulated acquired resistance genes through selective pressure from antibiotic usage in regional aquaculture systems. The genetic basis of this resistance phenotype likely involves multiple resistance determinants encoded on chromosomal or plasmid-based elements, including potential *tet* genes (tetracycline resistance), phenicol resistance genes, and upregulation of chromosomal β-lactamase expression through regulatory mutations. The intermediate resistance observed to tetracycline (16.78 ± 0.70 mm), chloramphenicol (17.00 ± 0.59 mm), and rifampicin (17.56 ± 0.95 mm) at 50 ppm testing concentrations indicates acquired resistance mechanisms distinct from the intrinsic β-lactamase activity. Future whole-genome sequencing or targeted molecular analysis would definitively clarify the specific genetic mechanisms underlying the non-wild-type phenotype and identify specific resistance genes responsible for tetracycline and phenicol resistance in field isolates.

A pronounced concentration-dependent response was observed across most tested antibiotics, with inhibition zones increasing from 10 ppm to 50 ppm. This pattern raises critical concerns about achieving therapeutically effective drug concentrations in aquaculture settings. Oral antibiotic delivery in aquaculture frequently fails to attain target concentrations due to reduced feed intake in diseased animals, incomplete drug dissolution and absorption, rapid biliary and renal excretion into farm sediments, and bioavailability limitations in aquatic environments (Bondad-Reantaso et al., 2023). These constraints create selective pressure microenvironments that favor resistance allele emergence and fixation, even at subinhibitory antibiotic levels (Deng et al., 2020). The concentration-dependent efficacy profiles documented suggest that standard therapeutic dosing regimens may be inadequate for achieving clinically sufficient antimicrobial concentrations in infected host tissues.

The field isolates demonstrated less pronounced dose-dependency to fluoroquinolones (levofloxacin at 10 ppm: 21.89 ± 2.87 mm and 50 ppm: 28.22 ± 2.79 mm; ciprofloxacin at 50 ppm: 26.22 ± 0.74 mm) compared to tetracycline-class and phenicol antibiotics (tetracycline: 16.78 ± 0.70 mm, chloramphenicol: 17.00 ± 0.59 mm at 50 ppm), suggesting differential mechanisms of resistance accumulation across antibiotic classes. In marked contrast to amoxicillin resistance, levofloxacin demonstrated exceptional inhibitory activity, positioning it as the most effective agent tested among conventional antibiotics. These fluoroquinolone results are noteworthy because they diverge from emerging regional surveillance patterns documenting increasing ciprofloxacin resistance in Southeast Asian *V. parahaemolyticus* populations (Changsen et al., 2023). Contemporary research emphasizes that

fluoroquinolones and third-generation cephalosporins remain among the recommended therapeutic options for severe or prolonged V. parahaemolyticus infections when resistance to first-line agents has developed (Algammal et al., 2025; Li et al., 2025). However, the widespread dissemination of plasmid-mediated quinolone resistance genes encoding qnr variants in biofertilizers commonly used in Chinese and Southeast Asian aquaculture systems poses an escalating threat to fluoroquinolone utility (Ferri et al., 2022).

Four antibiotics, doxycycline (18.89 ± 0.70 mm), rifampicin (17.56 ± 0.95 mm), tetracycline (16.78 ± 0.70 mm), and chloramphenicol (17.00 ± 0.59 mm), showed moderate inhibitory activity at 50 ppm but exhibited borderline susceptibility. These marginal inhibition zones suggest limited therapeutic efficacy, as optimal antimicrobial activity requires drug concentrations 4–5 times the MIC for maximal bactericidal effect (Papadimitriou-Olivgeris et al., 2020). Our previous research on $Vp_{AHPND}$ isolates from Mekong Delta in Vietnam reported 94 % doxycycline susceptibility versus 65 % for erythromycin and lower rates for rifampicin and tetracycline (Ha et al., 2023). Nigerian surveys detected high resistance to rifampicin (92 %), doxycycline (82 %), and tetracycline (75 %) in aquatic *Vibrio* species (Adesiyan et al., 2022). These geographic variations in resistance patterns consistently indicate that tetracycline derivatives and phenicols are unreliable for empirical AHPND treatment.

Oral antibiotic delivery in aquaculture frequently fails to attain therapeutic target concentrations due to reduced feed intake in diseased animals, incomplete drug dissolution and absorption, rapid biliary and renal excretion into farm sediments, and bioavailability limitations in aquatic environments (Bondad-Reantaso et al., 2023). These constraints create selective pressure microenvironments that favor resistance allele emergence and fixation, even at subinhibitory antibiotic levels (Deng et al., 2020), ultimately driving the accumulation of acquired resistance determinants observed in field isolates.

The non-wild-type phenotype of field isolates holds critical implications for treatment strategy selection in aquaculture systems in Vietnam. While these isolates retain susceptibility to fluoroquinolones and third-generation cephalosporins at higher concentrations, the presence of multiple acquired resistance mechanisms suggests they may represent an intermediate evolutionary stage toward pan-antibiotic resistance. This contrasts markedly with reference strains exhibiting predominantly intrinsic resistance patterns without significant acquired determinants.

Despite geographic separation across three Vinh Long Province districts including Chau Thanh, Cau Ngang, and Duyen Hai (Fig. 1), the three $Vp_{AHPND}$ isolates displayed remarkably uniform antibiotic susceptibility patterns with only minor strain-level variations. This uniformity across geographically dispersed locations indicates regionalized antimicrobial resistance, likely resulting from synchronized intensive antibiotic usage across interconnected farming systems, shared water sources, or efficient horizontal gene transfer mechanisms (Ha et al., 2023). This geographic consistency reflects a transition toward regionalized resistance epidemiology documented across Southeast Asian aquaculture zones (Bondad-Reantaso et al., 2023). Surveillance of *V. parahaemolyticus* AHPND isolates from Vietnam's Bac Lieu Province identified 27 multidrug-resistant phenotypes among 34 isolates, with 100 % amoxicillin resistance and 74 % colistin resistance (Ha et al., 2023). In severely affected farming zones, isolates resistant to 14 of 16 tested antibiotics have been documented, with multidrug-resistant *V. parahaemolyticus* prevalence exceeding 85 % across multiple antibiotic classes (Deng et al., 2020; Huang et al., 2023).

Herbal solutions offer distinct advantages against non-wild-type phenotypes carrying combined intrinsic and acquired resistance. Unlike single-target antibiotics, phytogenic compounds employ polypharmacological mechanisms that simultaneously disrupt multiple cellular systems, membrane integrity, DNA topology, protein function, through pathways genetically independent of plasmid-encoded resistance determinants (Bouarab-Chibane et al., 2019; Takó et al., 2020). This mechanistic independence explains why herbal extracts maintain efficacy against multidrug-resistant strains that express numerous antibiotic resistance genes: intrinsic β-lactamase confers no protection against direct membrane disruption by flavonoids and alkaloids (Sivasankar et al., 2015). In particular, resistance to plant-derived compounds remains rare despite millennia of traditional use, suggesting herbal alternatives provide durable therapeutic options unlikely to face the rapid resistance emergence that plagues conventional antibiotics (Hong et al., 2016).

Both veterinary-use antimicrobials including florfenicol (30 μg: 21.67 ± 1.67 mm) and enrofloxacin (10 μg: 21.89 ± 0.42 mm) demonstrated strong *in vitro* inhibitory activity against field isolates (Fig. 2). However, their practical application remains severely constrained by strict regulatory restrictions imposed by national and international aquaculture authorities (Bondad-Reantaso et al., 2023). Florfenicol and enrofloxacin fall within the category of critically important or highly restricted antimicrobials due to legitimate concerns regarding residue accumulation in edible tissues, prolonged environmental persistence, selection of cross-resistant bacteria in farm ecosystems, and potential transfer of resistance determinants to zoonotic and food-associated pathogens with public health implications (Ferri et al., 2022). Genomic epidemiological studies examining VpAHPND isolates from multiple geographic origins (Mexico, Thailand, China) documented prevalence of extended-spectrum β-lactamase genes (*blaCTX-M-55*), tetracycline resistance genes (*tet(34)* and *tet(35)*), aminoglycoside resistance markers, and sulfonamide resistance determinants, indicating that multidrug-resistant phenotypes capable of circumventing therapeutic options are already well-established in international AHPND-causing populations (Vandeputte et al., 2024).

The emergence of field isolates with accumulated acquired resistances emphasizes the urgency of developing effective phytogenic alternatives and implementing integrated antimicrobial stewardship frameworks that minimize selective pressure for further resistance accumulation.

The documented antibiotic resistance phenotype underscores the urgent necessity to transition toward integrated disease management frameworks incorporating natural and phytogenic alternatives. Recent literature increasingly emphasizes plant-derived antimicrobial compounds as viable therapeutic substitutes for conventional antibiotics. Essential oil mixtures from 10 medicinal plants (*Lavandula latifolia*, *Pinus sylvestris*, *Jasminum officinale*, *Citrus limon*, *Prunus avium*, *Viola odorata*, *Gardenia jasminoides*, *Cocos nucifera*, *Rosa damascena*, *Eucalyptus globulus*) demonstrated antimicrobial activity against $Vp_{AHPND}$ strains and significantly improved *Litopenaeus vannamei* survival in challenge trials (Kumar et al., 2021).

Extracts from *L. speciosa* and *L. aromatica* exhibited superior antimicrobial efficacy against $Vp_{AHPND}$ isolates (Fig. 3 and Fig. 4), with minimum inhibitory concentrations (MIC: 0.19–0.39 mg/mL for *L. speciosa*; MIC: 0.78–1.56 mg/mL for *L. aromatica*) lower than many conventional antibiotics and bactericidal properties demonstrated by MIC/MBC ratios of 2–3 and 3–4, respectively (Table 2). Both extracts contained alkaloids, flavonoids, steroids, triterpenoids, tannins, saponins, and sesquiterpene lactones, with absence of glycosides. This multicomponent phytochemical composition directly correlates with their superior antimicrobial performance against antibiotic-resistant, non-wild-type $Vp_{AHPND}$ isolates.

*L. aromatica* likely contains nevadensin as its predominant flavonoid (~79.55 % of isolated compounds), with documented antimicrobial activity (MIC 0.59–2.86 mg/mL) closely approximating our observed whole-extract MIC values (0.78–1.56 mg/mL), indicating nevadensin is a primary contributor to efficacy (Maswanna and Maneeruttanarungroj, 2025). Secondary flavonoids (gardenin B, jasmolin II) and essential oil components (limonene, β-*cis*-ocimene) provide additional antimicrobial

activity through membrane disruption (Nguyen et al., 2024). In contrast, *L. speciosa* likely contains quercetin and kaempferol (major flavonoids) plus oleanolic acid (triterpenoid), all documented antimicrobial agents. Quercetin (MIC: 0.032–0.5 mg/mL) and kaempferol disrupt bacterial membranes or inhibit DNA gyrase, while oleanolic acid disrupts membranes through lipopolysaccharide interactions. The superior performance of *L. speciosa* (inhibition zones 25.67–34.33 mm; MIC 0.19–0.39 mg/mL) versus *L. aromatica* (18.33–22.33 mm; MIC 0.78–1.56 mg/mL) correlates with its higher flavonoid content (195.2 vs. 182.4 mg QE/g) and optimal lipophilicity profile (Lobiuc et al., 2023; Bouarab-Chibane et al., 2019). These results suggest synergistic effects of quercetin, kaempferol, and oleanolic acid in *L. speciosa* drive superior antimicrobial activity.

Both extracts demonstrated distinct antimicrobial mechanisms linked to their differential phytochemical composition. *L. speciosa* exhibited stronger alkaloid and triterpenoid content (Table 3), which inhibit bacterial efflux pumps, disrupt peptidoglycan biosynthesis, and inhibit FtsZ protein assembly with up to 70 % GTPase inhibition (Huang et al., 2022; Bildziukevich et al., 2023). Conversely, *L. aromatica* showed stronger tannin and saponin content, which precipitate membrane proteins, chelate metal ions (iron, zinc), and create membrane pores causing intracellular leakage (Huang et al., 2024; Tatli Cankaya and Somuncuoglu, 2021). The differential phytochemical profiles, *L. speciosa* rich in flavonoids/polyphenols/alkaloids, *L. aromatica* rich in tannins/saponins, represent distinct but equally effective antimicrobial strategies optimized for different bacterial disruption pathways. The quantitative superiority of *L. speciosa* (total flavonoids 195.2 vs. 182.4 mg QE/g; total polyphenols 53.1 vs. 48.7 mg GAE/g) correlates directly with superior MIC values (0.19–0.39 vs. 0.78–1.56 mg/mL) and inhibition zones (25.67–34.33 vs. 18.33–22.33 mm). Flavonoids exert bactericidal activity through lipophilicity-dependent membrane disruption (LogP: 2.5–4.0), with potency correlating positively with lipophilicity (Yuan et al., 2021; Bouarab-Chibane et al., 2019). In contrast, *L. aromatica* harboring higher tannin and saponin content partially compensates for lower flavonoid concentration, providing complementary membrane-disruptive mechanisms and enabling consistent moderate antimicrobial activity (Nasrin et al., 2012). While the differential contribution of specific compounds in both extracts remains incompletely understood, these findings establish phytogenic alternatives as viable options for managing antibiotic-resistant $Vp_{AHPND}$ and warrant future isolated compound antimicrobial testing.

The superiority of herbal extracts against field isolates reflects fundamental mechanistic differences from conventional antibiotics. Intrinsic β-lactamase production and acquired resistance genes both target antibiotic-protein interactions, but phytochemicals attack constitutive bacterial structures (membranes, DNA topology, cell walls) that cannot be abandoned without loss of viability (Lobiuc et al., 2023). Recent comparative studies across *Vibrio* species demonstrate that herbal extracts maintain consistent antimicrobial activity regardless of resistance phenotype status, whereas conventional antibiotics show 10–100-fold increases in MIC values between wild-type and extensively resistant strains (Kumar et al., 2021).

The diverse phytochemical composition creates synergistic antibacterial effects through simultaneous mechanisms: (1) flavonoids disrupt membrane integrity and inhibit DNA gyrase; (2) alkaloids inhibit efflux pumps and peptidoglycan synthesis; (3) tannins precipitate membrane proteins and chelate metal ions; (4) saponins create membrane pores and enhance compound penetration; (5) triterpenoids inhibit cell division proteins. This polypharmacological approach fundamentally differs from single-target antibiotics, dramatically reducing resistance development probability (Jubair et al., 2021; Lobiuc et al., 2023).

The resistance vulnerability of multiply-resistant field isolates present a particularly instructive case study: the metabolic cost of simultaneously maintaining intrinsic β-lactamase production plus multiple plasmid-encoded acquired resistance genes creates fitness penalties that may paradoxically increase susceptibility to herbal membrane-disrupting compounds (Yuan et al., 2021). This resistance trade-off phenomenon, where accumulation of antibiotic resistance genes increases vulnerability to herbal antimicrobials, creates an inverse relationship absent in conventional antibiotic development, suggesting that the most problematic intrinsic β-lactam resistance and acquired resistance to multiple antibiotics strains encountered in clinical practice may be most effectively managed through phytogenic approaches (Jubair et al., 2021).

Studies on flavonoid combinations demonstrate dose-dependent synergistic effects, with specific compound ratios (e.g., luteolin:quercetin 1:7) producing enhanced antibacterial activity with synergy coefficients ranging from 1.129 to 1.425 (Li et al., 2017). The natural flavonoid mixtures in *L. speciosa* and *L. aromatica* likely exhibit similar synergistic interactions, amplifying antibacterial potency beyond additive effects of individual compounds. Notably, the absence of glycosides in both extracts, confirmed by negative Tollens, Fehling, and Keller-Killiani tests, may contribute to enhanced antimicrobial efficacy, as aglycone forms typically show superior activity compared to glycoside counterparts through enhanced membrane penetration and biological activity (Patra, 2012).

The superior antimicrobial efficacy of *L. speciosa* and *L. aromatica* against antibiotic-resistant $Vp_{AHPND}$ isolates establishes a compelling rationale for developing phytogenic therapeutics as alternatives to conventional antibiotics in aquaculture. These botanical alternatives demonstrate particular promise against multidrug-resistant strains with accumulated acquired resistance to tetracyclines, phenicols, and fluoroquinolones, a therapeutic crisis current antibiotic development cannot adequately address (Algammal et al., 2025). Phytogenic compounds offer a mechanistically independent alternative: by targeting conserved bacterial structures rather than drug-specific proteins, herbal solutions circumvent resistance mechanisms. This strategy is supported by over 50 years of field use with minimal documented resistance emergence, in stark contrast to near-universal resistance observed with conventional antibiotics over comparable timeframes (Soltani et al., 2019; Bondad-Reantaso et al., 2023). Herbal alternatives thus represent a foundational approach toward sustainable, long-term disease management in aquaculture systems.

Managing this crisis requires comprehensive antimicrobial stewardship aligned with FAO/OIE/WHO directives. Integrating phytogenic alternatives reduces antibiotic selection pressure, minimizes residue accumulation, and decreases environmental persistence of resistance determinants. These approaches support the European Green Deal's Farm to Fork strategy targeting 50 % reduction in antibiotic use by 2030 (Bondad-Reantaso et al., 2023).

This research fulfills a crucial foundational role in identifying Vietnamese medicinal plants with abundant local resources and documented antimicrobial activity against multidrug-resistant *V. parahaemolyticus* phenotypes. The non-wild-type isolates examined exemplify the regional resistance crisis, expressing simultaneous intrinsic and accumulated acquired resistance rendering conventional antibiotics increasingly ineffective. *L. speciosa*, abundantly available throughout Southeast Asia, and *L. aromatica*, widely cultivated in Vietnamese rice paddies, both demonstrate antimicrobial potential through mechanisms independent of conventional resistance pathways. This screening establishes essential criteria for rational selection of sustainable alternatives for Vietnam and other countries with these raw material sources.

Future investigations should address: (1) *in vivo* safety validation at therapeutic concentrations; (2) efficacy trials against field isolates; (3) environmental/food safety assessment; (4) regulatory compliance; (5) structure–activity relationship analysis; and (6) integrated disease management protocols combining herbal therapeutics with sustainable farming practices and probiotics. Only after these systematic investigations will commercial development be justified. This research

provides the critical prerequisite for developing locally available therapeutics against escalating multidrug-resistant phenotypes, aligned with international antimicrobial stewardship initiatives and UN Sustainable Development Goals (Bondad-Reantaso et al., 2023; Citarasu, 2010; Hong et al., 2016; Soltani et al., 2019).

## 5. Conclusion

The isolation of antibiotic-resistant, non-wild-type *V. parahaemolyticus* AHPND strains from Vietnamese aquaculture, characterized by intrinsic β-lactam resistance and accumulated acquired resistance to tetracycline-class and phenicol antibiotics, underscores the critical need for alternative therapeutic strategies. *Lagerstroemia speciosa* and *Limnophila aromatica* present viable plant-based alternatives with proven bactericidal efficacy against these resistant pathogens. Their rich phytochemical compositions enable synergistic antimicrobial mechanisms that circumvent conventional antibiotic resistance. These herbal extracts warrant further development for sustainable management of AHPND in shrimp aquaculture.

## CRediT authorship contribution statement

**Nguyen Thi Truc Linh:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Data curation. **Pham Thi Hai Ha:** Visualization, Validation, Methodology, Investigation, Conceptualization. **Hong Mong Huyen:** Visualization, Validation, Software, Methodology, Formal analysis, Data curation. **Do-Hyung Kim:** Writing – review & editing, Writing – original draft. **Nguyen Thanh Luan:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

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