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Combination Therapy: Investigating the Combined Effects of *Zingiber officinale* and Azithromycin against *Vibrio cholerae*

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Abstract

Multi-drug resistant *Vibrio* species pose a significant global health threat, particularly in developing countries. The genes responsible for this resistance are often plasmid-encoded, making it a pressing concern. This study aimed to evaluate the antibacterial effect of *Zingiber officinale* (ginger) extract on various strains of *Vibrio* species isolated from stream samples. Stream samples were screened for *Vibrio* species using standard microbiological techniques. The phytochemical constituents of *Zingiber officinale* were quantitatively determined using gravimetric and spectrophotometric methods. The antibacterial activity was assessed using agar-well diffusion method. The study isolated three strains of *Vibrio cholerae*: VCC6, VCP2, and VCE7, with VCP2 being the most prevalent. *Zingiber officinale* extract exhibited significant ($p \leq 0.05$) antibacterial activity against all strains, which was further enhanced when combined with Azithromycin (AZR). Notably, the extract showed the most pronounced activity against VCE7. The findings suggest that *Zingiber officinale* extract possesses potent antibacterial properties against Vibrio species, making it a potential adjunct therapy to conventional antibiotics like Azithromycin.

Keywords: *Zingiber officinale, Vibrio species, antibacterial activity, multidrug resistance, phytochemical analysis*

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Introduction

The history of medicine is deeply intertwined with human civilization, with plant-based remedies dating back to ancient times (Gyawali and Ibrahim, 2013). Plants have been used therapeutically for various ailments, with ancient civilizations in China, India, Egypt, and Greece employing herbal remedies long before the advent of microbiological sciences. The extensive use of antibiotics has contributed to antimicrobial resistance (AMR), a pressing global public health concern (Abimbola *et al.*, 2013).

Plant extracts have been used to treat infectious diseases across cultures (Sofowora et al., 2012). Many plants are rich in antimicrobial constituents, and their extracts are widely used to combat bacterial and fungal infections (Rahmani *et al*., 2014). Ginger (*Zingiber officinale*), a medicinal and culinary plant, has been traditionally used to treat various ailments, including infectious diseases (Ali, 2008). Research has demonstrated its direct antimicrobial activity, suggesting its potential in treating bacterial infections (Zick *et al*., 2008).

The rise of antibiotic-resistant bacteria due to improper drug use has led to increased prevalence of difficult-to-treat infections. New and effective antimicrobial agents are therefore in high demand. This study aims to evaluate the inhibitory potential of *Zingiber officinale* extract against *Vibrio cholerae*, a formidable waterborne pathogen.

Vibrio cholerae is a Gram-negative, facultatively anaerobic, curved rod-shaped bacterium that causes cholera, a severe gastrointestinal disease primarily affecting populations in low-resource settings. It is typically transmitted via contaminated water and seafood. *V. cholerae* has been isolated from diverse environmental samples including seawater, sediments, plankton, and shellfish (Siggh *et al.*, 2011; Nasreldin et al., 2014).

This study aims to evaluate the antibacterial properties of ginger ethanolic extract against *Vibrio cholerae*, a waterborne pathogen. With the growing issue of multidrug-resistant bacteria, there is a need for novel, effective, and affordable antimicrobials (Martino *et al*., 2002). This research investigates the inhibitory potential of *Zingiber officinale* extract against *Vibrio cholerae*, exploring its potential as an alternative antimicrobial agent.

Materials and Methods

Isolation and Characterization of Test Isolates

Sample collection, handling and transportation: The samples used for this study were drawn from the rivers. A total of 100 freshwater samples were collected from five different streams used in Uli community. Samples were taken from twenty different sites, each site in triplicates. The stream samples were collected with sterile containers. The containers were thoroughly washed with detergent, rinsed with water, and then rinsed with 70% ethanol and final rinsed three times with distilled water. The containers were placed inverted in order to drain the water inside them. The container was inverted and lowered 5 cm below the river water sample, then placed vertically for the water sample to refill the sample container. This sample was covered immediately and kept in a cooler containing ice block, and this transported to the laboratory for immediate analysis.

Isolation of organisms: One milliliter (1.0 ml) water sample was aseptically transferred into a sterile test tube (Pyrex) containing 9.0 ml of the diluents (sterile normal saline), and from this, ten-fold serial dilutions were made up to 10^{-3}. One milliliter of the diluted sample (10^{-3}) was plated on Petri dishes containing Thiosulphate Citrate Bile Sucrose agar medium (TCBS/Biotech) using the pour plate method. All the plates in triplicate were incubated inverted at 37˚C for 24-48 hours.

Characterization and identification of the isolates

The isolates were sub cultured on nutrient agar (Biotech), incubated in inverted position at 37˚C for 24 hours. The isolates were characterized and identified using their colonial and morphological descriptions as described in the study published by Iheukwumere *et al*. (2018), Iheukwumere *et al*. (2025a), Iheukwumere *et al*. (2025b) biochemical reactions as described in the study published by Iheukwumere *et al*. (2020) and molecular characterization as described in the study published by Gabriela *et al*. (2014). The colonial description was carried out to determine the colours of the isolates on agar media plates, their sizes, edges, consistencies and optical properties of the isolates.

Preparation and extraction of the plant material

Preparations of ginger powder: The first step for extraction involved the preparation of dry powder from ginger rhizomes. For this, fresh gingers were purchased from the local market of Uli in Ihiala L.G.A of Anambra state, Nigeria. The Fresh ginger rhizomes were washed, peeled, sliced, shadow dried and dried in hot oven at 70° C. The dried ginger samples were ground into powder form using a sterile electric grinder and sieved to give a powdery form.

Ginger extracts preparation: This was done using the method published by Iheukwumere *et al*. (2025c) and Ekesiobi *et al*. (2017). Firstly, 50 grams of ginger powder was weighed into a 1000 mL conical flask (Pyrex) and then 50 mL of 99 % ethanol was added. This was thoroughly shaken and then made up to 500 mL using absolute ethanol. The flasks were incubated at room temperature for 72 hours with intermittent manual shaking. The crude extracts were filtered with Whatman No. 1filter paper. The extracts were concentrated using a rotary evaporator at 78℃ (Iheukwumere and Umedum, 2013).

Phytochemical analysis of the plant extracts

The phytochemical components (alkaloids, glycosides, flavonoids, phenolics, tannins, steroids and saponins) of the plant extracts were determined quantitatively using the methods described by Iheukwumere and Umedum (2013), Abiodum *et al*. (2024a), Iheukwumere *et al*. (2025d) and Abiodum *et al*. (2024b).

Alkaloids: Five milliliters of the sample was mixed with 96% ethanol and 20% tetraoxosulphate (VI) acid (1:1). One milliliter of the filtrate from the mixture was added to 5 ml of 60% tetraoxosulphate (VI) acid and allowed to stand for 5 minutes. Then, 5 ml of 0.5% formaldehyde was added and allowed to stand for 3 h. The reading was taken at an absorbance of 550 nm.

Glycosides: This was carried out using Buljet's reagent. One gram of the fine powder of the sample was soaked in 10 ml of 70% alcohol for 2 h and then filtered with Whatman No. 1 filter paper. The extract was then purified using lead acetate solution and disodium hydrogen tetraoxosulphate (VI) solution before the addition of freshly prepared Buljet's reagent. The absorbance was taken at of 550 nm.

Flavonoids: Five milliliters of the extract was mixed with 5 ml of dilute hydrochloric acid and boiled for 30 minutes. The boiled extract was allowed to cool and then filtered with Whatman No. 1 filter paper. One milliliter of the filtrate was added to 5 ml of ethyl acetate and 5 ml of 1% ammonia solution. The absorbance was taken at 420 nm.

Tannin: Ten milliliters was pipetted into 50 ml plastic containing 50 ml of distilled water. This was mixed for 1 h on a sterile mechanical shaker. The solution was filtered with Whatman No. 1 filter paper, and 5 ml of the filtrate was mixed with 2 ml of iron (III) chloride solution in 0.1 N hydrochloric acid. The absorbance was taken at 550 nm.

Steroids: The extract was eluted with normal ammonium hydroxide solution. Two milliliters of eluate was mixed with 2 ml of chloroform in a test tube. Three milliliters of ice cold acetic anhydride was added to the mixture and allowed to cool. The absorbance was taken at 420 nm.

Saponins: Five milliliters of the sample was dissolved in aqueous methanol. The 0.25 ml of aliquot was taken for spectrophotometric determination for total saponins at 544 nm.

In Vitro Antibacterial Activity

Preparation of the inhibitory substance for *in vitro* antibacterial susceptibility Tests

In this study the concentration of 100 mg/ml of the extract was used to screen for the antibacterial activity. This was carried out using the modified method described in the study published by Iheukwumere *et al*. (2018). Here, 2.5 g of the extract was dissolved in 25.0 ml of peptone water. Similarly equal concentration of the antibiotic was prepared, and then equal volume of the extract and antibiotic were mixed, and this was used for the study

In vitro antibacterial susceptibility test

This was carried out using the method described in the study published by Iheukwumere *et al*. (2018). Each labeled plate was uniformly inoculated with the test organism using pour plate method. A sterile cork borer of 5 mm diameter was used to make the wells on the medium. One tenth millilitre of the inhibitory substance was dropped into each labeled wells and then incubated at 37±2ºC for 24 h. Antibacterial activity was determined by measuring the diameter of the zones of inhibition (mm) produced after incubation

Statistical Analysis

The results of the data generated were expressed as mean, percentage and Table; Data were analyzed by two-way Analysis of Variance (ANOVA) to determine the significance of the main effects and interactions at 95 % confidence level. Pair wise comparison of mean was done by Student "t" test as described in the study published by Iheukwumere *et al* (2018), Ekesiobi (2025), Iheukwumere *et al*. (2025e), Abiodum *et al*. (2024c).

Results

The cultural and morphological characteristics of the isolates are shown in (Table 1). The study revealed that the isolates similar appearance on Thiosulfate citrate-bile salts-sucrose agar, similar Elevation, Edge and surface and also similar morphological characteristics on string test, Gram reaction, endospore, capsule and motile nature. The biochemical characteristics of the isolates revealed that the isolates were hydrogen sulphide production, methyl red, urease, arabinose, dulcitol negative as Shown in (Table 2). The isolates differ in their variation in utilization of sugar. They were all catalase, citrate, gelatin, oxidase, glucose and galactose positive but differ in their abilities to utilize inositol, xylose, sorbitol and lactose. The nucleic acid extracted from the isolates showed the ratio of their absorbances of wavelength of 260 nm and 280 nm using Nanodrop was at the range of 1.80 - 1.90, and this confirmed that the nucleic acids were DNA as shown in (Table 3). The molecular identities of the isolates revealed that isolate L, M and N were V*ibrio cholerae* 01 biovor EITor strain C6709 (VCC6), V*ibrio cholerae* 01 biovor EITor strain P27459 (VCP2) and V*ibrio cholerae* 01 biovor EITor E7946 (VCE7) as shown in (Table 4). The quantitative phytochemical constituents of the plant extract revealed the presence of alkaloids, phenolics, flavonoids, saponins, tannins, cardiac glycosides and steroids as shown in Table 5. It was also observed that alkaloids were mostly detected whereas steroids were detected least in the study. The study revealed that the plant extract showed significant activity against the *vibrio species*, and the activity was most against VCP2 as shown in Table 6. It was also observed that the activity of the plant extract was significant ($p < 0.05$) boosted with an Antibiotic (Azithromycin). The study also showed that there was synergistic activity between the extract and the Antibiotic as the activity of the combination increased more than the activity of either of the inhibitory substances.

Table 1: Cultural and morphological characteristics of the isolates.

Parameter	L	M	N
Appearance on TCBS	Yellow	Yellow	Yellow
Edge	Smooth	Smooth	Smooth
Elevation	Raised	Raised	Raised
Surface	Smooth	Smooth	Smooth
String Test	+	+	+
Gram reaction	-	-	-
Shape	Rods/Comma	Rods/Comma	Rods/Comma
Endospore	-	-	-
Capsule	-	-	-
Motility	+	+	+

Table 2: Biochemical characteristics of the isolates

Parameter	L	M	N
Catalase	+	+	+
Citrate	+	+	+
Gelatin	+	+	+
H2S	-	-	-
Methylred	-	-	-
Oxidase	+	+	+
Urease	-	-	-
Arabinose	-	-	-
Glucose	+	+	+
Galactose	+	+	+
Inositol	-	+/-	-
Dulcitol	-	-	-
Xylose	+/-	-	+/-
Sorbitol	-	+/-	-
Ketose	+/-	-	+/-

Table 3: Verification of the extracted nucleic acids

Sample ID	Conc (Ng/ml)	26nm	280nm	260/280
L	121.20	3.0120	1.6194	1.86
M	125.70	3.1082	1.6801	1.85
N	132.80	3.2110	1.7643	1.82

Table 4: Molecular identities of the isolates

Parameter	L	M	N
Max Score	5686	5686	5686
Total Score	7295	7295	7295
Ovary Cover (%)	100	100	100
E-Value	0.0	0.0	0.0
Identity (%)	100	100	100
Accession Length	1070351	1073537	1071008
Accession Number	Cp047298	Cp047300	Cp047304
Description	*Vibrio cholerae* 01biovar EITor strain C6709 (VCC6)	*Vibrio cholerae* 01biovar EITor strain P27459 (VCP2)	*Vibrio cholerae* 01biovar EITor strain E7946 (VCE2)

Table 5: Phytochemical constituents of *Zingiber officinale* rhizome extract

Parameter	Value (g/100g)
Alkaloids	6.20 ± 0.21
Phenolics	1.21± 0.01
Flavonoids	5.41± 0.14
Tannins	4.56± 0.17
Saponins	0.92 ± 0.01
Glycosides	1.02 ± 0.01
Steroids	0.02 ± 0.00

Table 6: Antibacterial activity

	Diameter Zone of inhibition [x ± SD] mm		
Inhibitory substance	VCC6	VCP2	VCE7
EEZ	8.80 ± 0.14	9.33 ± 0.67	8.20 ± 0.17
AEZ	6.77 ± 0.21	7.10 ± 0.08	6.20 ± 0.03
CPX	14.00 ± 017	17.30 ± 0.82	14.56 ± 0.07
AZR	15.80 ± 0.22	18.80 ± 0.82	16.70 ± 0.11
EEZ + AZR	17.10± 0.11	26.10 ± 0.14	21.22±0.13
AEZ + AZR	15.84 ± 0.19	20.70 ± 0.14	19.40 ± 0.11

EEZ- Ethanolic Extract of *Zingiber officinale* (Ginger)
AEZ – Aqueous Extract of *Zingiber officinale*
CPX – Ciprofloxacin AZR- Azithromycin

Discussion

The occurrences of the studied isolates in the stream samples supported the findings of many researchers (Lutz *et al*., 2013; Ekelozie *et al.*, 2018; and Adabe *et al*., 2022). The study also revealed that the studied isolates were significantly seen most in the stream samples, and this corroborated the findings of Ekelozie *et al.*, (2018) and Lut *et al.*, (2013). Ekelozie *et al*. (2018) reported that *Vibrio cholerae strain* was predominant in surface water among stream samples analysed in Uli, Anambra state, Nigeria. Lutz *et al.* (2013) reported that *Vibrio cholerae strains* survive in aquatic environments by entering a viable. The occurrences of *Vibrio cholerae strain* C6709 (VCC6), *Vibrio cholerae strain* P27459 (VCP2), and *Vibrio cholerae strain* E7946 (VCE7) in stream samples supported the findings of many researchers (Huq *et al*., 2012; Ekelozie *et al*., 2018; Islam *et al*., 2020 and Adabe *et al*., 2022). Huq *et al.* (2012) stated that the presence of *Vibrio cholerae strains* in these samples reaffirms the need for monitoring in order to minimize the risks of infection to exposed persons.

The highest occurrences of *Vibrio cholerae strain* in the stream samples observed in the present study could be attributed to the diversity of this strain within the study area. Studies have shown that genetic variation, genomic heterogeneity, antigenic variation, horizontal gene transfer and presence of dispensable (strain-specific) genes which confer fitness advantages to a particular strain, are associated with diverse epidemiological settings among different strains of microorganisms (Yap *et al*., 2014). Also, the occurrence of *Vibrio cholerae strain* mostly in the stream samples could be attributed to poor sanitation, indiscriminate sewage disposal, dumping of refuse, uncontrolled flooding and other anthropogenic activities within the study area. A similar conclusion was drawn by Huq *et al*. (2012), Kumar *et al*. (2013) and Lutz *et al*. (2013).

The study further revealed the presence of cardiac glycosides, steroids, alkaloids, tannins, flavonoids, phenolics and saponins from the rhizome extract of *Zingiber officiale* (ZO), and these agree with the findings of many researchers (John-Dewole *et al*., 2012; Aguoru *et al*., 2016; Ogbuagu *et al*., 2020; Fategbe *et al*., 2021 and Arora and Sen, 2023). The researchers also pointed out that these phytochemical constituents could be responsible for the activities and other ethnomedical potentials attributed to the above-studied extracts. Also, the variations in the amount of the phytochemical constituents associated with the extract could be attributed to the source and species of the plant involved. The pronounced activities of *Zingiber officinale* against the resistant strains of C6709 (VCC6), P27459 (VCP2), and E7946 (VCE7) could be attributed to the activities of the phytochemical constituents present in the extracts. The significant reduction in the number of resistant strains observed in this study corroborated the findings of many researchers (Kumar *et al*., 2013; Njimoh *et al*., 2015; Oguoma *et al*., 2020; Musbal *et al*., 2024). Several studies have reported that the horizontal spread of antibiotic-resistant genes among bacteria is driven by bacterial plasmid, which promotes the evolution of resistance.

Conclusion

This study identified *Vibrio cholerae* strains (VCC6, VCE7, and VCP2) in stream samples, with VCP2 being predominant. Notably, *Zingiber officinale* extract exhibited significant antimicrobial activity against these strains, which was enhanced when combined with Azithromycin, suggesting a potential therapeutic approach.

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Competing interest

Authors declare no competing interest.

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