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Studies on Phytochemical Screening, GC-MS Analysis and their Antibacterial Property Against *Vibrio cholerae*

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Abstract

In the current study, two different MTCC strains of *Vibrio cholera*, 3904 and 3906, were used to determine the anti-bacterial properties of *Cinnamomum verum* (*C. verum*) leaves (Family: Lauraceae). This was done through qualitative phytochemical compound screening and quantitative identification of volatile compounds using gas chromatography and mass spectrometry (GC-MS). Methanolic extract was subjected to phytochemical screening in order to determine the active components. Through qualitative examination revealed that the substance contained alkaloids, total carbohydrates, cardiac glycosides, flavonoids, glycosides, phenols, saponins, and tannins. Due to the abundance of phytochemical agents in the extract, GC-MS experiments were conducted. Based on their retention times and coverage percentages in mass spectra, these studies identified 42 volatile chemicals. The maximum zone of inhibition for the antibacterial activity of methanol extract were 15.9 mm for the *V. cholera* MTCC 3904 strain and 16.3 mm for the *V. cholera* MTCC 3906 strain, respectively, at concentrations of 100µL, Azithromycin showed 22.30 mm, a positive control, at a dosage of 30µg. Total antibacterial activity was determined to be 15.05 ±1.15 mm and 15.10±1.15 mm, respectively, in the examined bacterial strains. These values are equivalent to conventional azithromycin. In conclusion, *C. verum* can be a potential therapeutic agent against *V. cholera* strains, this is due to strong antibacterial nature of active biomolecules present in the medicinal plant.

Keywords: *Cinnamomum verum,* Phytochemicals, GC-MS analysis*, V. cholera,* Azithromycin

Introduction

The investigation of novel anti-microbial compounds from plant origin is gained lot of interest in researchers; also, it is inevitable due to multi drug resistance of microorganism, regardless of molecular mechanisms involved in the antibiotics and to combat any level of infection. For the centuries, in order to find the novel antibiotic and resistance of epidemic spreads, scientists and medical practitioners have developed the appropriate application of antibiotics (1). Efficient application of any antibiotics in humans depends upon the problem level and scenario of infection, which was always better under physician prescription established in their regulation against the infection and the responsibility of patient's during the therapeutic time. In anti-microbial findings, bacteria are major fo-

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cus to face with fierce struggle which were developed the several molecular mechanisms as multidrug resistance against synthetic anti-microbial drugs whose function mainly involved in enzymatic inactivation (2, 3), changing the drug route, and utilization of lower concentration of drug in cells as per the membrane permeability or cell specific overexpression at the efflux pumps (4). During the reaction of efflux pumps antibiotics segregated in self-defense mode which are removed actively from the cell. For any new antibacterial developments, lethality of drug concentration in active sites of cells results into predispose of pathogenic organism which is under the threat of over resistance (5). Knowing such scenario of pathogens, efflux pump reaction is inevitable to target the organisms and this major challenge is to tackle by the research by developing newer drugs, which is less toxic and higher rate of efficacy of potent molecules, in single or in synergistic approach with other usual antibiotic drugs combating effectively against any infections of multidrug-resistance. Medicinal plants are the treasure for fighting any infective agents and it has been used in all the countries from centuries to various diseases. In today's context, there is an extreme revival of medicinal plant interest and almost exploited the sources for identification of novel drugs (6) which shown pharmacological significance in various approach (7). The present investigation is to explore the *C.verum* phytochemicals qualitatively, identification of volatile compounds by GC-MS analysis and their biological action against *V. cholera* strains under *in vitro* conditions.

Materials and Methods

Plant material

The University of Trans-disciplinary Health Sciences and Technology in Bengaluru, Karnataka, India, certified the *Cinnamomum verum*, or Daalchini, leaves that were obtained from the botanical garden of the Agriculture University in Bengaluru.

Preparation of plant extracts

The leaves of *C. verum* were collected, cleaned thoroughly in double distilled water, chopped into small pieces and for the removal of moisture shade dried in room temperature. Around 200 g of leaves material was accurately weighed, crushed, powdered and Soxhlet extraction in methanol at 50°C were carried out. Buchi's rotary vacuum was used to obtain the concentrated extract and the resulted extract was preserved in refrigerator until use of experimental studies (8-10).

Screening of phytochemicals

Qualitative screening of phytochemicals was conducted in order to investigate the primary metabolites present in the methanol extract of *C. verum* leaves. This further aimed to identify the volatile compounds by GC-MS analysis.

The *C. verum* extract was screened for phytochemical analysis by following modified method of Sharangouda and Patil, (9), with the comparison of standard method of Harnborne, (10) and Fransworth, (11) to investigate the secondary metabolites such as alkaloids, total carbohydrates, cardiac glycosides, flavonoids, glycosides, phenols, saponins, tannins, terpenoids and total proteins. For the preparation of phytochemical reagents for the tests followed standard method of Harnborne (12). To make the concentration to obtain the proper solution of methanol extract further dissolute in water (Milli-Q ultra-pure distilled water) and filtered for removal of residues.

Gas chromatography and mass spectroscopy (gc-ms) analysis

Preparation of extract

Filtered10mg/ml of aromatic medicinal plant extract of *C. verum* leaves (methanol) was prepared further in methanol due to high polarity of the solvent and for which 1µl plant extract was employed to quantify the volatile compounds by GC-MS analysis.

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Instruments and chromatographic conditions

GC-MS analysis of *C. verum* extract was performed using a Thermo GC-MS Clarus 500 (Perkin Elmer). For MS detection, the MS DSQ II electron ionization mode with an ionization energy of 70 eV was used, with a mass range at m/z 50-650. Zebron capillary column 2B-XLB. The analysis was conducted using a column with the following specifications: 30 meters in length, 0.25mm (I.D), and 0.25 film thickness (m). The initial column temperature was set to 60°C for five minutes. Temperatures of 280°C and 290°C, respectively, were established for the GC injector and MS transfer line. The GC was carried out in spitless mode. As the carrier gas, helium (1.0 ml/min flow rate) was employed. There was an injection volume of 1.0 µL. The plant extract was diluted in methanol, passed through a polymeric solid phase extraction (SPE) column, and then subjected to GC-MS analysis to determine its constituent parts. Compounds in the plant sample were found using computer searches on the NIST REFPROP Version 9.1 database and comparisons of the GCMS spectrum.

Bioactive constituents' identification

The National Institute of Standard and Technology's (NIST's) database, which contains more than 62,000 patterns were used for the interpretation of the Mass Spectrum GC-MS. A comparison was made between the spectra of the unknown components and the spectrum of known components kept in the NIST collection. The test materials' constituent parts' names, molecular formulas, weights, and chemical structures were determined.

Anti-bacterial activity

The extract of *C. verum* applied to antibacterial activity by disk diffusion using Agar plate method (Muller-Hinton (MH)) using the standard azithromycin for control (positive) and methanol (negative) for the comparison. The strains used in the present investigations are *Vibrio cholera* MTCC 3904 and *V. cholera* MTCC 3906. Test strains are allowed to grow in around 14 hours in Luria Broth (LB) froth medium at around 30 °C. The incubated culture was then placed in MH medium. Each strain swabbed in MH Agar plates by sterile lab grade cotton swabs. Wells were made 6mm diameter on each plate by using standard lab grade gel puncture tool. The methanol extract of *C. verum* leaves of different concentrations 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µl were poured on each well by using calibrated micropipette. Results of antibacterial activity were measured by zone of inhibition which obtained clearly around the well, where extract loaded and incubated at 37 ˚C constantly for 24hrs.

Statistical analysis

All experiments were performed in triplicates and the mean difference was statistically calculated. The values are expressed as mean ± SEM. Students "t" test was used and $p<0.01$ was considered as statistically significant.

Results and Discussion

Phytochemicals screening of cinnamomum verum methanol extract of leaves

The phytochemical screening of *C. verum* methanol extract of leaves exhibited good results for alkaloids, total carbohydrates, cardiac glycosides, flavonoids, glycosides, phenols, saponins, and tannins but negative results for terpenoids and total proteins (Table 1). Many researchers in the field of medicinal plants have reported similar findings employing various types of plant parts and extracts (13-15).

Table.1 Screening of phytochemicals qualitatively in leaves of methanolic extract of C. verum

Screening of Qualitative Phytochemical Analysis	
Tests	C. verum
Total Carbohydrates	+ ve
Cardiac glycosides	+ ve
Flavonoids	+ ve
Terpenoids	- ve

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Glycosides	+ve
Tannins	+ve
Alkaloids	+ve
Phenols	+ve
Saponins	+ve
Total Proteins	-ve

Gc-ms analysis of methanolic extract of c. Verum leaves

The GC-MS analysis of the methanolic extract results were shown in figure 2 and it has eluted 42 different active compounds. Identification of volatile compounds were assessed with the comparison of standards of NIST and Wiley 9.1. Most of the phytochemical were characterized by their respective area (%) depends on the availability and elution on particular retention time. The obtained peaks of the chromatograms, shown highest % of area by 10-Nonadecanone (11.84), Apiol (7.02), Pentadecanoic acid (6.26), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (5.02), α-Tocopherol-β-D-mannoside (4.78), Hexadecanoic acid, methyl ester (2.53), Diisooctyl phthalate(2.44), 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (1.87), 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (1.66), Phytol (1.77), 8,11-Octadecadienoic acid, methyl ester (1.47), 1,2-Bis (trimethylsilyl) benzene(1.40), Tricyclo (4.3.1.1(3,8))undecan-1-amine(1.37), 3β,6α,20β-Trihydroxy-5 α-pregnane (1.20), 1,3-Dioxane-5-methanol, 5-ethyl- (1.13), Androst-11-en-17-one, 3-formyloxy-, (3.α, 5.α.)- (1.09), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (1.01), Hydrazine, N-(N-methyl-1-azacyclotridecan-2-ylidene)-N'-(di(methylthio)methylidene)- (1.00), and rest other 27 volatile compounds shown less than 1 % of area as per the elution on particular retention time. As per the literature many of the volatile compounds which were present in the plant shown potent cytotoxicity activities and also several researchers reported for various pharmacological applications on these compounds in different plants, hence these volatile compounds listed with various characters to understand their biological action in table 2.

Table.2 Quantification of volatile compounds by GC-MS analysis of methanolic extract of *C. verum* leaves based on retention time, peak area (%) and their names

Sl. No.	Compound Name	Retention Time	M/Z	Peak Area (%)
1	cis-Aconitic anhydride	4.03	40	0.258605
2	3-tert-Butyl-5-chloro-2-hydroxybenzophenone	4.226	40	0.458313
3	5-Nitro-3-cyano-2(1H)-pyridone	4.29	44	0.286222
4	4-Carbamoyl-5-methylhexanoic acid, methyl ester	4.729	40	0.25252
5	2-Deoxy-D-galactose	6.735	57	0.36625
6	1,3-Dioxane-5-methanol, 5-ethyl-	6.79	57	1.13271
7	1,1-Difluorocyclohexan-3-ol	6.815	57	0.98037
8	Apiol	8.26	222	7.02984
9	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	10.64	68	5.02365
10	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	11.04	95	0.41302
1	2-Cyclopenten-1-one, 4-hydroxy-3-methyl-2-(2,4-pentadienyl)-, (Z)-(+)-	11.313	40	0.166978
12	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	11.379	81	1.01896
13	1,2-Benzenedicarboxylic acid, 2-butoxyethyl butyl ester	11.592	149	0.77162
14	Hexadecanoic acid, methyl ester	12.357	74	2.53206
15	Pentadecanoic acid	13.126	73	6.26521
16	8,11-Octadecadienoic acid, methyl ester	15.405	81	1.47058
17	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	15.545	79	1.66363
18	Phytol	15.629	71	1.78180
19	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	16.351	79	1.87666

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20	Tricyclo(4.3.1.1(3,8)) undecan-1-amine	21.665	44	1.38974
21	Diisooctyl phthalate	22.282	149	2.446947
22	3.beta.-Acetoxy-bis-nor-5-cholenamide	24.25	73	0.66110
23	6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol	24.651	159	0.39112
24	8-Pregnene, 3-acetoxy-20-hydroxymethyl-4,4,14-trimethyl-	24.802	341	0.34201
25	1,3-Dioxolane, 4-((octadecyloxy) methyl)-2-phenyl-	25.925	149	0.14055
26	Silanamine, N-(2,6-dimethyl-4-((trimethylsilyl)oxy) phenyl)-1,1,1-trimethyl-	25.965	73	0.96934
27	.3beta.,6.alpha.,20.beta.-Trihydroxy-5.alpha.-pregnane	26.103	73	1.20526
28	Hydrazine, N-(N-methyl-1-azacyclotridecan-2-ylidene)-N'-(di(methylthio) methylidene)-	26.475	44	1.00675
29	7-Hydroxy-6,9a-dimethyl-3-methylene-decahydro-azuleno(4,5-b) furan-2,9-dione	27.03	107	0.25691
30	Haloxazolam	27.26	281	0.98480
31	Pregnane-3,11,20,21-tetrol, cyclic 20,21-(methylboronate), (3.alpha.,5.alpha.,11.beta.,20R)-	27.329	93	0.35701
32	T-2 Tetraol	28.798	67	0.32008
33	10-Nonadecanone	29.189	71	11.84532
34	Silane, diphenylisobutoxy(5-methoxy-3-methylpentyloxy)-	29.575	281	0.94791
35	Ethanethioic acid, S-(8-(diethylphosphono)octyl) ester	30.143	281	0.92556
36	2,4-Di-tert-butyl-6-(tert-butylamino) phenol	30.289	208	0.52400
37	.alpha.-Tocopherol-.beta.-D-mannoside	30.384	165	4.78939
38	Androst-11-en-17-one, 3-formyloxy-, (3.alpha.,5.alpha.)-	30.478	207	1.09485
39	Kauran-18-oic acid, 7-(acetyloxy)-15,16-epoxy-, methyl ester, (4.alpha.,7.beta.,15.alpha.)-	30.632	159	0.30512
40	1,2-Bis(trimethylsilyl) benzene	31.94	207	1.40814
41	Acrylophenone, 3,3-diphenyl-, semicarbazone	32.338	44	0.58697
42	5-Chloropentanoic acid, 2-butyl ester	32.373	55	0.30752
43	2,3-Diphenylquinoxaline 1-oxide	32.499	282	0.56888
44	Cyclooctasiloxane, hexadecamethyl-	33.668	147	0.84829

GC-MS analysis of *C. verum* leaves methanolic extract (Figure 2) revealed a total of 42 volatile compounds based on peak detection and retention time on each compound, indicating the presence of forty-four different volatile compounds with various biological properties. These compounds were discovered by their characterization in GC-MS analysis by differentiating retention duration in each compound as well as their peak (%) in the examined plant extract. These features are characterised based on the retention period of each concentration of the peak area in percent area in the entire spectrum (Table 2). The characterized results represented as per the peak and percent area of the volatile compounds, i.e., 10-Nonadecanone (11.84), Apiol (7.02), Pentadecanoic acid (6.26), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (5.02), α-Tocopherol-β-D-mannoside (4.78), Hexadecanoic acid, methyl ester (2.53), Diisooctyl phthalate (2.44), 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (1.87), 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (1.66), Phytol (1.77),

8,11-Octadecadienoic acid, methyl ester (1.47), 1,2-Bis (trimethylsilyl)benzene (1.40),Tricyclo (4.3.1.1(3,8))undecan-1-amine(1.37), 3β,6α,20β-Trihydroxy-5 α-pregnane (1.20), 1,3-Dioxane-5-methanol, 5-ethyl- (1.13), Androst-11-en-17-one, 3-formyloxy-, (3.α, 5.α.)- (1.09), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (1.01), Hydrazine, N-(N-methyl-1-azacyclotridecan-2-ylidene)-N'-(di(methylthio)methylidene)- (1.00) and remaining 27 volatile compounds shown less than 1 % with respect retention time and peak area. Patil *et al*., revealed the active principal compounds of petroleum ether of *Citrus medica* seeds and their biological action on *in vivo* models (16). Eramma and Patil, (17) revealed 41 distinct volatile compounds from crude and TLC fractions in *Flacourtia indica* root extract of methanol, GC-MS analysis indicated the presence of, including Heneicosane (25.945), Squalene (20.51), Cholesterol (33.525), Cycloheptasiloxane, tetradecamethyl-(14.864), 2, 4-Di-tert-butylphenol-(16.032), Cycloheptasiloxane hexadecamethyl (16.848), Cyclononasiloxane octadecamethyl (20.733), and n-Hexadecanoic acid (22.092). Kolgi et al., reported alkaloid and flavonoid present in *Leucas aspera* leaves of chloroform and ethanol extracts revealed antioxidant and anticancer property (18, 19). Similar compounds present in *Simarouba glauca* seed petroleum and ethanol extracts reported for antioxidant property (20, 21).

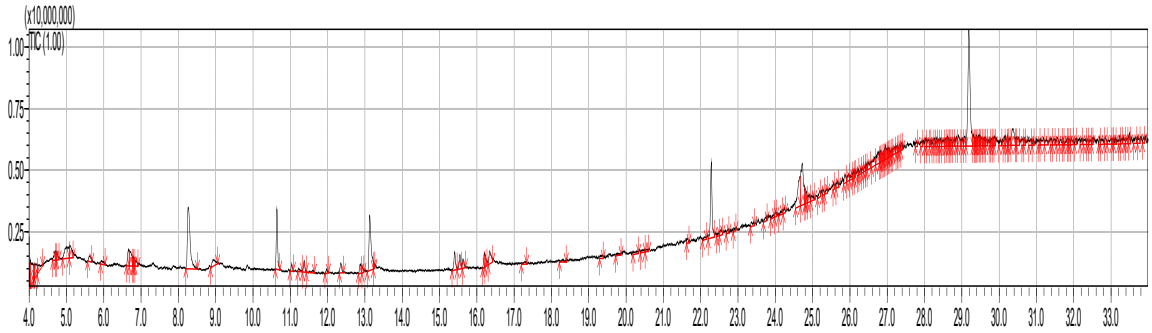

Figure 1**:** GC-MS analysis spectrum of *C. verum* leaves of methanolic extract.

Antimicrobial activity of C. verum leaves methanolic extract

The antibacterial activity was tested *in vitro* against the 24 h *V. cholera* two strains of bacteria utilising the disc diffusion plate method of Muller-Hinton (MH) agar, with conventional azithromycin and methanol serving as positive and negative controls, respectively. The disc diffusion method was used to test the antibacterial susceptibility of *C. verum* leaves extract against *V. cholera* MTCC-3904 and 3906 strains. The maximum inhibitory zone observed on higher concentrations at 100% methanol extract was 15.8 mm and 16.5 mm, respectively, while the minimum at 60% methanol extract was 10.5 mm and 11.8 mm. The positive control azithromycin (30 g) demonstrated the greatest inhibitory zone on *V.cholera* strains MTCC-3904 and 3906 at 40% concentration (Figure 2). Srivastava et al., (22) discovered that several spices have extremely strong antibacterial efficacy against *Vibrio* bacterium species isolated from pond water, with Black pepper having the highest zone of inhibition at 100% ethanol extract and 70% activity on methanol extract. Coriander had the greatest zone of inhibition at 85% extract and the lowest activity at 70% on ethanol extract. Cinnamon showed the greatest zone of inhibition at 85% acetone extract and the least at 70% action on ethanol extract. Green cardamom was discovered to have a strong potential against isolated *Vibrio* species (Table 3).

Table 3: Total antibacterial activity and zone of inhibition of methanol extract of *C. verum* leaves

Organisms		**Zone of Inhibition (mm)**										**Total Antibacterial Activity**
	Concentration of Methanol extract of *C. verum* leaves										**Zone of Inhibition (mm)**	
	10	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**	**100 Conc.**	
V. cholera 3904		-	-	-	-	-	10.5	10.8	12.4	13.8	15.8	15.05±1.15 mm
-		-	-	-	-	10.5	12.5	12.8	14.5	15.9		
V. cholera 3906		-	-	-	-	-	12.4	12.8	13.5	13.8	16.8	15.10±1.15 mm
-		-	-	-	-	11.5	11.2	14.5	13.8	16.2		
Positive Control (Azithromycin)	V. cholera 3904	22.5	22.8	22.4	22.9							13.22±0.05 mm
	V. cholera 3906	22.8	22.6	22.8	22.7							14.44±1.15 mm
Negative Control (Methanol)	V. cholera 3904	-	-	-	-							
	V. cholera 3906	-	-	-	-							

Based on the minimum inhibitory concentration at higher zone of inhibition on 100% methanol extract, total antimicrobial activity was tested in triplicates on the same organisms and found zone of inhibition 15.05±1.15 mm and 15.10±1.15 mm in *Vibrio cholera* MTCC-3904 and 3906 strains, respectively, whereas positive control showed 13.22±0.05 mm and 14.44±1.15 mm and it was significant increase in the methanol extract of *C. verum* the date was presented in Table 3. Previous research has clearly shown that active components such as camphene, limonene, caryophyllene, and others have excellent bactericidal activities (23). Ragasa et al., (24) demonstrated antibacterial activity of sapwood extract (Dracontomelon dao) against *Staphylococcus typhimurium, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis, Candida albicans*, and *Aspergillus niger* using ethanolic extract, and they revealed these activities were due to the of 54 compounds which were analysed through GC-MS of the extract, among GCMS analyses of Rhus semialata chloroform extract of seeds identified active ingredients such as Tridecane, Decane, Anethole, (Z)6, (Z)9-Pentadecadein-1-ol, and Squalene as a more volatile molecule, as well as antibacterial capabilities in diverse species (25). Several other studies on the pharmacological properties of the plant Dracontomelon dao revealed potent antimicrobial activity with various organisms, antioxidant activity with various scavenging potential, anti-inflammatory in quantitative method, anti-diabetic in in vitro studies, and anti-trypanosomal activities (26-29). On the same plant, isolation and characterisation of other plant components proposed for medicinal and therapeutic activities (30, 31).

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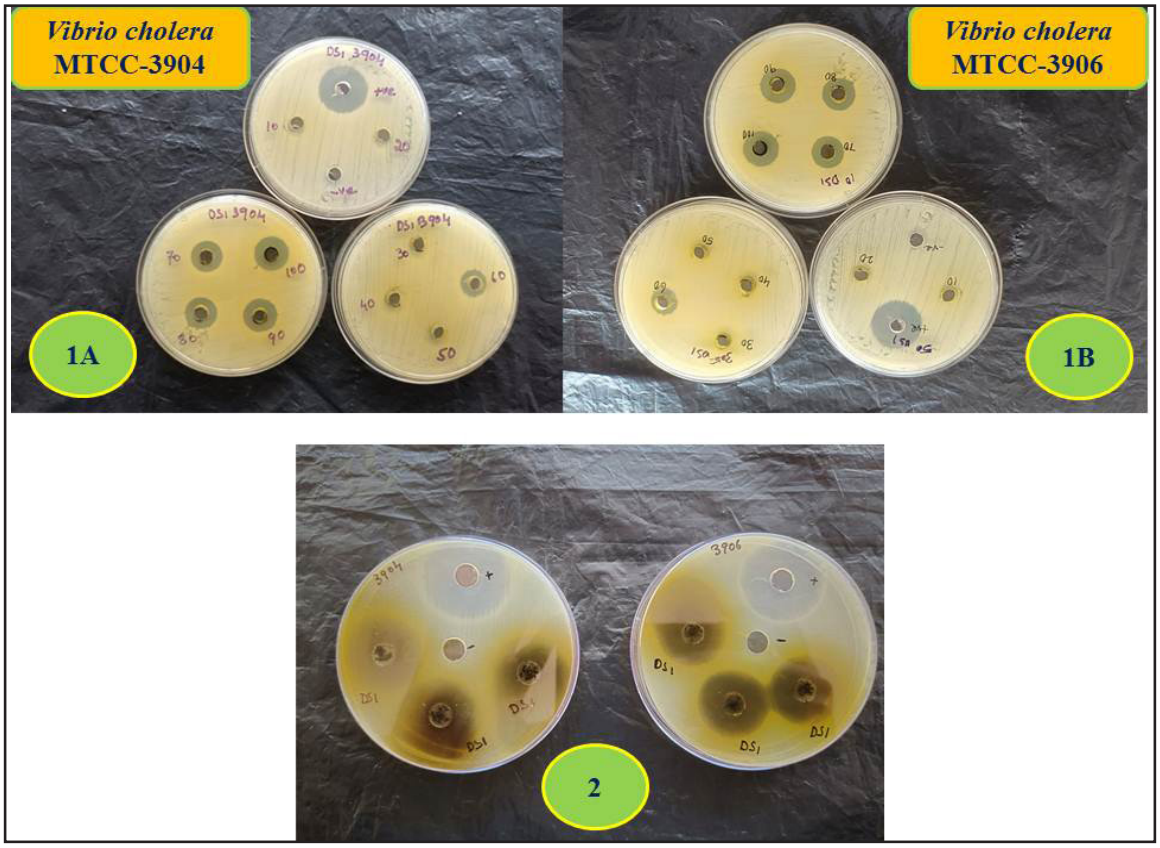

Figure 2**:** Antibacterial activity of methanol extract of *C. verum* leaves resulted zone of inhibition (mm) of bacterial strains 1A) *V. cholera* MTCC-3904, 1B) *V. cholera* MTCC-3906 2) *V. cholera* MTCC-3904 & 3906 showing total antibacterial activity with the comparison of azithromycin positive control and methanol negative control

Conclusion

The experimental research of C. verum leaves extract revealed significant secondary metabolites and antibacterial activity against two different strains of *V. cholera*, employing ten different doses of the methanol extract (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 l). In a qualitative analysis of phytochemicals, screening study results showed that alkaloids, total carbohydrates, cardiac glycosides, flavonoids, glycosides, phenols, saponins, and tannins had antimicrobial properties against *V. cholerae* strains at grade dependent concentrations in disc diffusion method. GCMS analysis revealed 42 volatile chemicals in the methanol extract, with higher concentrations of the 15 compounds exhibiting antibacterial capabilities and maybe other biological actions. Other substances in the literature exhibit antioxidant, anti-inflammatory, anticarcinogenic, antifertility, and antisteroidegenic activities in addition to antimicrobial. These findings point to Cinnamomum verum leaves having powerful natural antibacterial properties.

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Conflict of Interest

The authors declare no conflict of interest.

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