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Original article

Exploring microalgal lipids as anti-virulence agents targeting *MDR Vibrio cholerae infection:* A Step Toward Developing Herbal Oral Rehydration Salt (ORS) Formulations

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ABSTRACT

Introduction: Cholera, a life-threatening diarrheal disease caused by Vibrio cholerae, remains a major global health issue, especially in tropical regions. Increasing antibiotic resistance in V. cholerae strains poses a significant threat to effective treatment. Novel antivirulence strategies that do not promote resistance are urgently needed.

Objectives: This study investigated the potential of lipid extracts and crude biomass derived from *Chlorella variabilis* (CV) and *Chlorococcum* sp. (CCM) microalgae to inhibit cholera toxin (CT) production by a multidrug-resistant V. cholerae strain (SRK-19), without affecting bacterial viability.

Materials and Methods: Lipid extracts and crude freeze-dried biomass from CV and CCM were tested against *V. cholerae* SRK-19 *in vitro* for their effects on CT production (measured via ELISA) and bacterial viability. For *in vivo* validation, a rabbit ileal loop assay was performed, and fluid accumulation (FA ratio), colony-forming units (CFU), and CT levels were measured.

Results: Both lipid extracts and crude biomass significantly reduced CT production *in vitro* in a dose-dependent manner, with up to 97.9% inhibition observed using CCM lipid extract at 150 µg/ml. Crude extracts achieved comparable inhibition, with CV and CCM biomass reducing CT levels by 93% and 97%, respectively, at 1 mg/ml. *In vivo* studies confirmed reduced FA ratios and CT levels in treated ileal loops, without affecting bacterial growth (CFU counts remained unchanged), indicating that bacterial viability was not compromised.

Conclusion: Crude and lipid extracts from CV and CCM demonstrate potent antivirulence activity against *V. cholerae* by inhibiting CT production without promoting antimicrobial resistance. These findings support

the development of a microalgae-based oral rehydration formulation as a promising alternative to conventional antibiotic therapy for cholera.

KEYWORDS: CHOLERA TOXIN; MICROALGAE; LIPID EXTRACTS; IN-VIVO STUDY; ANTI-VIRULENCE

INTRODUCTION

Vibrio cholerae is a gamma-proteobacterium responsible for the watery diarrhoea which is transferred feco-orally through contaminated food and water (Safa et al., 2010). Pathogens produce virulence factors to exhibit virulence, which subsequently results in severe clinical condition. Pathogenic strains of *V. cholerae* harbour toxin-coregulated pili (*TCP*) for colonisation in intestine and a cholera toxin prophage (*CTXϕ*) which encodes the cholera toxin (CT), a key virulence factor that is directly responsible for the major clinical symptoms of cholera (Safa et al., 2010; S. Chatterjee et al., 2021). In 2023, WHO received cholera data from 103 countries. Of these, 45 nations reported 4007 fatalities and 535,321 cases, yielding a 0.7% case-fatality rate (CFR). Notably, there was 13% increase in cholera cases and a 71% increase in reported deaths compared to 2022 (WHO,2023). Although 23 countries used oral rehydration points (ORPs) during outbreaks, the persistence of community deaths underscores the urgent need to enhance treatment access at the community level (WHO,2023). Treatment of Cholera primarily involves oral rehydration solution (ORS) or intravenous fluids to prevent dehydration, which significantly improves and is crucial for patient survival. Antimicrobial agents are used as a secondary treatment, but the growing problem of multidrug-resistant (MDR) *V. cholerae* strains poses a serious threat to effective disease management.

Antibiotics have long been the cornerstone of treating infectious diseases in modern medicine, greatly improving quality of life by effectively combating bacterial infections. However, the growing threat of antibiotic resistance (AR) now poses a significant global health challenge (Cepas et al., 2021). Emergence of AR is a natural phenomenon in bacteria; however, the selection of AR is guided by indiscriminate and frequent use of antibiotics in healthcare, agriculture, and industrial fields (Hamza & Zinjarde, 2018; Rolff et al., 2024). A study involving 44 clinical *Vibrio cholerae* isolates reported a widespread presence of virulence genes and evidence of infection by classical CTXΦ. The isolates also exhibited broad-spectrum antibiotic resistance, supported by the detection of resistance genes (Goel et al., 2010). As a result, it has become critical to explore other novel therapeutic approaches for combating AR in clinical pathogens like MDR *V. cholerae*. One of such way is anti-virulent therapy, where anti-virulent compound that disarms the bacteria by targeting their virulence factors without affecting their viability (S. Chatterjee et al., 2021; Dehbanipour & Ghalavand, 2022). Targeting virulence pathways that are not essential for bacterial growth offers a promising strategy to limit the development of resistance, making it a potential approach for the development of next-generation anti-infective therapies. In our previous *in-vitro* study, we have established *Chlorella variabilis*, a type of green edible microalgae, is one of the potential candidates for anti-virulence in various *V. cholerae* strains (S. Chatterjee et al., 2021). Microalgae are rich in nutrients and biologically active compounds, such as proteins, polysaccharides, lipids, polyunsaturated fatty acids, vitamins, pigments, phycobiliproteins, enzymes, etc (Barkia et al., 2019; Encarnação et al., 2015), which exhibits antioxidant, antibacterial, antiviral, antitumor, regenerative, antihypertensive, neuroprotective and immune-stimulating effects (Dolganyuk et al., 2020). Microalage like *C. ellipsoidea* are also used for biodiesel production, heavy metal bioremediation(Satpati & Pal, 2021).

To our knowledge, this is the first study to demonstrate a dose-dependent inhibition of cholera toxin production by both lipid extracts and crude biomass of *Chlorella variabilis* and *Chlorococcum sp.* against a toxigenic *V. cholerae* strain, without impacting bacterial viability.

In-vitro (CT-ELISA) and *in-vivo* (rabbit ileal loop model) evaluations have been conducted using both microalgae crude extracts and their lipid fractions. This study addresses a significant gap by validating the

antivirulence potential of GRAS-status microalgae using both *in vitro* and *in vivo* models, thereby paving the way for alternative, resistance-free interventions. The *in-vivo* study uses rabbit models of CT-induced intestinal fluid secretion to investigate anti-virulent therapy employing possible anti-virulent drugs for cholera (Sawasvirojwong et al., 2013). The amount of fluid accumulation in the ileal loop decreases if extracts with anti-virulent properties are applied concurrently and limit CT production. This makes it possible to evaluate the compound's efficiency in actual infection cases.

MATERIALS AND METHODS

MATERIALS

Luria-Bertani media, Thiosulphate citrate bile-salt sucrose (TCBS), AKI (composition: peptone- 15 gms/l, yeast extract- 4 g/l, sodium chloride-5 g/l, final pH 7.4±.02 at 25°C), purchased from Himedia, India. Phosphate buffer saline (PBS), bovine serum albumin (BSA), cholera toxin b subunit ≥95% (SDS-PAGE),lyophilized powder, anti-cholera toxin antibody produced in rabbit whole antiserum, monosialoganglioside-GM 1 from bovine brain lyophilized powder, anti-rabbit IgG (whole molecule)–peroxidase antibody produced in goat, 3,3',5,5'-tetramethybenzidine (TMB) liquid substrate system, peroxidase substrate, Supelco37 F.A.M.E Mix were purchased from Sigma Aldrich, India. Isoflurane volatile anesthesia, scalpel blades & holder, nylon suture, catgut absorbable suture, betadine solution, non- ratcheted tissue forceps, straight blunt scissors, forceps, surgical drape / green cloth, cotton, gauge, analgesics (meloxicam injection) were purchased from local medical store. All the necessary materials were sterilized before *in-vivo* study.

Methods

Microalgae culture and growth conditions

Two microalgae, *Chlorococcum* spp. (CCM) and *C. variabilis* (ATCC PTA 12198) (CV), were collected from the microalgal collection of Department of Applied Phycology and Biotechnology, CSIR-CSMCRI Bhavnagar, Gujarat (Chatterjee, S et al., 2021). *C. variabilis* was grown in modified Zarrouk's media (refer the supplementary materials; table S1) and incubated for 21 days (S. Chatterjee et al., 2021; Pancha et al., 2015). *Chlorococcum spp.* was cultured in BG11 (refer the supplementary materials table S2) and incubated for 15 days at room temperature in a 12/12 (dark/light) regime with an aerator (Harwati et al., 2012). Biomass was harvested after being centrifuged at 4°C for 10 min at 8000 rpm; pellets were washed with distilled water and freeze-dried (lyophilized) for further use (Puspanadan et al., 2018).

Lipid Extraction and Transesterification

Microalgae use polar lipids, including phospholipids and glycolipids, to form cell membranes, whereas non-polar lipids are used as energy sources (Sati et al., 2019). For lipid extraction, Bligh and Dayer's method was followed with a few modifications (Bligh & Dyer, 1959). Wet biomass of 5g was crushed using a mortar and pestle using 20 ml of ethanol and incubated for an hour, followed by 20 ml of n-Hexane and incubation in the same condition (Ranjan et al., 2010) Subsequently, supernatant was collected after centrifugation at 3000 rpm for 5 min and heat dried at 50°C. Fatty acid methyl ester (FAME) is the widely used solvent-mediated microalgal lipid extraction method. The sample was treated with 0.5M methanolic KOH (prepared freshly) in a saponification flask, heated using a water bath, followed by the addition of 0.5M methanolic HCl (prepared freshly). Then, n-hexane was added to the solution, and esters were collected. The esters were transferred into a test tube; the upper layer of hexane was taken into a glass vial, and the lower layer of crude was taken back into the previous flask to add n-hexane. Lipids are then labelled and stored at 4°C before GC/MS analysis (Carrapiso & García, 2000).

GC/MS Analysis

The GC/MS analysis was performed according to previous methodology with slight modifications (Kumaran et al., 2023). The lipid extracts were analyzed by Shimadzu-made HS-20 headspace sampler model GC/MS TQ8040. ZB5

MSi column with 30 m length, 0.5 µm thickness, and 0.25 µm diameter and helium as carrier gas. The sample injection temperature was 300°C at split injection mode. The initial oven temperature was 40°C for 3 min, then progressively increased to 230°C at a rate of 10°C/min, and finally reached 300°C at 20°C/min for 15 min. The MS ion source temperature was maintained at 330°C, the interface temperature was at 300°C, and the solvent cut time was 4 min. Supelco37 F.A.M.E Mix, manufactured by Sigma Aldrich, was used as the standard.

Anti-virulence activity of CV & CCM - *in-vitro*

Preparation of bacterial inoculum and CFS collection

V. cholerae serogroup O1, El Tor biotype SRK-19 strain was chosen for the study (S. Chatterjee et al., 2021). The bacterial culture was inoculated in Luria-Bertani (LB) broth medium for overnight at 37°C and 120 rpm. The overnight culture was adjusted for $OD600_{nm}$ to 0.1 of ~10^5 colony forming unit (CFU/ml) (S. Chatterjee et al., 2010). A100 µl culture was then inoculated in fresh AKI medium with different concentrations of lipids and crudes, as mentioned in table 1, all the experiments were done in triplicate and incubated for 8h at 37°C (4 h static condition followed by 4 h shaking condition). Colony Forming Unit (CFU) was estimated in TCBS and LB agar, and the cell-free supernatant (CFS) (refer the supplementary materials S.2.5.1) was collected for enzyme-linked immunosorbent assay (ELISA)(S. Chatterjee et al., 2010, 2021).

CT assay

The cholera toxin (CT) assay was performed by GM1 ELISA. In brief, the 96-well plate was coated with 1µg/ml GM1 diluted in 0.5M of bicarbonate buffer and kept at 4°C overnight, followed by washing in between each step with washing buffer (0.05% tween 20 in 1X PBS), twice, and once with 1X PBS, each for 2 min. Blocking was performed with a 3% BSA solution dissolved in PBS and kept at 37°C for 6 h. Standard cholera toxin (Sigma Aldrich, India) was diluted in different concentrations (2, 4, 8, 16, 32, & 100, ng/ml) and used as a standard. The collected CFS were loaded and kept at 37°C for 1 h. The primary antibody (1:2000) was loaded in each well and incubated for 1 h at 37°C, followed by the secondary antibody (1:8000) and incubated at 37°C for 1hr. Then 3,3',5,5' tetramethyl benzidine (TMB) substrate in dark condition was added and kept for 20–30 seconds for colour production, followed by the addition of 2M H_2SO_4 (stop solution). Immediately, the reading was taken at 450 nm (S. Chatterjee et al., 2010, 2016, 2021). ELISA was performed, and a standard graph was prepared with the CT standard to compare the CT production in the absence and presence of microalgal extracts. Inhibition of CT production (%) was calculated using the formula given below. ELISA was performed in triplicate for all the samples.

$$CT\ inhibiton\ (\%) = \frac{CT\ production\ in\ PC - CT\ production\ in\ test}{CT\ production\ in\ PC} * 100$$

PC: Positive control (*V. cholerae* without extract)
Test: *V. cholerae* with extract

Anti-virulence activity of CV & CCM - *in-vivo*

Preparation of bacterial inoculum

The *V. cholerae* bacterial culture was inoculated in LB broth medium for overnight at 37°C and 120 rpm. The culture was then inoculated in LB media for 4-5 h to get 10^6 CFU/ml (E. Chatterjee & Chowdhury, 2013). The freshly prepared *V. cholerae* strain was pelleted down at 10000 rpm for 10 min at 4°C and then washed twice with phosphate-buffered saline (PBS, pH 7.0) by centrifugation at 5,000 rpm for 5 min at 4°C. The cells were re-suspended in PBS for the inoculum (10^6CFU/ml) (E. Chatterjee & Chowdhury, 2013).

Ethical clearance

For all *in-vivo* studies ethical clearance (IAEC approval number IAEC/21/09) were taken and all the experiments were carried out following all the norms at iCARE facility of CSIR-IMTECH (https://www.imtech.res.in/facilities/icare).

Passage assay

A passage assay was performed using lab-grown *V. cholerae* strain SRK-19 to enhance cholera toxin (CT) production by passaging through the rabbit ileal loop model (refer the supplementary materials; Figure S1). Cholera toxin levels were quantified using an enzyme-linked immunosorbent assay (ELISA). SRK-19 strains demonstrating elevated CT production were selected for subsequent experiments (S. Chatterjee et al., 2010, 2021).

Rabbit Ileal Loop Assays

For the experiment, one New Zealand rabbit was allocated per algal sample. In brief, New Zealand white rabbits were kept fasted for 12–24 h prior to surgery, with access to *water ad libitum*. Prior to the experiment, rabbits were anesthetized using isoflurane- a volatile aesthetic as per dose prescribed by a veterinarian, under aseptic conditions (TF et al., 2025). A midline incision was made below the centre of the abdomen and a section of the ileum was exteriorized by incising through the abdominal muscles and peritoneum. Further, the ileal loop was carefully tied up with sterilized silk ligatures to create loops, avoiding any damage to the blood vessels or mesentery (E. Chatterjee & Chowdhury, 2013; De & Chatterjee, 1953).

To avoid cross-interference between different microalgal treatments, separate rabbits were used for each microalgal strain. For each rabbit, a total of 5 loops (~6cm long) for injecting *V. cholerae* and microalgal extracts, in addition to 4 inter-loops (~3cm long), and each (~3 cm each) were created. The first loop was injected with 500 µl of freshly prepared *V. cholerae* SRK-19 (10^6 CFU/ml) as a positive control. Two loops were injected with mixtures containing 10^6 CFU/ml of *V. cholerae* and two different concentrations of lipid extracted from microalgae. A fourth loop received a mixture of crude microalgal biomass and *V. cholerae* (10^6 CFU/ml). The fifth loop was injected with PBS and served as the negative control. (See Table 1; refer the supplementary materials Figure S2) (E. Chatterjee & Chowdhury, 2013). Following, the abdomen was closed using two layers of sutures, and 1 ml of veterinary Meloxicam was administered subcutaneously for analgesia. After regaining consciousness, animals were maintained without food or water. Approximately 18 hours post-surgery, the ileal loops were excised, and the animals were euthanized via cardiac puncture (Faruque et al., 2004). The accumulated fluid in each loop was collected separately, and the fluid accumulation (FA) ratio—defined as the volume of fluid (ml) per centimetre of loop—was calculated (Figure S3) (E. Chatterjee & Chowdhury, 2013; De & Chatterjee, 1953).

A colony count was done on TCBS plates after overnight incubation. Further, ELISA was then performed for the quantification of cholera toxin (CT), and a standard graph was prepared (Almeida et al., 1990; Paulie et al., 2023).

Table 1 Dosage of compounds in *in-vitro* testing & *in-vivo* testing

Microalgae	*In-vitro* dosage concentration		*In-vivo* dosage concentration	
C. variabilis (CV)	Lipid concentration (µg/ml)	Crude concentration (mg/ml)	Lipid concentration (mg/ml)	Crude concentration (mg/ml)

	10,50,100,150, 200	1	1, 2	2
Chlorococcum (CCM)	10,50,100,150, 200	1	2.5, 5	5

Statistical analysis

All the assays were carried out in triplicate to validate the reproducibility. The data was analyzed by using XLSTAT. The p-value for in-vitro Assay was calculated using Independent two-sample t-test. A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

GC-MS Analysis

Lipids, acids, alcohol, and some unknown components were identified in both CV and CCM lipid samples (Figures S4, S5, and S6). Some lipid peaks were found to be common in both CV and CCM (Table 2). Neophytadiene, methyl 18-fluoro-octadecanoate, methyl 3-cis9-cis12cis-octadecatrienoate, hexadecanoic acid, methyl heneicosanoate, tridecanoic acid, methylmyristate, cis-10-heptadecenoic acid, palmitic acid, TMS derivative, 9,11-Ooctadecadienoic acid, methyl esters were solely found in CCM, and methyl 4,7,10,13-hexadecatetraenoate, methyl stearate, pentadecanal, cis-10-heptsdecenoic acid, Octadecanoic acid, 9,12,15-octadecatrienoic acid, heptadecanoic acid, ethyl ester, cis-5,8,11,14,17-eicosapentaenoic acid were exclusively found in CV.

Table 2 Comparaisons of fatty acids with standards.

R. Time	15.897	18.306	19.504	19.969	20.616	21.316	21.482	22.587	23.442	23.53	24.998	25.107	25.398	27.321
Name	Methyl Undecanoate	Methyl Tridecanoate	Methyl Tetradecanoate	Methyl Myristate	Methyl Pentadecanoate	Methyl Palmitoleate	Methyl Palmitate	Methyl Heptadecanoate	Gamma-Linolenic acid	Methyl Stearate	Methyl cis 5,8,11,14 eicosapntaenoate	Methyl Arachdonate	Methyl Heneicosanoate	Cis 4,7,10,13,16,19-Docosahexanoate
C. variabilis	-	-	-	+	+	+	+	+	+	-	+	-	-	-
Chlorococcum sp.	-	-	-	-	-	+	+	-	+	+	-	-	-	-

+ Present, - Absent

Assessment of CT production- *in-vitro*

In the present study, MDR *V. cholerae* SRK 19 was treated with lipid extracts (10–200 µg) and crude freeze-dried biomass derived from two microalgae species – *C. variabilis* (CV) and *Chlorococcum* sp. (CCM) - to evaluate their effects on bacterial viability and cholera toxin (CT) production. No significant growth inhibition was observed with either the lipid extracts or crude biomass, as confirmed by viability assays. However, a marked reduction in CT production was observed following treatment with both the lipid extracts and crude biomass of the microalgae (refer the supplementary materials, Table S3).

As shown in Figure 1(a), increasing concentrations of lipid extracts correlated with a dose-dependent decrease in CT production, indicating an inverse relationship. CT inhibition was calculated using the formula described earlier. At the highest concentration of lipid extract (200 µg), CT inhibition was 85.73% for CV and 98.07% for CCM, with statistically significant differences between the treated and untreated controls (**$p < 0.01$**, two sample t-test). *In vitro* assay, both the lipid extract and crude biomass from CCM resulted in a more pronounced reduction in CT production compared to CV. The highest inhibition rate (97.9%) was observed with 150 µg/ml of CCM lipid extract (Table 3), also statistically significant (**$p < 0.01$**, refer the supplementary materials Table S6), indicating a potent anti-virulence effect. Significant differences between CV and CCM values were observed at concentrations of **50, 100, 150, and 200 µg/ml** ($p < 0.05$). The strongest evidence for difference was at **150 µg/ml ($p = 0.0089$)**. However, no significant difference was found at **10 µg/ml ($p = 0.0607$)** or with **1 mg crude extract ($p = 0.0815$)**.
These findings suggest that crude biomass of CCM can be employed as an effective antivirulent agent, potentially reducing the need for labour-intensive and costly lipid extraction processes.

Importantly, neither the microalgal lipid extracts nor crude biomass exhibited antimicrobial activity against *V. cholerae*, as evidenced by the absence of log reduction in bacterial growth (Figure 1b). This indicates that the reduction in CT production is not attributable to decreased bacterial viability, but rather to a specific anti-virulence effect.

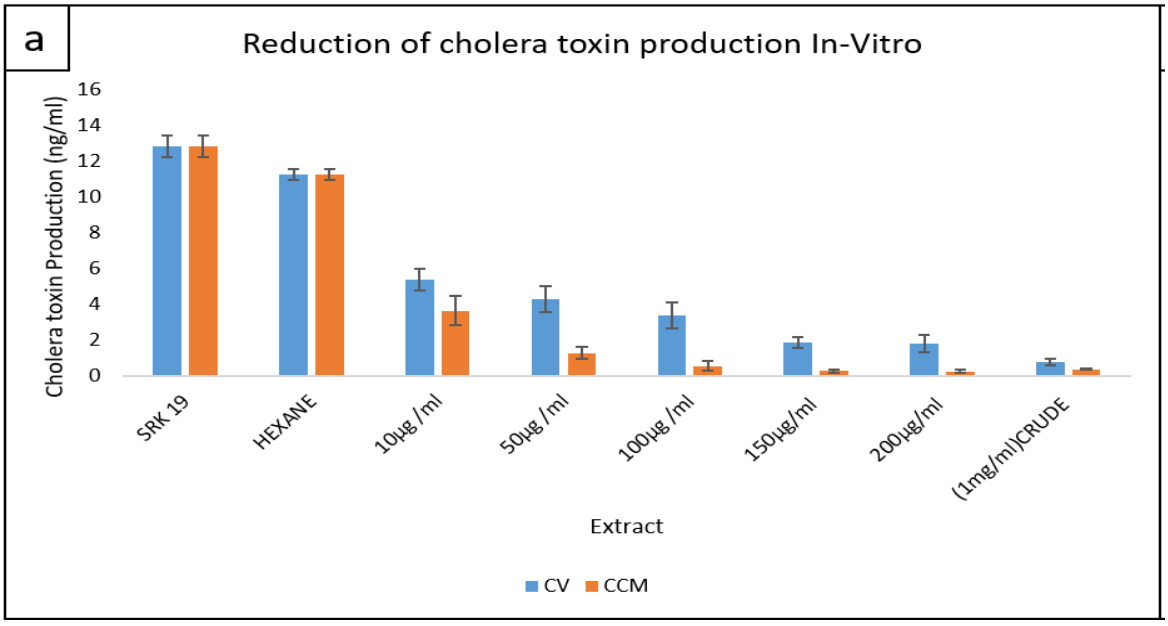

Figure 1(a) Shows the dose dependent assay with lipid extracts of CV and CCM along with the crude biomass on the cholera toxin production in *V. cholerae* SRK 19 strain. Blue and orange bars indicate CT production in presence and absence of lipid extracts and crude biomass from CV and CCM respectively. Error bars represent mean ± SD.

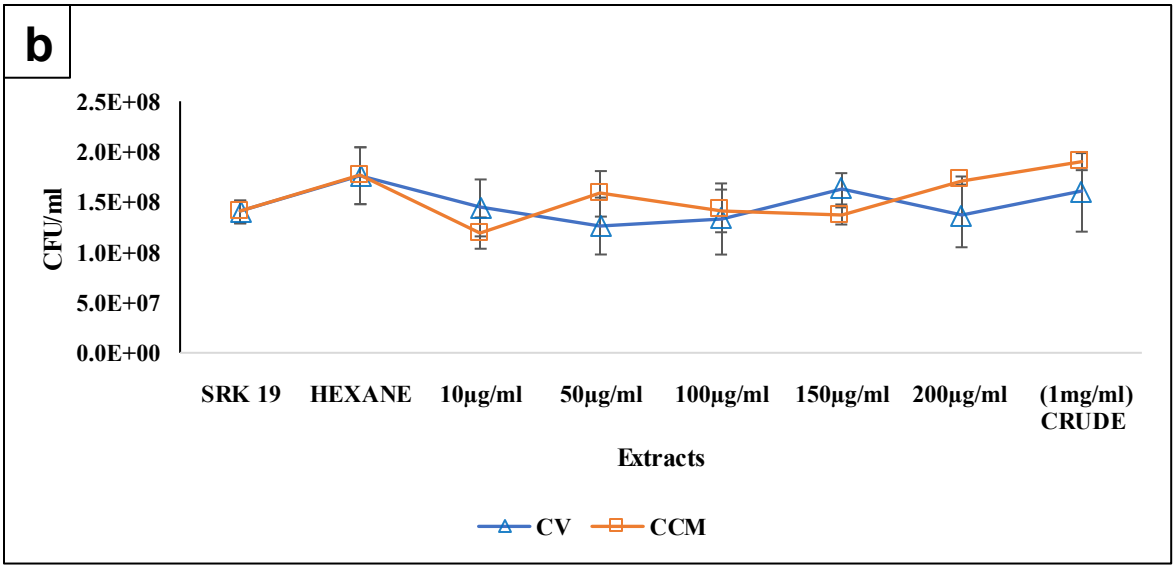

Figure 1 (b) Growth (CFU/ml) of SRK19 in presence and absence of lipid extracts and crude biomass of CV (blue line) and CCM (orange line). Error bars represent mean ± SD

Table 3 *in-vitro* CT inhibition (%) by total lipid extracts and crude biomass of CV and CCM

Extracts(µg/ml)	#CT inhibition (%)		P-value (independent two-sample t-test)
	CV	CCM	
10	57.88±6.47	71.66±6.56	0.0607
50	66.44±7.22	90.23±2.27	0.0214*
100	73.56±6.54	95.67±2.31	0.0188*
150	85.49±2.35	97.91±0.54	0.0089*
200	85.62±4.22	98.08±0.59	0.0340*
1 mg crude (contains 3 µg of total lipid)	93.99±1.66	97.08±0.23	0.0815

#Values represent the mean of triplicates.

Assessment of CT production- *In- vivo*

The fluid accumulation (FA) assay was performed in rabbits using various concentrations of microalgal lipid extracts and crude biomass mixtures, alongside positive and negative controls. The FA ratio (measured as ml of fluid per cm of loop length) was calculated for each condition (Table 4). In almost all treatment groups, fluid accumulation was lower compared to the positive control, which received *V. cholerae* SRK-19 alone. Notably, except for the negative control, all loops showed the presence of blood-tinged fluid (refer the supplementary materials, Figure S3).

In the rabbit ileal loop assay (RILA) for CCM, rupture of one loop was observed at the highest lipid concentration (5 mg/ml), although sufficient fluid was collected for subsequent analysis (Figure 2a). Loop rupture under similar experimental conditions has also been reported in previous studies (Everest et al., 1993). In contrast, all loops in the RILA for CV were intact and successfully retrieved (Figure 2b).

ELISA results demonstrated a decrease in CT production by *V. cholerae* in the ileal loops, correlating with increasing concentrations of lipid extracts, and most significantly with crude biomass treatment. This trend closely mirrored *in vitro* results (Figures 3 and 4). Importantly, colony-forming unit (CFU) counts showed no significant reduction across treatments with CCM or CV, suggesting that bacterial growth was not compromised. This indicates that the reduction in CT production was not due to bactericidal effects, but rather a specific antivirulent action, avoiding the potential risk of triggering antibiotic resistance mechanisms (refer the supplementary materials, Table S4 and S5).

Crude biomass co-cultured with *V. cholerae* reduced CT production by 72.68% in case of 2 mg CV, and 73.71% in case of 5 mg CCM. In contrast, treatment with total lipid extracts resulted in CT inhibition of 12.79% (CV, 1 mg/ml), 35.19% (CV, 2 mg/ml), and 40.17% (CCM, 2.5 mg/ml) (Table 4). These findings suggest that crude biomass may be a more reliable and cost-effective antivirulent agent compared to purified lipid extracts.

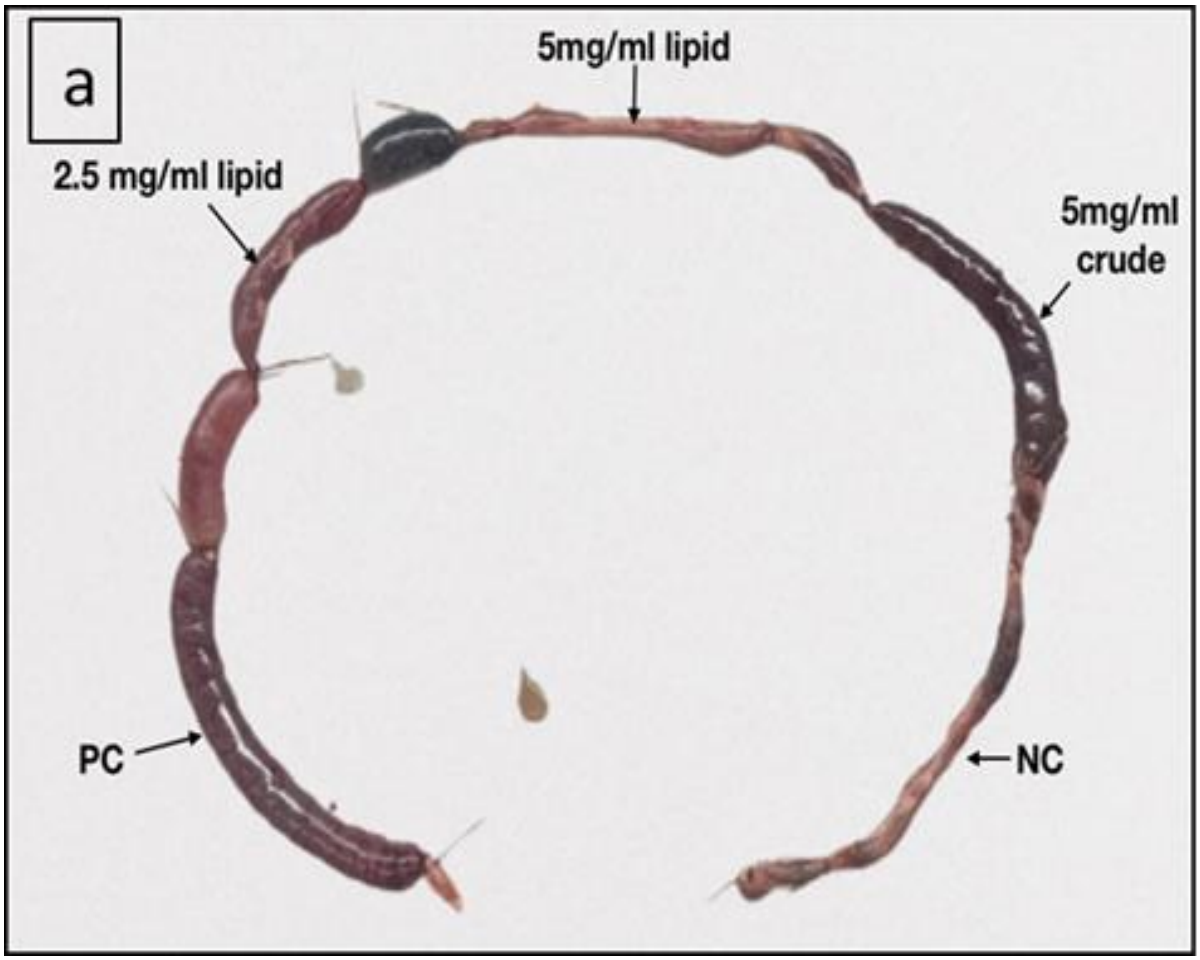

Figure 2 (a) *In-vivo r*abbit ileal loop showing fluid accumulation by SRK-19 with and without the presence of lipid extract and crude biomass by CCM.

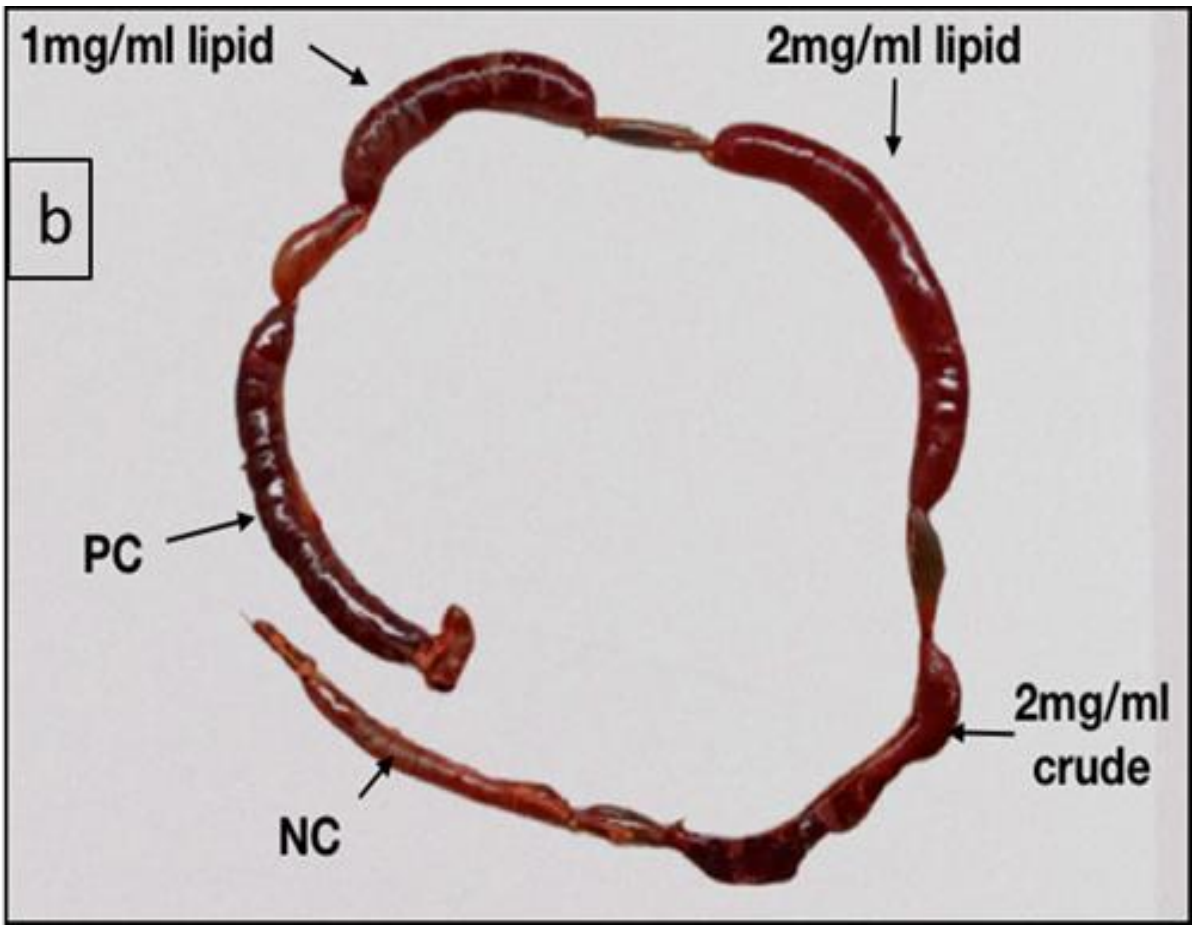

Figure 2 (b) *In-vivo r*abbit ileal loop showing fluid accumulation by SRK-19 with and without the presence of lipid extract and crude biomass by CV.

Table 4 *in-vivo* FA ratio CT inhibition (%) by total lipid extracts and crude biomass of CV and CCM

Microalgae	Concentration (/ml)	FA ratio	CT inhibition (%)
CV	Total Lipid 1mg	1.22	12.79
	Total Lipid 2mg	1.50	35.19
	Crude 2mg	0.73	72.69
CCM	Total Lipid 2.5mg	0.76	40.16
	Crude 5mg	0.31	73.51

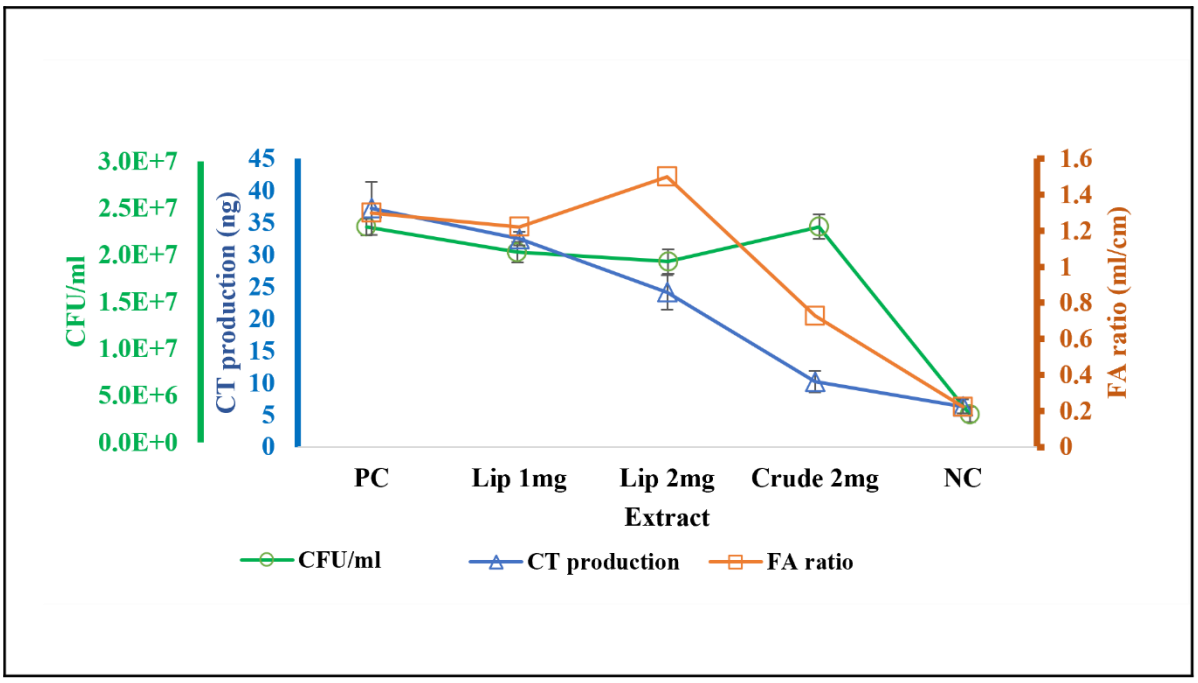

Figure 3 Shows the anti-virulence activity of CV with lipids and crude biomass on the O1 EL Tor *V. cholerae* SRK19 strain. The green line indicates CFU/ml, the blue line indicates CT production and the orange line indicates FA ratio. Error bars represent mean ± SD.

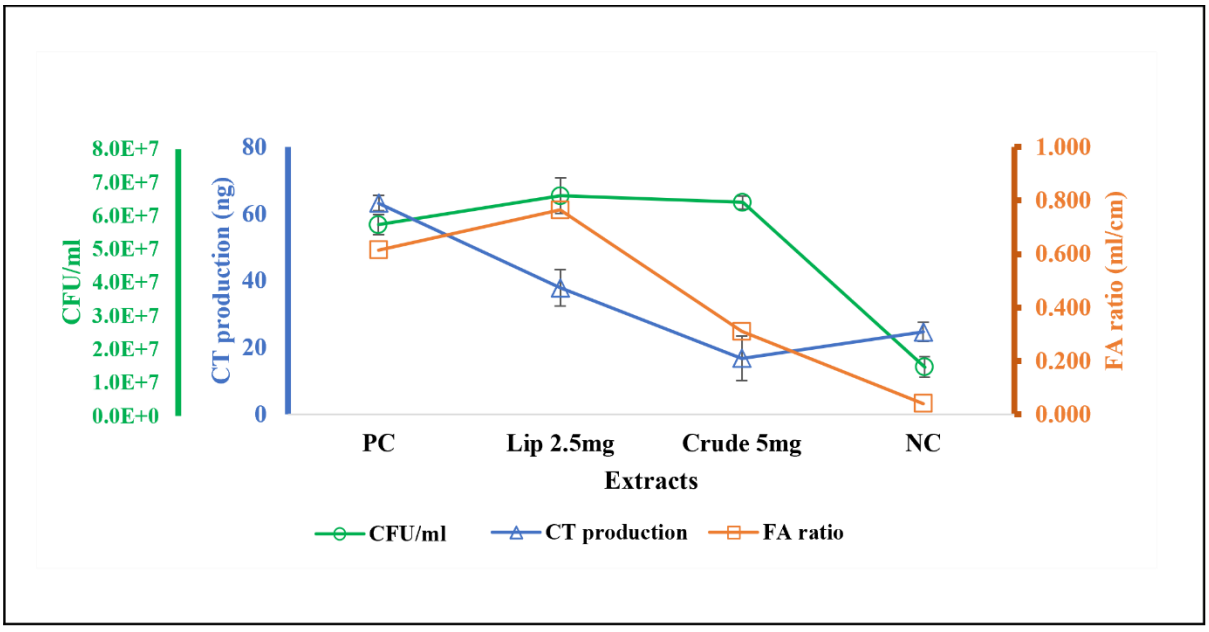

Figure 4 Shows the anti-virulence activity of CCM with lipids and crude biomass on the O1 EL Tor *V. cholerae* SRK19 strain. The green line indicates CFU/ml, the blue line indicates CT production and the orange line indicates FA ratio. Error bars represent mean ± SD.

The emergence of multidrug-resistant (MDR) *Vibrio cholerae* strains has severely limited the efficacy of conventional antibiotic treatments, necessitating alternative strategies that target bacterial virulence instead of viability. This study investigated the antivirulent properties of lipid extracts and crude biomass derived from two microalgae—*C. variabilis* (CV) and *Chlorococcum* sp. (CCM)—on CT production in the MDR strain *V. cholerae* SRK-19.While no significant bacterial growth inhibition was observed following treatment with either lipid extracts or crude biomass, both interventions resulted in a marked and dose-dependent reduction in CT production, with CCM consistently outperforming CV. Notably, at the highest tested concentration (200 µg/ml), CT inhibition reached 85.73% for CV and 98.07% for CCM. Independent two sample t-test analysis confirmed the statistical significance of these findings ($p < 0.01$), supporting the hypothesis that lipid extracts act via a specific anti-virulence mechanism without inducing selective pressure on bacterial survival.

The *in-vivo* rabbit ileal loop assay further validated these results. Fluid accumulation (FA) ratios were significantly reduced in treated groups compared to the positive control, indicating effective suppression of toxin-induced pathophysiological effects. In several cases, loops treated with high-dose CCM lipid ruptured, a phenomenon previously noted in literature (Everest et al., 1993), possibly due to rapid neutralization of enterotoxins and mucosal irritation.

Microalgae such as CV and CCM are known reservoirs of bioactive compounds, including saturated and polyunsaturated fatty acids (PUFAs) like oleic acid, gamma-linolenic acid, palmitic acid, and linolenic acid—all of which may interfere with *V. cholerae*'s virulence pathways (Zhong et al., 2008). Previous studies have suggested that fatty acids such as palmitoleic and gamma-linolenic acid inhibit *toxT*, a transcriptional activator of the *ctxAB* and *tcpA* genes (Lowden et al., 2010). These genes are essential for CT and pilus expression, which are most important virulence factors and central to the organism's pathogenicity.

The current study reinforces earlier findings (S. Chatterjee et al., 2021) that lipid extracts from *C. variabilis* and related species reduce colonization and suppress virulence gene expression in *V. cholerae*. Moreover, crude biomass from both CV and CCM showed higher efficacy than isolated lipids in reducing CT production—over 95% inhibition was observed with 1 mg of crude biomass, suggesting that other, non-lipid bioactives may contribute to this effect. These findings not only emphasize the therapeutic potential of microalgae but also highlight the advantage of using crude extracts over purified fractions in terms of cost, scalability, and efficacy.

Crucially, *Chlorella* spp. are FDA-designated GRAS substances and have a longstanding history of safe use in humans. Their established safety profiles, coupled with their demonstrated anti-virulence effects, position microalgae as attractive candidates for future development of sustainable, resistance-free therapies for cholera and potentially other bacterial infections.

CONCLUSIONS

This study demonstrated that both lipid extracts and crude biomass from *Chlorococcum* sp. (CCM) and *Chlorella variabilis* (CV) significantly inhibited cholera toxin (CT) production *in-vitro* and *in-vivo*, without affecting bacterial viability. Crude biomass showed greater efficacy than lipid extracts, suggesting the involvement of additional bioactive compounds.

Given the GRAS status of *Chlorella* spp. and the sustainability of microalgal cultivation, these findings highlight the potential of microalgae-based antivirulent therapies as an alternative to antibiotics. Large-scale coastal cultivation could support sea agriculture while reducing pressure on land resources and minimizing antibiotic resistance.

Future work will focus on developing an ORS formulation enriched with optimal concentrations of these microalgal extracts as a novel, natural treatment against cholera, characterization of the specific bioactive compounds involved, delineation of the molecular pathways affected, and validation of these effects in higher animal models or clinical settings.

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Data availability

The data that has been used is confidential.

Conflict of interest

The authors report no commercial or proprietary interest in any product or concept discussed in this article. The authors declare no conflict of interest.

Authors contributions

Sweta Jaiswal, Nikita Vadadoriya and Abdul Nasir: Investigation; Data curation; Formal analysis. **Ramalingam Dineshkumar**: Supervision. **Neeraj Khatri and Sachin Raut**: Methodology; Formal analysis; Supervision. **Saumya Raychaudhuri:** Funding acquisition; Project administration; Resources; Writing - original draft. **Shruti Chatterjee:** Conceptualization; Methodology; Funding acquisition; Writing -original draft; Writing - review & editing. **Soumya Haldar:** Conceptualization; Methodology; review & editing; Funding acquisition; Investigation; Methodology; Project administration; Resources.

Supplementary Materials

S.2.Materials and Methods

S.2.1 Microalgae culture and growth conditions: Chlorococcum spp. (CCM) and Chlorella variabilis (ATCC PTA 12198) (CV), were collected from the microalgal collection of Department of Applied Phycology and Biotechnology, CSIR-CSMCRI Bhavnagar, Gujarat (Chatterjee et al., 2021). C. variabilis was grown in modified Zarrouk's media (supplementary table S1) under conditions like a 12/12 (dark/light) regime at 28±2°C with aerators. Single culture was maintained, and 1.5 L of microalgal inoculum was added to 10 L of fresh media (Mohammadi & Arabian, 2022; Puspanadan et al., 2018) and incubated for 21 days (Pancha et al., 2015). Chlorococcum spp. was cultured in BG11 (Table S2) and incubated for 15 days at room temperature in a 12/12 (dark/light) regime with an aerator (Harwati et al., 2012). Biomass was harvested after being centrifuged at 4°C for 10 min at 8000 rpm; pellets were washed with distilled water and freeze-dried (lyophilized) for further use (Puspanadan et al., 2018).

Supplementary table S1: Components of Zarrouks media

Components	g/ltr
$NaNo_3$	2.5
$MgSo_4 7H_2O$	0.2
$CaCl_2\ 2H_2O$	0.04
NaCl	1.0

$NaHCO_3$	16.08
K_2HPO_4	0.5
$FeSO_47H_2O$	0.01
K_2SO_4	1.0
EDTA	0.08

Supplementary table S2: Components of BG11 media

Components	g/ltr
$NaNo_3$	1.5
$MgSo_47H_2O$	0.075
$CaCl_2$ $2H_2O$	0.036
Citric Acid	0.006
Na_2Co_3	0.02
K_2HPO_4	0.04
EDTA	0.001
$(NH_4)_5Fe(C_6H_4O_7)_2$	0.006
A5 Components (g/ltr) H_3BO_3 2.8 $MnCl_24H_2O$ 1.81 $ZnSO_47H_2O$ 0.222 $NaMaO_42H_2O$ 0.39 $CuSO_45H_2O$ 0.079	1.0ml

S.2.4.1 Cell-free Supernatant (CFS) Collection:
Cell-free supernatant (CFS) was collected by inoculating a single colony in AKI broth overnight at 37°C and 120 rpm. OD was then taken at 600 nm, adjusting it to 0.1. The inoculum was then diluted 10 times in PBS, followed by fresh inoculation by taking 100 µl of culture, mixed with 3 ml of freshly made AKI broth, and kept for 4 hours in a stationary condition at 37°C, followed by 4 hours at a shaking condition at 37°C and 120 rpm. This 8-hour grown culture was centrifuged at 20,000 rpm for 20 min, the supernatant was taken, filtered by syringe filter (0.2µm pore size), used for ELISA (if not immediately stored at -20 °C). The pellet was discarded (López et al., 2022).

2.5.3 Passage assay
A passage assay was conducted using the lab-grown Vibrio (SRK-19) to increase the virulency.
New Zealand white rabbits were prepared by fasting them for 12–24 hours prior to surgery and being fed only water ad libitum. on the day of the experiment, rabbits were anesthetized with isoflurane, a volatile anesthetic (NCBI, 2023), maintaining aseptic conditions. A midline incision was made below the center of the abdomen. By dissecting through the muscles and peritoneum, a fragment of the ileal was carefully isolated and tied up with silk ligatures to create intestinal loops, avoiding any damage to the blood vessels or mesentery (Chatterjee et al., 2013; DE S N et al., 1953). Two loops (~8 cm long) and one interloop (~4cm

long) were created. One of the loops was injected with 500 µl of lab-grown SRK-19 inoculum (10^7 CFU/mL) while the control loop was injected with 500 µL of phosphate-buffered saline (PBS). The abdomen was then sutured with two layers of thread and 1 ml of Meloxicam vet analgesic was given subcutaneously. Following recovery from anesthesia, the animal was kept without food or water. Approximately 18 hours post-surgery the ileal loop was excised, fluid accumulation in each loop was examined. The rabbits were then humanely euthanized via cardiac puncture (Chatterjee et al., 2013; Anvari Sh et al., 2012). The fluid accumulated in each loop was collected in separate tubes, and the fluid accumulation (FA) ratio was calculated as milliliters of fluid per centimeter of loop length (Chatterjee et al., 2013; DE S N et al., 1953).
100µl of the collected fluid was then serially diluted until 10^4 CFU/ml for a viable bacterial count and plated onto LB agar and TCBS agar, followed by overnight incubation at 37°C. Colonies of SRK-19 grown on TCBS plates were subsequently inoculated into AKI broth for toxin production. The resulting culture supernatant (CFS) was then collected for toxin quantification.

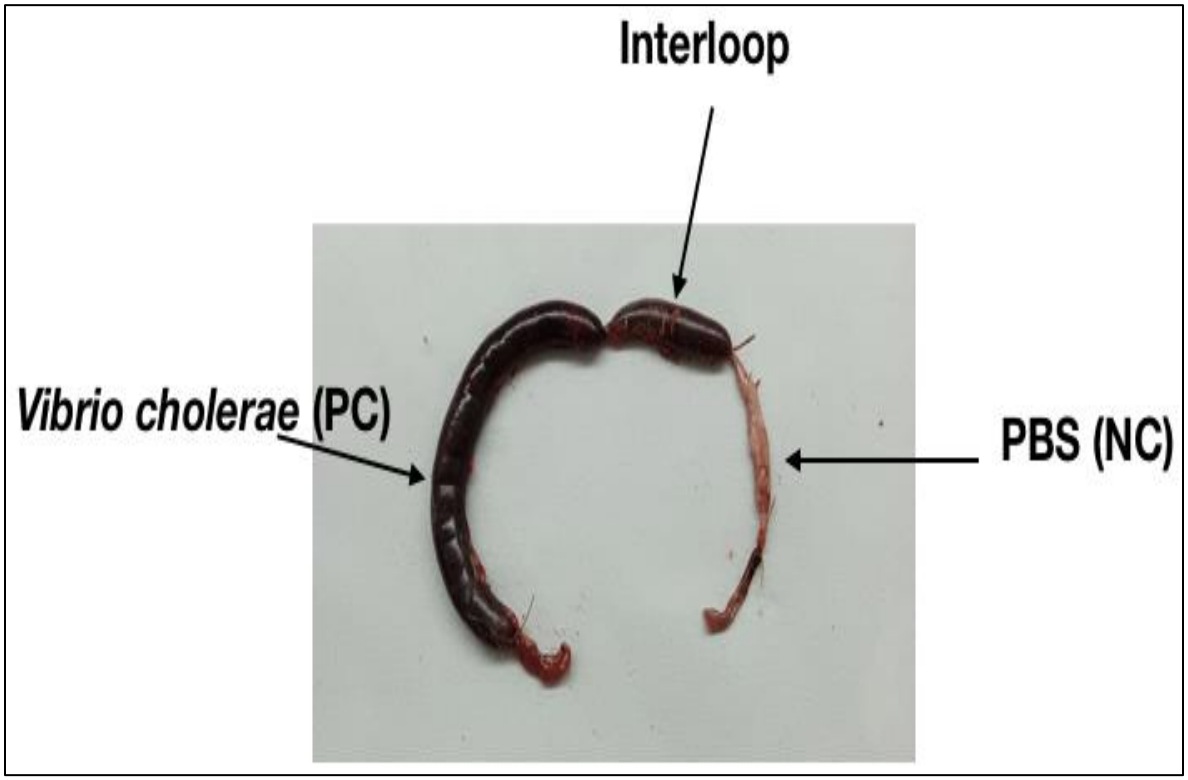


Figure S1: Passage Assay

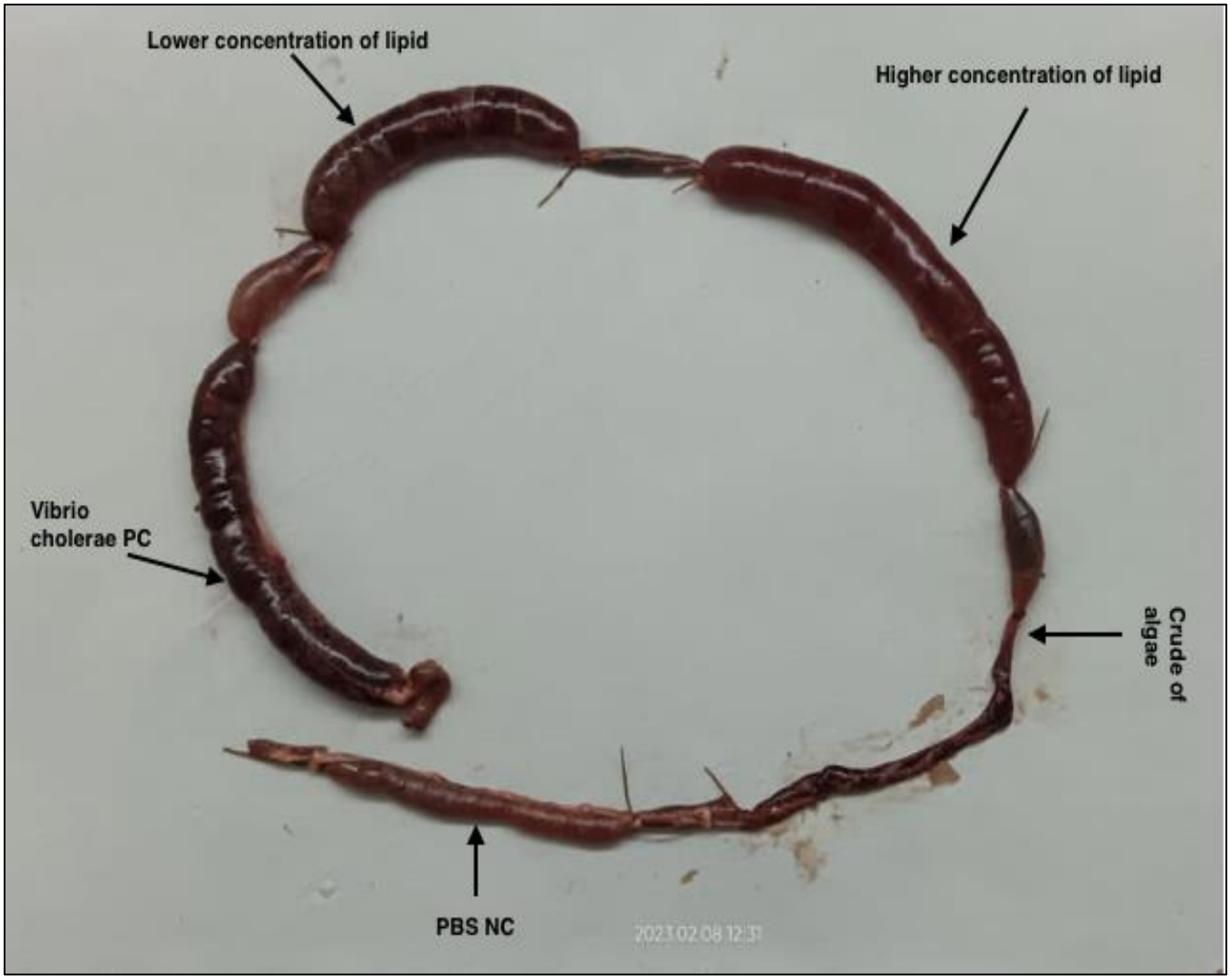
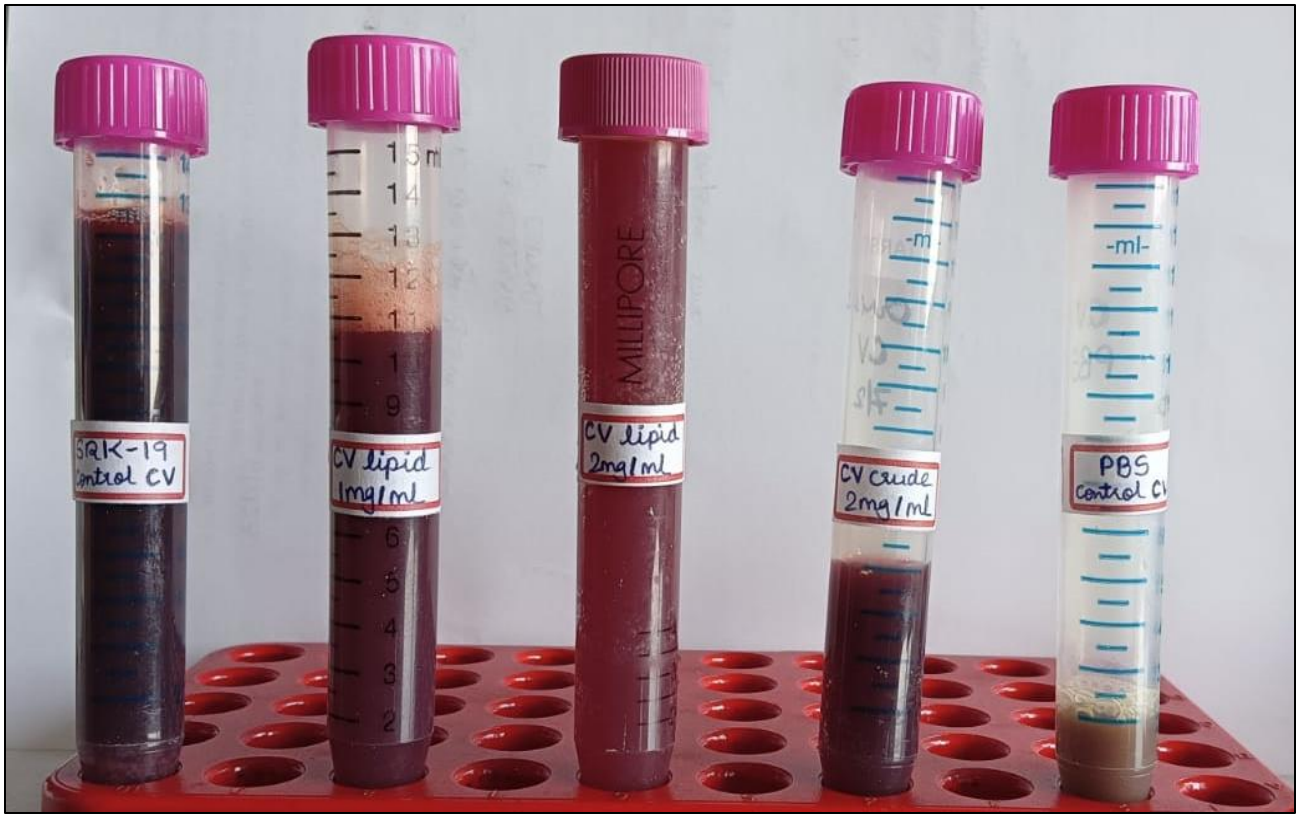


Figure S2: Order of inoculation of V. cholerae with and without compounds



1. **Figure S3: Fluid accumulation in CV RILA**

S3. Results:
S3.1 GC-MS Analysis

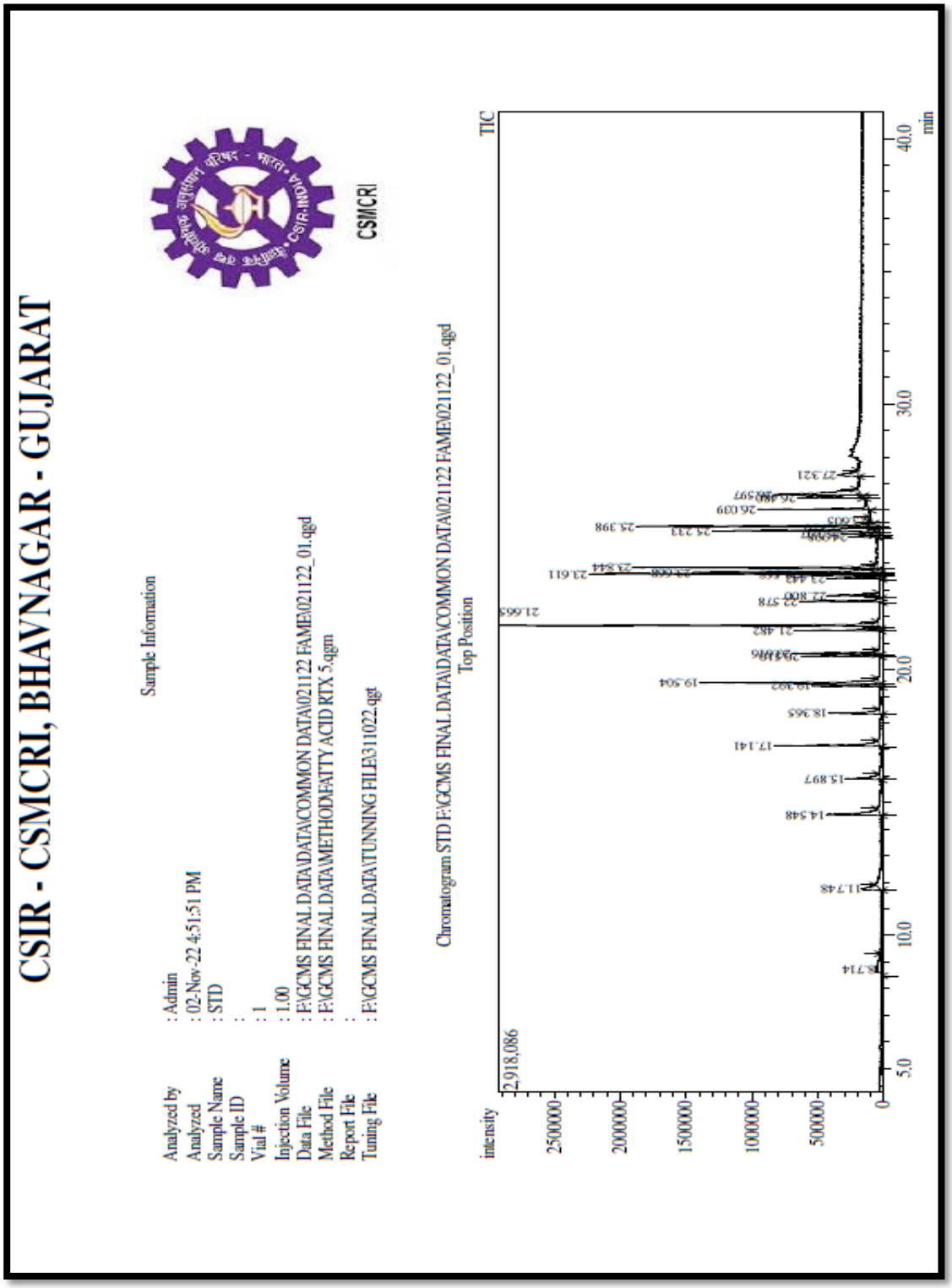


Figure S4: GC-MS profile of standard lipid (Supelco37 F.A.M.E MIX)

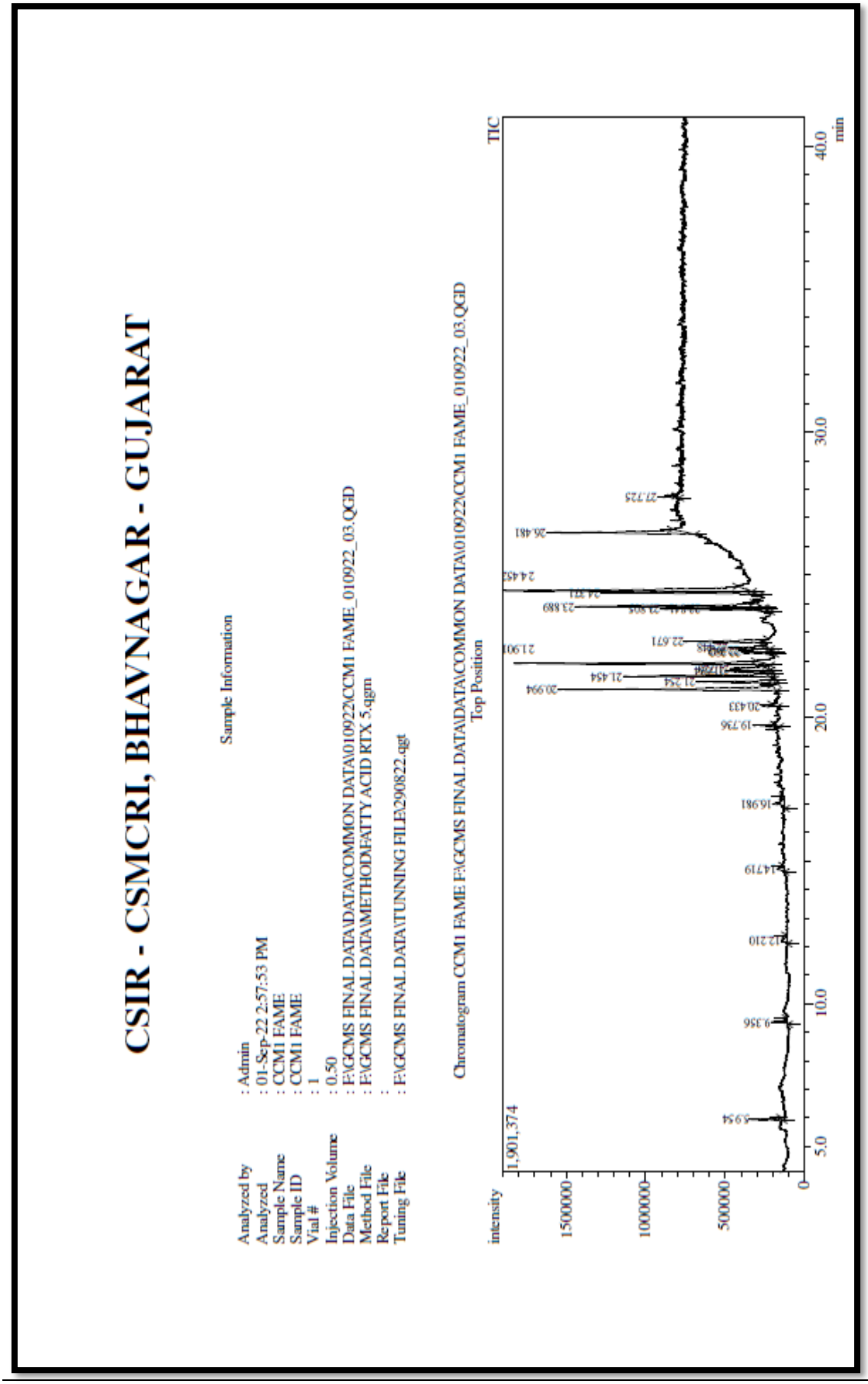


Figure S5: CCM Lipid GC/MS Sample report

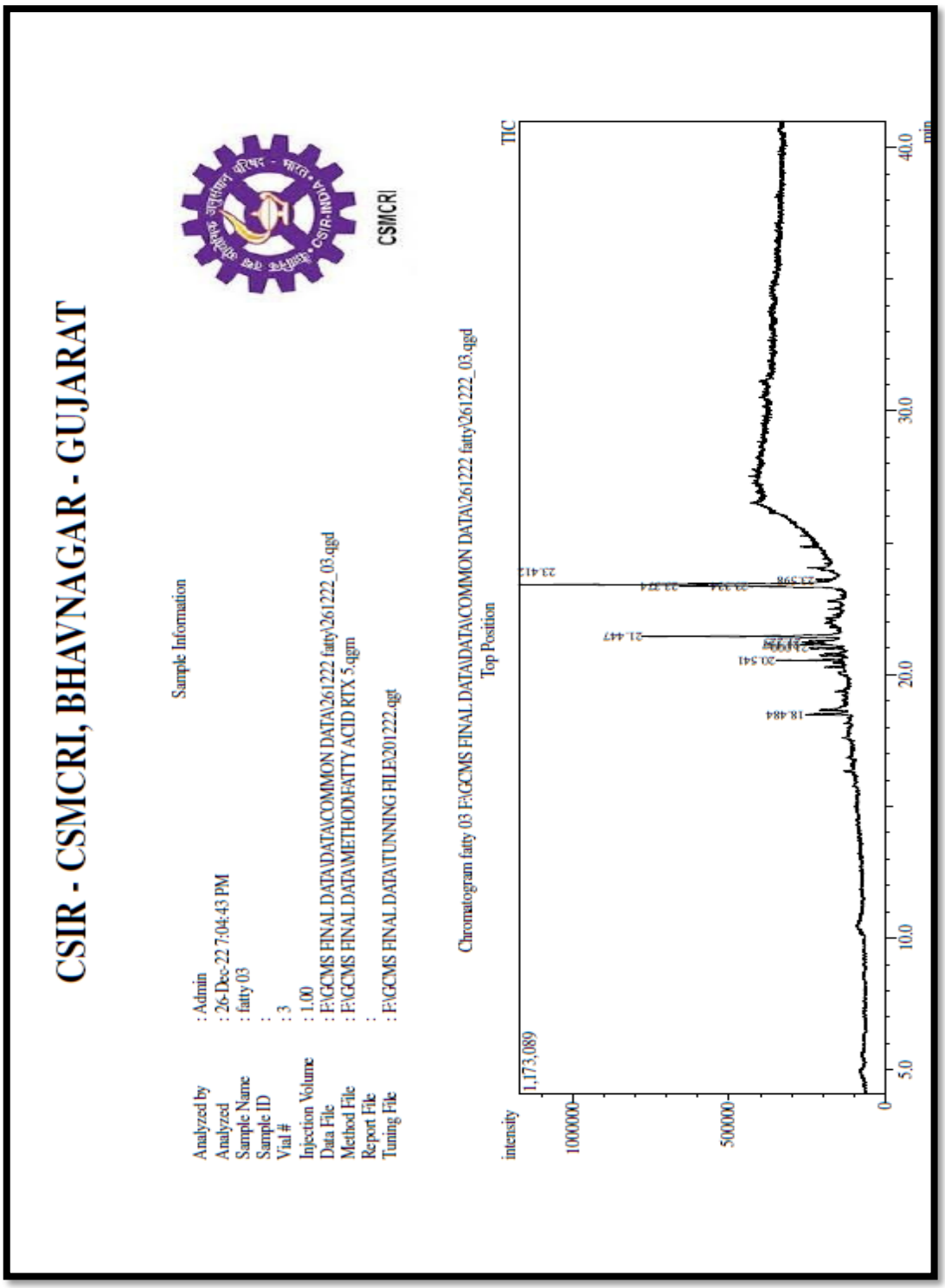


Figure S6: CV Lipid GC/MS Sample report

Table S3: *In-vitro* CT production and CFU/ml in presence and absence of extracts

Extracts	CV		CCM	
	CT (ng)	CFU/ml	CT (ng)	CFU/ml
SRK 19	12.80899	132000000	12.80899	132000000
HEXANE	11.22097	156000000	11.22097	156000000
10µg	5.370787	164000000	3.625468	130000000
50µg	4.269663	106000000	1.258427	142000000
100µg	3.363296	158000000	0.546816	156000000
150µg	1.857678	152000000	0.269663	130000000
200µg	1.827715	114000000	0.247191	174000000
CRUDE	0.764045	188000000	0.374532	196000000

Table S4: *In-vivo* CT production, CFU/ml and FA ratio in presence and absence of extracts and crude biomass of CV

	CFU/ml (10^6)	CT production	FA ratio
PC	23	37.277	1.3
Lip 1mg	20.3	32.508	1.222222
Lip 2mg	19.3	24.158	1.5
Crude 2mg	23	10.182	0.727273
NC	3	6.337	0.222222

Table S5: *In-vivo* CT production, CFU/ml and FA ratio in presence and absence of extracts and crude biomass of CCM

Fluid	CFU/ml (*10^6)	CT production	FA ratio
PC	57	63.265	0.615
Lip 2.5mg	66	37.854	0.765

Crude 5mg	64	16.758	0.310
NC	15	24.635	0.040

Table S6: p-value for ***in-vitro*** experiment

Concentration (µg/ml)	CV_mean	CV_sd	CCM_mean	CCM_sd	n	t-statistic	degrees of freedom	p-value
10	57.88	6.47	71.66	6.56	3	-2.59	4	0.0607
50	66.44	7.22	90.23	2.27	3	-5.444	2.39	0.0214
100	73.56	6.54	95.67	2.31	3	-5.521	2.49	0.0188
150	85.49	2.35	97.91	0.54	3	-8.922	2.21	0.0089
200	85.62	4.22	98.08	0.59	3	-5.065	2.08	0.034
1 mg crude	93.99	1.66	97.08	0.23	3	-3.194	2.08	0.0815

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