THE UNSTABLE ANTI-VIBRIO ACTIVITY OF GARLIC ESSENTIAL OIL

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(Received: May 05, 2025; Revised: June 12, 2025; Accepted: June 15, 2025)

DOI: 10.31130/ud-jst.2025.23(10B).648E

Abstract - Vibriosis, caused by *Vibrio cholerae* and *Vibrio alginolyticus*, causes mass mortalities and economic losses in aquaculture. With rising antibiotic resistance, sustainable alternatives are needed. This study evaluated garlic essential oil (GEO) for antibacterial activity and stability against these pathogens using agar disk diffusion and broth microdilution assays. GEO showed strong activity, with MICs of 5.6 mg/mL for *V. cholerae* and 1.4 mg/mL for *V. alginolyticus*. In agar well diffusion tests (100 mg/mL), GEO produced 42 mm inhibition zones for *V. alginolyticus* and 3 mm for *V. cholerae*. However, activity declined after 36 hours due to air exposure and storage, indicating volatility. These results highlight GEO's potential as an eco-friendly antibiotic alternative, but its instability requires stabilized formulations. Future studies should focus on delivery systems to enhance practical use in vibriosis control and sustainable disease management in aquaculture.

Key words - Aquaculture; garlic essential oil; *Vibrio cholerae; V. alginolyticus;* unstable anti-bacterial activity

1. Introduction

Aquaculture plays a vital role in global food security by significantly contributing to economic revenue and meeting the increasing demand for seafood. In 2022, global aquaculture production reached approximately 94.4 million tonnes, highlighting its economic and nutritional importance [1]. However, the industry faces substantial challenges from bacterial diseases, particularly vibriosis, which is caused by Vibrio species such as *Vibrio cholerae* and *Vibrio alginolyticus*. Vibriosis is a devastating disease in shrimp aquaculture that can lead to mass mortality and economic losses estimated in the billions of dollars annually. These Gram-negative, halophilic bacteria thrive in marine environments and infect shrimp and other aquatic species through toxin and chitin degradation [2].

The conventional approach to managing vibriosis relies heavily on antibiotics, such as oxytetracycline and fluoroquinolones. However, their widespread and often indiscriminate use has driven the emergence of antibiotic-resistant Vibrio strains, posing risks to environmental health, aquatic ecosystems, and human food safety [3]. Studies have demonstrated that conventional cooking methods, such as grilling or sautéing, are insufficient to completely eliminate tetracycline-resistant microorganisms in the gut of shrimp [4]. Antibiotic resistance genes harbored by bacterial strains in the shrimp gut, including *Vibrio* species, can be transferred via horizontal gene transfer to commensal gut microbiota in humans. This transfer increases the risk of persistent colonization by resistant bacteria in the human gastrointestinal tract, potentially leading to long-term health consequences, including reduced efficacy of antibiotic treatments and increased susceptibility to infections. Consequently, there is an urgent need for sustainable, eco-friendly alternatives to control *Vibrio* infections in aquaculture.

Plant-derived essential oils have emerged as promising candidates due to their potent antibacterial properties, biodegradability, and minimal environmental impact [5]. Garlic (*Allium sativum*) essential oil (GEO), in particular, has garnered attention for its strong antibacterial activity, attributed to organosulfur compounds such as diallyl disulfide and diallyl trisulfide. These compounds disrupt bacterial cell membranes, inhibit enzyme activity, and interfere with quorum sensing, a key virulence mechanism in *Vibrio* species [6]. Despite its potential, commonly essential oil's practical application in aquaculture is hindered by its high volatility, poor water solubility, and susceptibility to oxidative degradation under environmental conditions such as air, light, and heat exposure [7, 8]. These factors lead to a rapid loss of antibacterial activity, limiting essential oils' efficacy in field settings.

While previous studies have demonstrated GEO's antibacterial effects against various pathogens, the stability of its anti-*Vibrio* activity remain poorly understood. Furthermore, the variability in antibacterial assessment methods, such as agar-based versus liquid-based assays, complicates the evaluation of GEO's efficacy due to differences in compound diffusion and bacterial physiology. Addressing these knowledge gaps is critical for developing stable GEO formulations and optimizing their use in aquaculture.

This study aimed to evaluate the antibacterial activity and stability of GEO against *V. cholerae* and *V. alginolyticus* using multiple assessment methods. Specifically, we investigated GEO's potency, its degradation, and the influence of methodological differences on observed antibacterial effects. These findings provide a foundation for developing sustainable, antibiotic-free strategies to mitigate vibriosis in shrimp aquaculture, contributing to environmentally responsible disease management practices.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Two *Vibrio* species, *Vibrio cholerae* and *Vibrio alginolyticus*, were used in this study and provided by Cell Laboratory, Institute of Biotechnolgy, Hue University.

Bacterial strains were maintained on alkaline peptone water (APW) (Himedia) supplemented with 2% agar at 4°C. For experiments, overnight cultures were grown in APW at 30°C with shaking at 200 rpm. Bacterial suspensions were adjusted to an 0.5 McFarland standard, corresponding to approximately 1 × 10^8 CFU/mL.

2.2. Preparation of Essential Oils

All essential oils was obtained commercially (Heber, Natural Life, Vietnam) with a purity of ≥98%. Solubility tests showed that garlic essential oil achieved homogeneity in ethanol and 20% Tween 80, but not in 10% DMSO or 10% Tween 80. In contrast, other essential oils was soluble in all tested solvents, including 10% DMSO, ethanol, and 10% or 20% Tween 80. Consequently, 20% Tween 80 was chosen as the solvent for diluting both GEO and BEO in antibacterial assays to ensure consistency. The essential oils were emulsified in sterile distilled water containing 20% Tween 80 (Sigma-Aldrich, USA) to prepare stock solutions of 100 mg/mL. Solutions were vortexed vigorously and used immidiately after preparation.

The garlic essential oil, sourced from India and identified as *Allium sativum*, was obtained through steam distillation, as specified by the supplier.

For the fresh garlic extract, dried bulbs of *Allium sativum* cultivated in Hai Duong, Vietnam, were used. This cultivar is noted for having the highest allicin content among garlic varieties surveyed in Vietnam [9]. Dried garlic bulbs, peeled and trimmed of roots and stems, were ground using a sterile mortar. The resulting material was filtered by pressing through a sterile muslin cloth to obtain the fresh garlic extract. This crude extract was diluted in 20% Tween 80 at various concentrations for antibacterial activity testing.

2.3. Agar Well Diffusion Assay

The antibacterial activity of essential oils was evaluated using the agar well diffusion method, as described by Balouiri et al. (2016) [10]. Mueller-Hinton agar (MHA; Difco, USA) plates supplemented with 2% NaCl and adjusted pH=8.4 were inoculated with 100 µL of bacterial suspension (1 × 10^8 CFU/mL) spread evenly using a sterile swab. Sterile 6-mm holes punched in MHA agar were filled with 100 µL of essential oils (100 mg/mL). Holes filled with 20% Tween 80 served as negative controls. Plates were incubated at 30°C for 24 h, and the diameter of inhibition zones was measured in millimeters using a digital caliper. Experiments were performed in triplicate.

2.4. Minimum Inhibitory Concentration (MIC) Determination

The MIC of garlic essential oil (GEO) and basil essential oil (BEO) was determined using a broth macrodilution assay, following the Clinical and Laboratory Standards Institute (CLSI, 2018) guidelines [11]. Two-fold serial dilutions of GEO or BEO were prepared in APW containing 20% Tween 80. Each tubes contained 2.5 mL of essential oil dilution and 25 µL of bacterial suspension (~1 × 10^6 CFU/mL). Negative controls (medium only), growth controls (bacteria without essential oil), and solvent controls (20% Tween 80) were included. Tubes were incubated at 30°C for 24 h. Bacterial viability was assessed using a resazurin-based assay in 96-well microtiter plates. Each well, containing 200 µL of bacterial suspension transferred from overnight cultures and treated with varying concentrations of essential oil, was supplemented with 20 µL of resazurin solution (0.015% w/v; Sigma-Aldrich, USA). The plates were incubated at 30°C for 2 h. A color change from blue to pink indicated viable bacteria, while the absence of color change denoted inhibition. The MIC was defined as the lowest concentration preventing a color change. Medium alone and medium containing different concentrations of essential oil were used as negative controls. Experiments were conducted in triplicate.

2.5. Stability of Garlic Essential Oil Under Normal condition after Air Exposure

The stability of GEO's antibacterial activity was assessed following brief air exposure. Vials were briefly opened to initiate air exposure at room temperature (25 ± 2°C) under ambient laboratory lighting (~500 lux). Following air exposure during sampling from the storage container, the GEO vial was sealed with the manufacturer-supplied cap and stored under ambient laboratory conditions (temperature: 25 ± 2°C, relative humidity: 50 ± 10%, lighting: ~500 lux). At 0, 6, 12, 24, and 36 h, samples were collected, and antibacterial activity against *V. cholerae* and *V. alginolyticus* was assessed using the agar well diffusion method and broth macrodilution assay described above. The MIC values were recorded to assess changes in activity over time. Experiments were performed in triplicate.

2.6. Statistical Analysis

Data from the agar well diffusion and MIC assays were expressed as mean ± standard deviation (SD). Differences in inhibition zone diameters and MIC values between 0h and 36h, were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Stability data were analyzed using Two-way ANOVA. Prior to conducting the ANOVA test, the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances were tested indicating that the ANOVA assumptions were satisfied, with $p > 0.05$ for both tests. Statistical analyses were performed using Real Statistics Resource Pack software (Release 8.9.1, Copyright (2013 – 2023), Charles Zaiontz, www.real-statistics.com), with a significance level of $p < 0.05$.

3. Results

The antibacterial activity of garlic (G), lemongrass (L), and basil (B) essential oils against 2 *Vibrio* spp. strains, including *Vibrio cholerae* and *Vibrio alginolyticus*, was evaluated using the agar well diffusion method at 0 h (immediately after opening) and after 36 h of storage at room temperature.

At a concentration of 100 mg/mL, garlic essential oil (G) exhibited the highest activity against *V. alginolyticus,* with an inhibition zone diameter of 42 mm, compared to 3 mm for *V. cholerae*. Lemongrass essential oil (L)

showed inhibition zones of 23 mm (*V. alginolyticus*) and 10 mm (*V. cholerae*), while basil essential oil (B) recorded 16 mm (*V. alginolyticus*) and 17 mm (*V. cholerae*). Basil essential oil maintained stable antibacterial activity over 36 h for both strains, whereas garlic and lemongrass essential oils displayed a significant reduction in activity ($p < 0.05$) after 36 h of storage. Notably, garlic essential oil retained its activity when stored in a dark, airtight container as per the manufacturer's instructions (Figure 1 and Table 1).

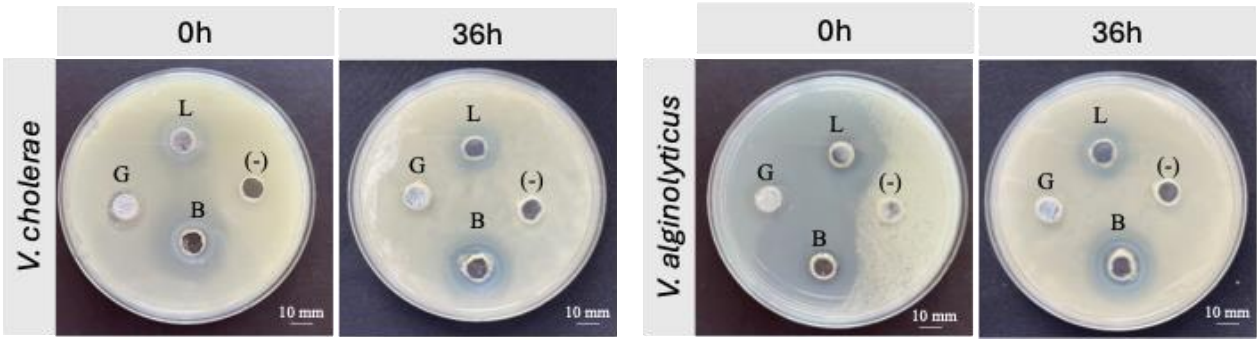

Figure 1. *Inhibition zones of essential oils (100 mg/mL) against V. cholerae and V. alginolyticus using the agar well diffusion method, tested immediately after opening (0 h) and 36 h of storage at room temperature. Wells are labeled as G (garlic essential oil), L (lemongrass essential oil), B (basil essential oil), and (-) (negative control, Tween 80 20%)*

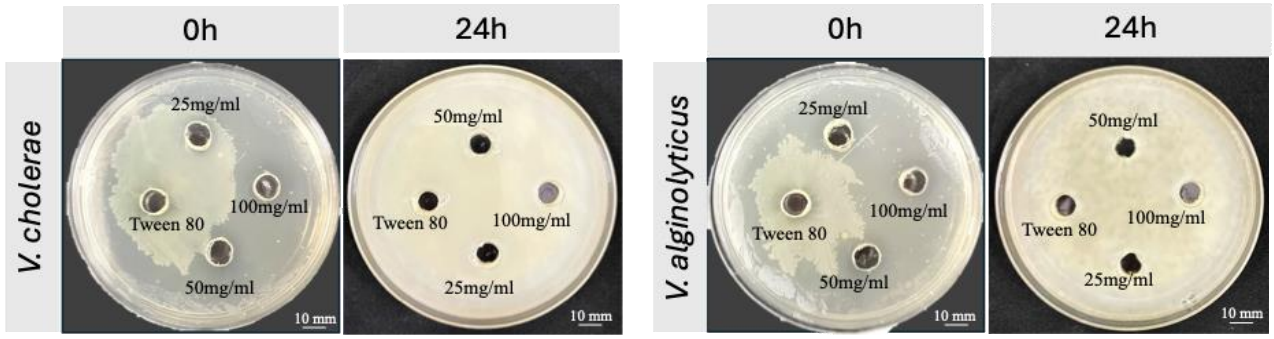

Figure 2. *Inhibition zones of the garlic extract by grinding fresh garlics at 25, 50 and 100 mg/mL against V. cholerae and V. alginolyticus using the agar well diffusion method, tested immediately after collecting (0 h) and 24 h of storage at room temperature. Wells are labeled as different concentration. 20% Tween 80 solution was a negative control*

Table 1. *Antibacterial activity of essential oils (100 mg/mL) on Vibrio spp. by agar well diffusion (0 h and 36h in storage at room temperature)*

Vibrio. spp	Essential oil	0h (mm)	36h (mm)
V. cholerae	Lemongrass	10 ±1	7 ± 1
	Basil**	17 ± 1	11 ± 2
	Garlic*	3 ±1	0
	Fresh garlic extract	27	0 (24h)
	Tween 80 20%	0	0
V. alginolyticus	Lemongrass**	23 ±1	7 ± 1
	Basil	16 ± 2	15 ± 2
	Garlic**	42 ± 2	0
	Fresh garlic extract	35	0 (24h)
	Tween 80 20%	0	0

Mean ± SD (in mm) represents the difference between the inhibition zone diameter and the agar hole size (8 mm), calculated from three replicates.

*$p<0.025$, **$p<0.001$ of Tukey's HSD for interaction test with Bonferroni correction following two-way ANOVA test showed the significant difference between the inhibition zone at 0h and 36h in storage.

To further assess stability, the antibacterial activity of fresh garlic extract was compared to commercial garlic essential oil using the same method (Figure 2). Fresh garlic extract initially exhibited activity comparable to the commercial product, with an inhibition zone observed in the first test. However, no inhibition zone was observed in subsequent tests using the same extract prepared 24 h earlier, confirming the instability of both fresh and commercial garlic essential oils (Figure 2 and Table 1). This instability limits their practical application in inductrial production.

To assess the stability of garlic essential oil (GEO) given its potent antibacterial activity, its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *V. cholerae* and *V. alginolyticus* were determined at 0 h and after 36 h of air exposure. At 0 h, GEO exhibited MIC values of 5.6 mg/mL and 1.4 mg/mL against *V. cholerae* and *V. alginolyticus*, respectively, with corresponding MBC values of 45.0 mg/mL and 22.5 mg/mL (Figures 3 and 4). After 36 h of air exposure, MIC values increased significantly to ≥90 mg/mL, and MBC values exceeded 180 mg/mL for both strains, indicating a substantial loss of inhibitory and bactericidal activity. Time-course MIC analysis revealed a rapid increase in MIC values within 24 h of air exposure, with *V. cholerae* and *V. alginolyticus* showing 16-fold and 32-fold increases, respectively, compared to baseline

(Figure 3C). Notably, repeated opening of GEO containers further exacerbated activity loss. In single-opening experiments, the MIC against *V. alginolyticus* reached 90 mg/mL after 36 h (Figure 3B), whereas multiple-opening experiments resulted in an MIC of 180 mg/mL after the same duration, despite consistent MIC values at 0 h. These findings suggest that repeated air exposure accelerates the oxidative degradation and volatility of GEO's organosulfur compounds, such as diallyl disulfide and diallyl trisulfide, leading to diminished antibacterial efficacy.

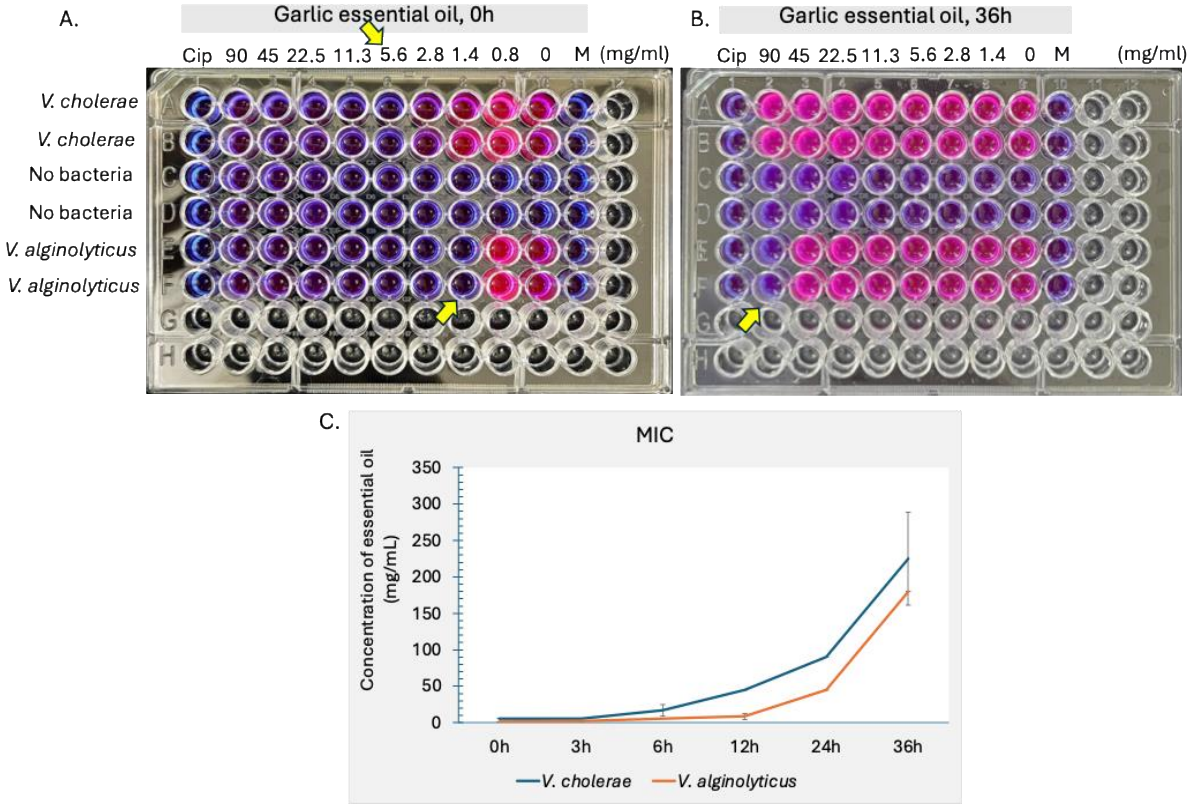

Figure 3. *Determination of MIC of garlic essential oil against* V. cholerae *and* V. alginolyticus *at 0 h and 36 h of storage at room temperature, with Ciprofloxacin (Cip, 1 µg/mL) as the positive control. (A) MIC determination using the resazurin assay, where the lowest concentration showing a color change from pink to blue indicates the MIC (yellow arrows mark the MIC values). Negative controls consisted of medium alone (wells in column M) and medium with garlic essential oil at various concentrations (wells in row No bacteria). (C) MIC determination following the time after open the lid and keep at room temperature. The MIC is the mean of 3 times experiment. Error bars are not visible at 12 and 24 hours due to zero standard deviation in MIC values across replicates*

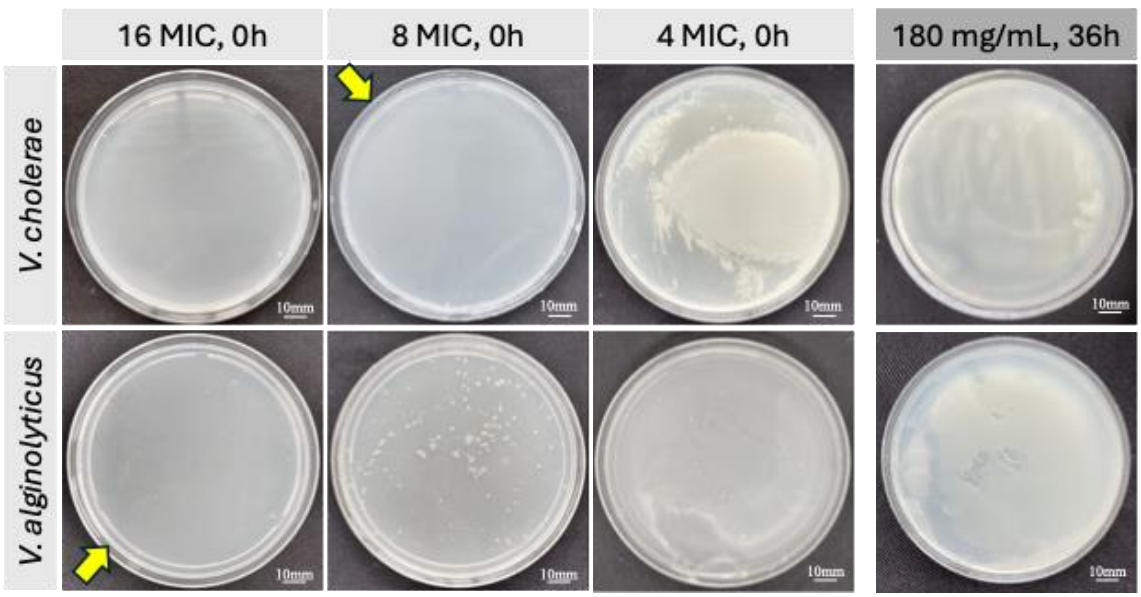

Figure 4*. Determination of MBC of garlic essential oil against* V. cholerae *and* V. alginolyticus *at 0 h and 36 h of storage at room condition*

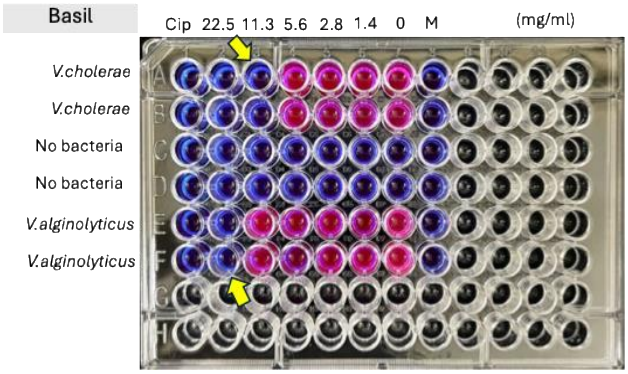

Figure 5. *Determination of MIC of basil essential oil against* V. cholerae *and* V. alginolyticus *at 0 h of storage at room temperature, with Ciprofloxacin (Cip, 1 μg/mL) as the positive control. MIC determination using the resazurin assay, where the lowest concentration showing a color change from pink to blue indicates the MIC (yellow arrows mark the MIC values).* *Negative controls consisted of medium alone (wells in column M) and medium with basil essential oil at various concentrations (wells in row No bacteria)*

4. Discussion

This study highlights the antibacterial efficacy of garlic essential oil (GEO) against *Vibrio cholerae* and *Vibrio alginolyticus*, two significant pathogens in shrimp aquaculture. GEO exhibited robust antibacterial activity, with a minimum inhibitory concentration (MIC) of 5.6 mg/mL against *V. cholerae* and 1.4 mg/mL *V. alginolyticus*. However, its practical application is limited by its instability, as the essential oil lost activity within 36 hours after brief air exposure. This observation aligns with previous reports on the volatility and oxidative degradation of essential oils [7]. The primary bioactive compounds in GEO, diallyl disulfide and diallyl trisulfide, are highly volatile organosulfur compounds prone to evaporation and chemical breakdown, which compromises their long-term efficacy in real-world settings [6].

The study revealed significant methodological differences in assessing antibacterial activity. The agar well diffusion assay, widely used for its simplicity and rapid qualitative screening of antimicrobial activity, measures inhibition zones to infer efficacy [10]. However, this method has limitations, particularly for hydrophobic compounds like essential oils. These compounds often exhibit poor diffusion through aqueous agar due to their lipophilic nature, which is essential for their interaction with bacterial membranes or intracellular targets [12]. In this study, GEO produced a modest inhibition zone of approximately 3 mm against *V. cholerae*, significantly smaller than the 17 mm observed for basil essential oil (BEO) at the same concentration (Figure 1). Conversely, MIC determination using a liquid culture assay with resazurin-based viability detection revealed that GEO was more potent than BEO, with an MIC value approximately twofold lower (Figures 3A and 5). GEO primarily contains sulfur-containing compounds such as allicin, diallyl disulfide, and diallyl trisulfide, characterized by thiosulfinate (-S(O)-S-) and disulfide (-S-S-) functional groups, respectively [13]. These groups confer high hydrophobicity due to their low polarity and limited ability to form hydrogen bonds with the aqueous agar matrix, thus restricting diffusion in the disk diffusion assay. In contrast, BEO contains compounds such as linalool, eugenol, and methyl chavicol, which possess hydroxyl (-OH) and methoxy (-OCH3) functional groups [14]. The hydroxyl groups in linalool and eugenol enable hydrogen bonding with water molecules in the agar, enhancing solubility and diffusion. Methyl chavicol, while less polar due to its methoxy group, still exhibits greater compatibility with aqueous environments compared to GEO's sulfur compounds. These differences in functional group interactions with the aqueous agar matrix account for BEO's better diffusion and observed antibacterial efficacy in the disk diffusion assay compared to GEO.

The notably larger inhibition zone observed for garlic essential oil (GEO) against *Vibrio alginolyticus* (42 mm) at 100 mg/mL in the agar well diffusion assay may be influenced by the superior motility of *V. alginolyticus*. Unlike *V. cholerae*, which relies solely on a polar flagellum, *V. alginolyticus* possesses both polar and lateral flagella, regulated by a sophisticated chemotaxis signaling system that enhances its navigational efficiency in complex environments [15]. Recent studies utilizing a multiscale 3D chemotaxis assay have demonstrated that *V. alginolyticus's* lateral flagella improve chemotactic performance in mechanically diverse settings, such as polymer solutions and hydrogels, by modulating drift speed and behavioral responses to chemical gradients [15]. This enhanced motility, coupled with swarming behavior, likely enables *V. alginolyticus* to rapidly move away from high concentrations of GEO's bioactive compounds, such as diallyl disulfide, in the agar matrix. Consequently, the observed inhibition zone may overestimate GEO's antibacterial efficacy against *V. alginolyticus* due to motility-driven dispersion, introducing potential measurement errors. These findings highlight the need for liquid-based assays, such as broth microdilution, to accurately assess GEO's antibacterial activity against motile pathogens like *V. alginolyticus*, as agar-based methods may be confounded by chemotactic and swarming behaviors. These findings underscore the limitations of the agar diffusion assay for both well and disk assay.

5. Conclusion

This study demonstrates the potent antibacterial activity of garlic essential oil (GEO) against *Vibrio cholerae* and *Vibrio alginolyticus*, two major pathogens in seafood aquaculture. GEO exhibited a minimum inhibitory concentration (MIC) of 5.6 mg/mL and 1.4 mg/mL, respectively. However, the rapid loss of GEO's antibacterial activity within 36 hours of brief air exposure highlights its instability. These results highlight GEO's potential as a sustainable, eco-friendly alternative to antibiotics for controlling vibriosis in shrimp aquaculture. However, its stability and solubility challenges necessitate the development of stabilized formulations, such as nanoencapsulation or emulsification systems, to enhance its efficacy and longevity in aquatic environments. By addressing these challenges, GEO could contribute to

antibiotic-free disease management strategies, supporting sustainable aquaculture practices and global food security.

Acknowledgment: This work was supported by The University of Danang – University of Science and Technology, code number of Project: T2024-02-35.

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