INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print); 2222-5234 (Online)
Website: https://www.innspub.net
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Vol. 27, Issue: 1, p. 1-7, 2025

RESEARCH PAPER OPEN ACCESS

In vitro Study of garlic extract's inhibitory effect on *Vibrio parahaemolyticus*: A potential alternative to antibiotics in aquaculture

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Key words: Garlic extract, *Allium sativum, Vibrio parahaemolyticus,* Antibacterial activity, Aquaculture

DOI: https://dx.doi.org/10.12692/ijb/27.1.1-7 Published: July 02, 2025

ABSTRACT

This study investigates the antibacterial activity of garlic extract (*Allium sativum*) against *Vibrio parahaemolyticus* using the agar well diffusion method. The results show that garlic extract effectively inhibits bacterial growth, forming a zone of inhibition (ZOI) with an average diameter of 21.6±4.3 mm. The minimum inhibitory concentration (MIC) was determined to be 0.15625 g/mL, while the minimum bactericidal concentration (MBC) was 0.625 g/mL. The antibacterial effect of garlic extract is attributed to bioactive compounds, particularly allicin, which disrupt bacterial membranes, inhibit DNA synthesis, and interferes with quorum sensing. These findings suggest that garlic extract has significant potential as a natural antibacterial agent against *V. parahaemolyticus*, offering a possible alternative to synthetic antibiotics in aquaculture. Further research should focus on optimizing extraction methods, improving compound stability, and conducting *in vivo* studies to assess its practical applications.

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INTRODUCTION

Bacterial infections caused by *V.parahaemolyticus* pose significant challenges in aquaculture and food safety. This opportunistic pathogen is commonly found in marine and estuarine environments and is a major cause of gastrointestinal illnesses in humans due to contaminated seafood consumption (Schmidt *et al.*, 2000). In aquaculture settings, *V. parahaemolyticus* contributes to severe disease outbreaks, leading to economic losses (Mishra, 2017). The increasing prevalence of antibiotic-resistant strains further complicates disease management, emphasizing the urgent need for alternative antibacterial strategies that are both effective and environmentally sustainable (Schmidt *et al.*, 2000).

Garlic (*A. sativum*) has been widely recognized for its potent antimicrobial properties, attributed to its rich content of bioactive organosulfur compounds, particularly allicin. Allicin exerts its antibacterial effects through multiple mechanisms: disruption of bacterial membrane integrity, inhibition of key metabolic enzymes like DNA gyrase, and interference with quorum-sensing mechanism Piyanut *et al.*, 2020. These diverse modes of action make garlic a promising candidate for combating antibiotic resistance. While previous studies have demonstrated the efficacy of garlic extract against various bacterial pathogens, including both Gram-positive and Gram-negative species (Sudalay *et al.*, 2013), comprehensive research on its activity against *V. parahaemolyticus*, specifically its MIC and MBC, remains limited.

This study aims to evaluate the antibacterial activity of garlic extract against *V. parahaemolyticus* through *in vitro* assays, specifically focusing on determining the inhibition zone, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) (Balouiri *et al.*, 2016). We hypothesize that garlic extract will effectively inhibit the growth of *V. parahaemolyticusin vitro*.The findings from this study will contribute to the development of natural antibacterial agents, potentially serving as an alternative to conventional antibiotics in controlling *V. parahaemolyticus* infections in both food safety and aquaculture contexts.

MATERIAL AND METHODS

Preparation of garlic extract

The garlic extract (*A. sativum*) was prepared using a manual extraction method. Fresh garlic cloves were cleaned thoroughly with distilled water and then finely ground using a porcelain mortar and pestle. The resulting mixture was filtered through multiple layers of cloth to remove solid residues. The obtained extract was then stored at 4°C to maintain its stability and bioactivity (Muhamad *et al.*, 2024).

This method was subsequently refined to optimize the extraction process and enhance the yield of the garlic extract. Specifically, fresh garlic was cleaned, peeled, and finely homogenized with distilled water at a ratio of 10 g/ml to ensure a uniform mixture. The resulting homogenate was then subjected to a filtration process using multiple layers of muslin cloth to effectively remove solid residues, including cell debris and unwanted impurities, yielding a highly purified extract. The final filtrate was designated as a 100% stock solution and was used directly in subsequent experiments without requiring additional processing steps.

Preparation of bacterial suspension

The *Vibrio parahaemolyticus* strain (ATCC 11218) was obtained from Can Tho University for this study. The bacteria were cultured in Tryptic Soy Broth (TSB) at 37°C for 24 hours to reach the stationary phase, achieving a density of approximately 10^8 CFU/mL. The culture was then diluted 100-fold with 0.85% sodium chloride solution to obtain a final concentration of 10^6 CFU/mL. The prepared suspension was used immediately or stored under appropriate conditions to ensure stability during experiments.

Antibacterial activity assay

The antibacterial efficacy of garlic extract (*A. sativum*) against *V. parahaemolyticus* was assessed using the agar well diffusion method, as described by Balouiri *et al.,* 2016.

A volume of 20 mL of sterile Tryptic Soy Broth (TSB) medium was poured into sterile Petri dishes and solidified. Subsequently, 100 µL of *V. parahaemolyticus* suspension at a concentration of 10^8 CFU/mL was evenly spread onto the agar surface using sterile swabs. Wells of 10 mm diameter were aseptically created using a sterilized cork borer. Each well was filled with 100 µL of 10 g/mLgarlic extract and applied under sterile conditions using a micropipette. As a negative control, 100 µL of sterile distilled water was introduced into a separate well.
The prepared plates were initially incubated at 4°C to facilitate extract diffusion, followed by incubation at 37°C for 24 hours.

The experiment was performed in six independent replicates to ensure reproducibility. The antibacterial activity was quantitatively evaluated by measuring the diameter of the inhibition zones around each well. The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guideline (Hindler and Richter, 2016).

Preparation of garlic extracts concentration and stock solution

In this study, garlic extract was prepared at a standardized concentration of 10g/mL for antibacterial assays. The extraction process involved finely crushing 1 g of fresh garlic and mixing it with 10 mL of sterile distilled water. The mixture was then filtered through a fine cloth to remove solid residues, obtaining a clear garlic extract.

All preparation steps were conducted under sterile conditions to prevent contamination and ensure reproducibility across experimental trials. The obtained garlic extract was stored at 4°C until use in antibacterial assays.

Preparation of 0.015% resazurin solution

To prepare a 0.015% resazurin solution, 15 mg of resazurin powder was dissolved in 100 mL of sterile distilled water. The solution was subsequently sterilized through filtration using a 0.22 µm membrane filter to eliminate potential contaminants. The filtered solution was stored at 4°C in a light-protected container to maintain its stability and prevent photodegradation. The prepared resazurin solution was utilized within two weeks for antibacterial assays, following the standardized methodology outlined by Elshikh *et al.,* 2016.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC of garlic extract against *V. parahaemolyticus* were determined using a 96-well microdilution assay, following the method described by Elshikh *et al.,* 2016. The experiment was conducted in a total volume of 200 µL per well, with three replicates for each concentration.

To prepare the test solutions, serial two-fold dilutions of garlic extract were prepared in sterile distilled water to achieve the desired concentrations. A negative control well contained only 200 µL of sterile 0.85% sodium chloride, while a positive control well contained 100 µL of Tryptic Soy Broth (TSB) and 100 µL of bacterial suspension at CFU/mL. Test wells contained 100 µL of the garlic extract solution and 100 µL of bacterial suspension.

The microplates were incubated at 37°C for 24 hours. Following incubation, 20 µL of 0.015% resazurin solution was added to each well, and the plates were further incubated for 2-4 hours. MIC was determined as the lowest concentration of garlic extract at which no colour change occurred, indicating complete inhibition of bacterial growth (Elshikh *et al.*, 2016).

For MBC determination, 50 µL of the bacterial suspension from wells corresponding to the MIC and two higher concentrations were spread onto Brain Heart Infusion Agar (BHIA) plates and incubated at 37°C for 24 hours. MBC was defined as the lowest concentration at which no bacterial colonies were observed. Each experiment was performed in triplicate to ensure accuracy and reproducibility.

Data Analysis

The collected data were statistically analyzed to assess the antibacterial efficacy of garlic extract against *V. parahaemolyticus*. The diameter of the inhibition zones was measured and expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc testwas performed to determine statistically significant differences among the tested concentrations, with a significance level set at 95% ($p<0.05$).

All statistical analyses were conducted using Minitab 18 software. The results were graphically represented to illustrate variations in inhibition zones, minimum inhibitory concentrations (MIC), and minimum bactericidal concentrations (MBC). Each experiment was performed in triplicate to ensure reproducibility and reliability of the findings.

RESULT AND DISCUSSION

The formation of an inhibition zone around the well-containing garlic extract indicates its ability to inhibit the growth of *V. parahaemolyticus* (Table 1).

This result confirms that garlic extract exhibits significant antibacterial activity, although it remains lower than the positive control.

Table 1. Inhibition zone diameter of experimental samples.

Experimental sample	Inhibition zone diameter (mm)
Negative control (distilled water)	0
Positive control (Tetracycline30 µg)	31.67±3.33
Garlic extract (10 g/mL)	21.6±4.3

The results of this study demonstrate that garlic extract has antibacterial activity against *V. parahaemolyticus*, with an average inhibition zone diameter of 21.6±4.3 mm (Figure 1). In a related study, Vu *et al.* (2023) reported that garlic essential oil exhibited a significantly larger inhibition zone, measuring 80±0 mm using fumigation. These findings indicate that different extraction methods influence the antibacterial efficacy of garlic, likely due to variations in the concentration and bioavailability of its active compounds. Compared to the study by Muhamad *et al.* 2024, the inhibition zone diameter in the present study was slightly smaller.

This variation may be attributed to differences in extract concentration in undiluted garlic extract at full strength, resulting in a larger inhibition zone (23.0±0.07 mm). In contrast, the present study employed a diluted extract with a 10g/mLratio of garlic to distilled water, which may have reduced the concentration of bioactive compounds, thereby leading to a lower antibacterial effect.

Additionally, the inhibition zonesize exhibited considerable variation (21.6±4.3 mm). This variability may be due to the uneven distribution of antibacterial compounds within the agar medium or differences in the sensitivity of individual bacterial cells. Muniesa *et al.* (2017) have also reported that variations in dilution techniques, bacterial colony age, and culture conditions could influence antibacterial assay outcomes.

When compared to the positive control (31.67±3.33 mm), the antibacterial activity of garlic extract was lower. This suggests that while garlic possesses antibacterial properties, its efficacy is not as potent as synthetic antibiotics or standard antimicrobial agents. However, the advantage of garlic extract lies in its natural origin, reduced risk of adverse effects, and potential application in aquaculture as an alternative to synthetic antibiotics.

Garlic (*Allium sativum*) exhibits antibacterial activity through multiple mechanisms, primarily via its organosulfur compound, allicin. Allicin has been shown to inhibit bacterial DNA gyrase, an essential enzyme for DNA replication, at concentrations comparable to the quinolone antibiotic nalidixic acid

(Reiter *et al.*, 2020). This inhibition occurs through the oxidation of a specific cysteine residue in the GyrA subunit of DNA gyrase, disrupting bacterial DNA synthesis and cell proliferation (Reiter *et al.*, 2020). This mechanism parallels the action of fluoroquinolones like ciprofloxacin, which also target DNA gyrase. Furthermore, garlic extract interferes with the bacterial cell wall by disrupting peptidoglycan cross-linking, similar to the effect of β-lactam antibiotics such as ampicillin. Studies have observed cell wall lysis in bacteria treated with garlic extract (Booyens *et al.*, 2014), and allicin has demonstrated a strong interaction with Penicillin-Binding Protein 3 (PBP3) in MRSA, suggesting an inhibition of peptidoglycan synthesis (Indira *et al.*, 2024). The dual action of garlic extract, targeting both DNA replication and cell wall integrity, underscores its potential as an alternative antimicrobial agent.

Table 2. Minimum inhibitory concentration (g/mL) and minimum bactericidal concentration (g/mL) for garlic extract against *V. parahaemolyticus*.

Experimental sample	MIC	MBC
Negative control (distilled water)	-	-
Positive control (Tetracycline)	+	+
Garlic extract	0.15625	0.625

Note: - The "-" symbol indicates that the sample exhibited no inhibitory or bactericidal activity at any tested concentration; The "+" symbol indicates that the antibiotic effectively inhibited and eliminated bacteria across all tested concentrations.

These findings highlight garlic extract as a promising natural antimicrobial agent with multiple inhibitory pathways, suggesting its potential application in controlling Gram-negative bacterial infections, particularly those caused by *V. parahaemolyticus*. The antibacterial activity of garlic extract (*A. sativum*) against *V. parahaemolyticus* was provided by Can Tho University, demonstrating a minimum inhibitory concentration (MIC) at the sixth dilution, equivalent to 0.15625 g/mL (Table 2). This result aligns with the broad-spectrum antimicrobial properties of garlic, as reported by Ramadhaniah *et al.* (2023). The study elucidated several mechanisms by which garlic exhibits its bacteriostatic and bactericidal effects.

Furthermore, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of garlic extract in this study were determined to be 0.15625 g/mL and 0.625 g/mL (Table 2), respectively. The MIC represents the lowest concentration of garlic extract required to inhibit the growth of *V. parahaemolyticus*, whereas the MBC is the minimum concentration necessary to eliminate the bacteria. The relatively small difference between MIC and MBC suggests that garlic extract exhibits both bacteriostatic and bactericidal properties. The MBC value of 0.625 g/mL was identified as the minimum concentration needed to completely eradicate *V. parahaemolyticus* in this study. This result aligns with the findings of (Vo *et al.*, 2021)observed an MBC range of 0.023% - 0.09 %for garlic extract when tested against *Vibrio* spp., reinforcing the reliability of the present study's findings.

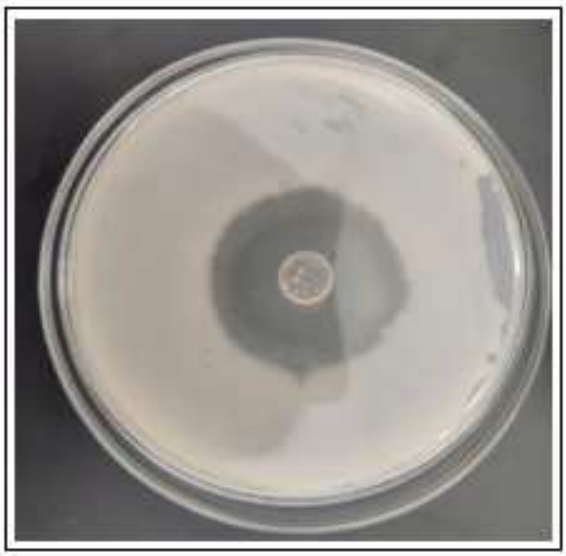

Fig. 1. Diameter of the inhibition zone of garlic extract against *Vibrio parahaemolyticus* of garlic extract at a concentration of 10 g/mL.

The bactericidal mechanism of garlic extract is primarily attributed to the presence of allicin, a sulfur-containing compound that disrupts bacterial cell membrane integrity. According to Bakri and Douglas (2005), allicin damages bacterial cell membranes, leading to leakage of intracellular components, disruption of osmotic balance, and eventual cell death. Furthermore, Maffei *et al.* (2011) elucidated the pharmacological properties of volatile plant compounds, including allicin, highlighting their potential antimicrobial effects.

These applications could help reduce reliance on synthetic antibiotics. Therefore, further research is necessary to optimize extraction methods, assess the stability of active compounds, and conduct *in vivo* trials in aquaculture systems.

CONCLUSION

This study demonstrates that garlic extract (*A.sativum*) exhibits significant antibacterial activity against *V. parahaemolyticus in vitro*. The MIC and MBC values obtained suggest its potential as a natural alternative to antibiotics in aquaculture. However, further research, including *in vivo* studies, is crucial to validate these findings and assess the practical applicability of garlic extract in controlling *V. parahaemolyticus* infections.

RECOMMENDATIONS

To enhance garlic extract's antimicrobial application against *Vibrio parahaemolyticus*, researchers should optimize extraction to preserve bioactive compounds, especially allicin. Future studies should clarify its antibacterial mechanisms for targeted therapies.

Exploring its synergy with antibiotics may boost efficacy and reduce resistance. *In vivo* trials in aquaculture are essential, as is assessing its potential as a natural food preservative.

For commercial use, safety, toxicity, and stability must be evaluated. Standardized formulations, such as antibacterial coatings or encapsulated systems, could enhance its applications in aquaculture and food safety.

ACKNOWLEDGMENT

The authors express their sincere gratitude to Ho Chi Minh City University of Education for its support. This research is funded by Ho Chi Minh City University of Education Foundation for Science and Technology.

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