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Antibacterial activity of *Cinnamomum verum* essential oil against antibiotic-resistant *Vibrio parahaemolyticus* isolated from whiteleg shrimp (*Litopenaeus vannamei*)

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Abstract

This study investigated multidrug-resistant (MDR) *Vibrio parahaemolyticus* strains and evaluated the antimicrobial activity of *Cinnamomum verum* (cinnamon bark) essential oil against these resistant strains, which are pathogenic to Pacific white shrimp (*Litopenaeus vannamei*). The results demonstrated that *Cinnamomum verum* essential oil exhibits significant antimicrobial activity against MDR *V. parahaemolyticus*. Specifically, the essential oil showed inhibition zone diameters ranging from 10 to 12 mm in disk diffusion assays, with a minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 12.5 µL/mL for both.

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Introduction

The whiteleg shrimp (*Litopenaeus vannamei*) aquaculture industry is a critical component of the global aquaculture economy, particularly in Vietnam, where shrimp farming significantly constitutes to seafood exports. However, the sustainability of this sector is increasingly threatened by the prevalence of infectious diseases. Bacterial pathogens, including *Aeromonas hydrophila*, *Edwardsiella tarda*, *Pseudomonas* sp., *Flexibacter columnaris*, *Vibrio* spp., and *Streptococcus* spp., are frequently implicated in aquaculture diseases (Nguyen *et al.*, 2022).

Among these, acute hepatopancreatic necrosis disease (AHPND), also known as early mortality syndrome (EMS), caused by certain strains of *Vibrio parahaemolyticus*, has emerged as a major cause of mortality and economic losses in shrimp farming worldwide (Dang *et al.*, 2022).

AHPND-affected shrimp typically display clinical signs such as lethargy, empty gastrointestinal tracts, pale coloration, and atrophy of the hepatopancreas. The widespread use of antibiotics to control bacterial diseases in shrimp aquaculture has led to a critical secondary problem: the proliferation of antibiotic-resistant bacterial strains (Nguyen *et al.*, 2022). The indiscriminate and often inappropriate use of antibiotics in aquaculture poses significant risks to the environment and human health. Antibiotic resistance compromises the effectiveness of disease treatment, threatens consumer health through the potential transfer of resistance genes, disrupts natural microbial ecosystems, and can harm non-target organisms. Furthermore, the presence of unauthorized antibiotic residues in commercial shrimp products can lead to trade restrictions and economic losses (Do *et al.*, 2023).

The limitations of current strategies for managing antibiotic-resistant bacteria highlight the urgent need for alternative, eco-friendly, and sustainable solutions. Essential oils derived from various plant species have gained attention due to their demonstrated antibacterial properties.

The use of essential oils represents a promising avenue for controlling *V. parahaemolyticus* and other shrimp pathogens in an environmentally responsible manner. *Cinnamomum verum* cinnamon bark essential oil has been shown to possess antimicrobial activity against a range of bacteria and fungi. For example, Nguyen (2017) reported that *Cinnamomum verum* essential oil at a concentration of 0.5% completely inhibited the growth of *Escherichia coli* and *Bacillus cereus* in culture media. While the antibacterial properties of cinnamon bark essential oil are well-documented, its specific effects on antibiotic-resistant *V. parahaemolyticus*, a key pathogen in whiteleg shrimp aquaculture, require further investigation.

Therefore, this study aimed to evaluate the antibacterial activity of *Cinnamomum verum* essential oil against antibiotic-resistant *V. parahaemolyticus* strains isolated from *Litopenaeus vannamei*. The findings are expected to contribute to the development of effective, safe, and sustainable strategies for preventing and treating diseases in whiteleg shrimp, thereby reducing the reliance on antibiotics in aquaculture practices.

Materials and methods

Essential oil sources

The cinnamon bark (*Cinnamomum verum*) essential oil was obtained from Vina Oils International JSC and stored at 4°C.

Bacteria inoculum preparation

Vibrio parahaemolyticus was obtained from the Institute of Food and Biotechnology, Can Tho University. Bacteria were cultured in Tryptic Soy Broth (TSB) supplemented with 1% NaCl.

Bacterial density was determined by measuring the optical density (OD) at 600 nm. A bacterial suspension with a density of 10^6 CFU/mL, corresponding to an OD_{600} of 0.1, was used for antibiotic resistance assays and the antimicrobial activity testing of cinnamon bark essential oil.

Antibiotic resistance assay

The antibiotic resistance profiles of five *V. parahaemolyticus* strains were determined using the Kirby-Bauer disk diffusion method (Balouiri *et al.*, 2016). This method allowed for identifying the strain exhibiting the highest level of antibiotic resistance. A bacterial suspension (10^6 CFU/mL) was evenly spread onto TSB agar plates supplemented with 1% NaCl (100 µL per plate). Ten commonly used antibiotics in aquaculture were selected for this assay: Ampicillin (AMP, 25 µg), Amoxicillin (AMO, 10 µg), Doxycycline (DOX, 30 µg), Erythromycin (ERYT, 15 µg), Ciprofloxacin (CIP, 5 µg), Tetracycline (TET, 30 µg), Clindamycin/Dalacin (DAL, 25 µg), Azithromycin (AZI, 30 µg), Cefpodoxime (CEF, 30 µg), and Clarithromycin (CLA, 20 µg).

Sterile paper disks were impregnated with 8 µL of each antibiotic solution or 10% DMSO (negative control). On each inoculated agar plate, 10% DMSO disk and five disks, each containing a different antibiotic, were placed with sufficient spacing. Each antibiotic was tested in triplicate across three separate agar plates. The plates were incubated at 28°C for 24 hours.

Antibiotic resistance was evaluated by measuring the diameter of the inhibition zones and interpreting the results according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2015; 2021) for *V. parahaemolyticus*.

Antibacterial activity assay

The antibacterial activity of 100% *Cinnamomum verum* essential oil was assessed using the Kirby-Bauer disk diffusion method (Balouiri *et al.*, 2016). A bacterial suspension (10^6 CFU/mL) was spread onto TSB agar plates supplemented with 1% NaCl (100 µL per plate). Sterile paper disks were impregnated with 8 µL of 100% cinnamon bark essential oil, 10% DMSO (negative control), and Doxycycline (DOX, 30 µg) (positive control). Disks were placed on inoculated agar plates with appropriate spacing. Doxycycline was used as positive controls to confirm the antibiotic resistance of the test strain, respectively. Each test was performed in triplicate on three separate agar plates. Plates were incubated at 28°C for 24 hours. Antibacterial activity was determined by measuring the zone of inhibition diameters.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of essential oil that inhibits visible bacterial growth. The MIC was determined using the two-fold serial dilution method in a 96-well microtiter plate (Ngo *et al.*, 2021). Control wells included TSB medium with 1% NaCl (100 µL and 50 µL), 30% DMSO (negative controls), and Doxycycline (30 µg) and Dalacin (25 µg) serially diluted in 10% DMSO (positive controls). Cinnamon bark essential oil was serially diluted in 30% DMSO from an initial concentration of 5%. A bacterial suspension (10^6 CFU/mL, 50 µL) was added to all wells except those containing 100 µL of TSB medium with 1% NaCl. The plate was incubated at 28°C for 5 hours. Following incubation, 30 µL of sterile resazurin solution (0.015%) was added to each well. The MIC was recorded as the lowest essential oil concentration that prevented a color change in the resazurin.

The MBC was defined as the lowest concentration of essential oil that kills 99.99% of the bacterial population. To determine the MBC, 100 µL aliquots from wells showing no resazurin color change were plated onto TSA agar plates and incubated at 28°C for 24 hours (Ngo *et al.*, 2021). The MBC was identified as the lowest concentration at which no bacterial growth was observed. The bactericidal or bacteriostatic activity of the essential oil was determined by calculating the MBC/MIC ratio: a ratio is ≤ 4 indicates bactericidal activity, while a ratio > 4 indicates bacteriostatic activity (Canillac and Mourey, 2001).

Results and discussion

Antibiotic resistance of bacteria

The antibiotic resistance profiles of the five *V. parahaemolyticus* strains against ten commonly used antibiotics in aquaculture are shown in Table 1. The results of the disk diffusion assays revealed a concerning trend of multidrug resistance among the tested isolates.

A high proportion of the isolates exhibited resistance to multiple antibiotics, most notably Ampicillin (AMP), Amoxicillin (AMO), and Doxycycline (DOX). This finding is consistent with the growing global concern regarding antibiotic resistance in aquaculture environments. The extensive use of these antibiotics in shrimp farming likely contributes to the selective pressure favoring the proliferation of resistant strains.

Table 1. Antibiotic resistance profiles of *V. parahaemolyticus* strains

Antibiotics	*Vibrio parahaemolyticus* strains isolated														
	V12			V14			V15			V16			V22		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
AMP	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
AMO	-	-	+	-	-	+	-	-	+	+	-	-	-	-	+
DOX	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
Eryt	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
CIP	-	+	-	-	-	+	+	-	-	+	-	-	-	+	-
TET	-	-	+	-	-	+	-	-	+	-	+	-	-	-	+
DAL	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
Azi	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
CEF	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-
CLA	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+

Note: S- Susceptible, I- Intermediate, R- Resistant

V. parahaemolyticus strain V14 demonstrated the most pronounced resistance phenotype, displaying the smallest inhibition zones across the majority of the tested antibiotics. This heightened resistance of strain V14 makes it a particularly relevant target for alternative antimicrobial strategies, such as the use of essential oils.

The observed resistance to older-generation antibiotics, such as Ampicillin, aligns with findings of Jiang *et al.* (2014), who documented a significant trend of *V. parahaemolyticus* resistance to these compounds. This suggests that the continued use of these antibiotics is increasingly ineffective and may exacerbate the problem of resistance.

Furthermore, our results corroborate the observation by Quach *et al.* (2024) that *V. parahaemolyticus* strains isolated from seafood and marine environments often exhibit higher antibiotic resistance compared to those from other sources. This phenomenon may be attributed to the complex interplay of factors in the aquaculture ecosystem, including horizontal gene transfer, selective pressure from antibiotic use, and the presence of diverse microbial communities. The uncontrolled and widespread use of antibiotics in aquaculture is a critical driver in the emergence and dissemination of antibiotic-resistant *V. parahaemolyticus* strains. This practice not only jeopardizes the efficacy of disease treatment but also poses risks to environmental and human health.

Antibacterial activity of Cinnamomum verum essential oil

The antibacterial activity of *Cinnamomum verum* essential oil against the multidrug-resistant *V. parahaemolyticus* strain V14 was evaluated using the disk diffusion method. The results, summarized in Table 2 and illustrated in Fig. 1, demonstrate the potent inhibitory effect of the essential oil.

Table 2. Zone of inhibition (ZOI) diameter of *Cinnamomum verum* essential oil against *V. parahaemolyticus* (V14)

Test substances	ZOI (mm)
DMSO 10%	0
DOX, 30 µg	9±0.5
Cinnamomum verum essential oil	15±1

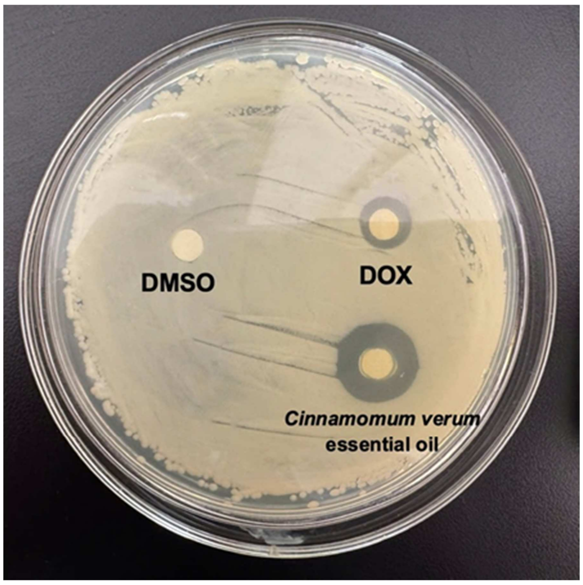

Fig. 1. ZOI diameter of *Cinnamomum verum* essential oil and control antibiotics against *V. Parahaemolyticus*

The cinnamon bark essential oil exhibited a substantial zone of inhibition with a diameter of 15±1 mm, indicating significant antibacterial activity against the resistant *V. parahaemolyticus* strain. In contrast, positive control antibiotic DOX showed a considerably smaller inhibition zone (9±0.5 mm), confirming the resistance of the V14 strain to this antibiotic. The 10% DMSO control showed no inhibition, confirming that the solvent did not contribute to the observed antibacterial effect.

The observed antibacterial activity of cinnamon bark essential oil is consistent with other studies that have reported the antimicrobial properties of this natural product. For instance, Nguyen *et al.* (2020) found that Moringa leaf extract had an inhibition zone diameter of 15.3±0.57 mm, which is comparable to our findings. However, Truong *et al. (*2023) reported a larger inhibition zone (17.9±1.5 mm) for Moringa leaf extract using 96% ethanol as the solvent. These variations in antibacterial activity may be attributed to differences in extraction methods, solvents used, or the specific composition of the plant extracts.

According to the classification by Faikoh *et al.* (2014), an inhibition zone diameter greater than 15 mm is indicative of strong antibacterial activity. Based on this criterion, our results suggest that cinnamon bark essential oil possesses strong antibacterial potential against the tested *V. parahaemolyticus* strain.

The primary compound responsible for the antibacterial activity of cinnamon bark essential oil is believed to be (E)-cinnamaldehyde. Phu *et al.* (2022) identified (E)-cinnamaldehyde as the main component contributing to the antibacterial effects of *Cinnamomum verum* essential oil. This finding is supported by research from Nguyen T. M. H. and Ho. (2017) and Liyanage *et al.* (2017), which demonstrated the antibacterial activity of (E)-cinnamaldehyde in other *Cinnamomum* species. *Cinnamomum verum* is known to have a high concentration of (E)-cinnamaldehyde, which may explain the potent antibacterial efficacy of its essential oil observed in this study.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Given the promising antibacterial activity of *Cinnamomum verum* essential oil against *V. parahaemolyticus*, we proceeded to determine its MIC and MBC. The MIC and MBC values provide crucial information about the concentration of the essential oil required to inhibit and kill the bacterial cells, respectively.

The results presented in Table 3, revealed that the MIC and MBC of cinnamon bark essential oil against the antibiotic-resistant *V. parahaemolyticus* strain V14 were both 12.5 µL/mL.

Table 3. Minimum inhibitory concentration and minimum bactericidal concentration of *Cinnamomum verum* essential oil

Bacteria	MIC (µL/mL)	MBC (µL/mL)	MBC /MIC
V. parahaemolyticus (V14)	12.5	12.5	1

The fact that the MIC and MBC values are identical indicates the strong bactericidal activity of cinnamon bark essential oil against *V. parahaemolyticus*. This is further supported by the

MBC/MIC ratio of 1, which, according to Canillac and Mourey (2001), confirms its bactericidal nature.

Our findings are consistent with previous research demonstrating the bactericidal potential of cinnamon essential oil against various pathogenic bacteria. For example, Hoque *et al.* (2008) reported the strong bactericidal activity of *Cinnamomum cassia* essential oil against foodborne pathogens. The low MIC and MBC values observed in our study suggest that relatively low concentrations of cinnamon bark essential oil can effectively control the growth and survival of antibiotic-resistant *V. parahaemolyticus*. This is a significant finding with potential implications for the development of therapeutic applications in aquaculture.

Overall discussion and implications

The emergence of antibiotic-resistant *V. parahaemolyticus* in aquaculture poses a significant threat to the sustainability of shrimp farming. The findings of this study highlight the potential of *Cinnamomum verum* essential oil as a promising alternative to conventional antibiotics for managing *V. parahaemolyticus* infections in whiteleg shrimp. The essential oil demonstrated significant antibacterial activity, with a large inhibition zone diameter and low MIC and MBC values. These results suggest that cinnamon bark essential oil could be used to develop effective and environmentally friendly treatment strategies to reduce the reliance on antibiotics and mitigate the spread of antibiotic resistance in aquaculture systems.

Further research is warranted to evaluate the *in vivo* efficacy and safety of cinnamon bark essential oil in shrimp. It is crucial to investigate optimal delivery methods, potential toxicity, and the long-term effects of essential oil treatment on shrimp health and the aquaculture environment. Additionally, exploring the synergistic effects of cinnamon bark essential oil with other natural compounds or antimicrobial agents could lead to the development of even more effective treatment strategies.

Conclusion

This study demonstrates the strong antibacterial activity of *Cinnamomum verum* essential oil against multidrug-resistant *V. parahaemolyticus* from whiteleg shrimp (*Litopenaeus vannamei*).

The essential oil showed a significant inhibition zone (15±1 mm) and potent bactericidal effects, with MIC and MBC values of 12.5 µL/mL and an MBC/MIC ratio of 1. These results suggest that *Cinnamomum verum* essential oil is a promising alternative to antibiotics for managing *V. parahaemolyticus* in aquaculture, potentially reducing antibiotic resistance. Further research should focus on optimizing its application and assessing its *in vivo* efficacy and safety.

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