0188NF2018

FORM 2

THE PATENTS ACT, 1970

(39 of 1970)

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The Patents Rules, 2003

PROVISIONAL SPECIFICATION

(See Section 10 and Rule 13)

ANTI-VIRULENCE FORMULATION FROM MICROALGAL LIPIDS AGAINST VIBRIO CHOLERAE AND PROCESS FOR THE PREPARATION THEREOF

COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH,

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The following specification describes the invention:

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FIELD OF THE INVENTION

The present invention relates to an anti-virulence formulation from microalgal lipids useful against *Vibrio cholerae* and a process for the preparation thereof. In particular, the present invention relates to a formulation comprising of 9-*cis*,12-*cis*-Linoleic acid (linoleic acid), 9,12 Octadecadienoic acid (linolenic acid), *cis,cis,cis*-6,9,12-Octadecatrienoic acid (γ-linolenic acid), *cis*-8,11,14-Eicosatrienoic acid (dihomo-gamma-linolenic acid) and cis-9-Octadecenoic acid (oleic acid) isolated from *Chlorella variabilis* which exhibits anti-virulence activity against enteropathogenic bacteria. More specifically, the developed formulation works against recently emerged variant of *Vibrio cholerae* strain causing severe diarrhea. Development of novel antibiotics has almost ceased, at least against Gram-negative bacteria, and the prospects for a reversal of this trend are bleak. Due to multi-drug resistance (MDR) occurrence, pathogenic Gram-negative *Vibrio cholerae* strains, which cause diarrhea, are resistant to most of the classical antibiotics. A possible novel approach is to target virulence regulation instead of viability of the bacteria. Strategies that specifically target virulence factors are those that are non-essential for growth of bacteria. Anti-virulence strategies have advantage to preserve the host endogenous microbiome, and exert less selective pressure, which may result in decreased resistance. The present invention relates to the utilization of formulation made from edible microalgae for the purpose of reducing infection caused by *Vibrio cholerae* especially in humans.

BACKGROUND OF THE INVENTION

Multi-drug resistance phenomena is very common in clinical strains. According to WHO by 2050, if no action is taken against MDR, it will kill more people each year than cancer. According to WHO estimation the prevalence of cholera is 1.3 to 4.0 million and 1,43,000 deaths per year (WHO, 2018). Humans get infected with *Vibrio cholerae via* contaminated water or food which causes severe diarrhea and dehydration known as cholera. Among 200 serogroups of *Vibrio cholerae*, the O1 (classical and El Tor biotypes) and O139 serogroups are mainly responsible for cholera epidemics. The strains belonging to other serogroups, referred as non-O1/non-O139, are associated with sporadic cases of diarrhea. The emergence of multi-drug resistant *Vibrio cholerae* in O1 and O139 serogroup is major concern. Recently, a new variant of

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Vibrio cholerae O1 El Tor biotype with attributes of classical biotype has been isolated from hospitalized patients in India and Bangladesh with more severe diarrhea than that caused by typical El Tor strains. WHO records show that variant strains rapidly spread to Asian countries as well as East African countries (www.who.int/cholera/2009).

The major virulence factors in *Vibrio cholerae* are cholera toxin (CT) and toxin-co-regulated pili (TCP), encoded by the *ctx* and *tcp*A genes, respectively. Cholera toxin belongs to the superfamily AB5 toxin and structurally similar to the *Escherichia coli* heat-labile enterotoxins: LT and LT-II. Whereas, toxin-co-regulated pili help in early colonization of bacteria in the host intestine and establish the infection. Antimicrobial agents are usually given to patients with severe cholera but, its effectiveness can be hampered due to the emergence of antimicrobial resistant *Vibrio cholerae* strains. The FDA has approved oral cholera vaccines, which are costlier and nevertheless the capacity of these vaccines to control rapidly spreading outbreaks is limited.

New therapeutics and/or prophylactics are required to tackle multi-drug resistant pathogens. It may include inhibition of major virulence factors like cholera toxin, toxin-co-regulated pili and function including inhibition of toxin production, regulation of virulence expression as well as bacterial adhesion etc., which are crucial to initiate an infection and cause disease. Anti-virulence strategies have advantage to preserve the host endogenous microbiome, and exert less selective pressure, which may result in decreased resistance.

Reference may be made to the paper entitled "Antimicrobial property of water and ethanol extract *Chlorella vulgaris*: A value-added advantage for a new wound dressing material" Microbiology (2015) 22, 399–401, which discloses the antimicrobial property of *Chlorella variabilis* towards three Gram-positive bacteria: *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, and two Gram-negative bacteria: *Pseudomonas aeruginosa* and *Escherichia coli*. Here bacterial growth inhibition has been studied by authors. Usually microalgae show antibacterial effect as reported. However, the present invention is not directed towards any formulation or compounds that exert any bacteriostatic or bactericidal activity, rather it relates to a novel formulation that exhibits anti-virulence properties against clinically

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active virulent cholera causing strains.

Reference may be made to the paper entitled "Uses of *Chlorella vulgaris* as antimicrobial agent and as a diet: the presence of bio-active compounds which caters the vitamins, minerals in general" (2015) 7, 185-190, which discloses *Chlorella vulgaris* contained flavanoids, tannin, phenolic compounds, terpenes, cardiac glycosides, saponins and carbohydrates, which has major role as a useful precursor to obtain various bioactive compounds. This algal species exhibits its maximum inhibition property against *Klebsiella* sp. This study show the different essential compounds present in the *Chlorella* sp. which are useful as bioactive components. However, the present invention is not directed towards any formulation or compounds that exert any bacteriostatic or bactericidal activity, rather it relates to a novel formulation that exhibits anti-virulence properties against clinically active virulent cholera causing strains.

Reference may be made to the paper 'Effect of microalgae commonly used in aquaculture on acyl-homoserine lactone quorum sensing', (2011), 317, which discloses that green microalgae *Chlorella saccharophilla* extracts inhibit quorum sensing regulated gene expression in three reporter strains. All these approaches are made to regulate virulence factors in pathogenic bacteria by quorum sensing regulation. In this present invention, microalgal species showed potential as anti-virulent agent but the approach of regulation of virulence factor is different. In *Vibrio cholerae* inhibition of virulence factors regulated by quorum sensing has not yet established by using any natural compounds so far.

Reference may be made to the paper entitled 'Small-molecule inhibitor of *Vibrio cholerae* virulence and intestinal colonization, Science, 2005, 310' which discloses the small-molecule virstatin interference with the homodimerization of the ToxT N-termini and thus blocks cholera toxin and toxin co-regulated pili production resulting in reduced colonization of *Vibrio cholerae* in mice. Unfortunately, virstatin-resistant *tox*T mutants containing a single amino acid substitution in the N-terminus have already been isolated. Virstatin inhibits dimerization of the transcriptional activator ToxT. Proc Natl Acad Sci USA 2007; 104. Virstatin is artificially synthesized and found resistant in non-O1/non-O139 strains. Resistance of a compound to

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particular virulence regulation may thus fail to develop future cholera drug. However, in the present invention total lipid extract of *Chlorella variabilis* does not have any potential to induce any resistance against virulence factor in *Vibrio cholerae* through generation study.

Reference may be made to the paper entitled 'Role of 6-Gingerol in reduction of Cholera Toxin activity *in vitro* and *in vivo*, Antimicrobs Agents & Chemotherapy, 2013, 57', which discloses the effect of ginger compound on the virulence of *Vibrio cholerae*. Natural compound is always required to mitigate this kind of problem, where side effect is less. However, induction of resistance has not been studied for gingerol.

Reference may be made to the paper entitled 'Capsaicin, a potential inhibitor of cholera toxin production in *Vibrio cholerae*' FEMS let, 2010, 306, which discloses capsaicin, which is capsaicinoids from chili pepper and can inhibit the cholera toxin production. However, working with capsaicin is very risky as it is the pungent component from chili and prolonged use may have negative effect on health.

Reference may be made to the patent, JP2011105715A, which discloses that different compounds *viz.* capsaicin, piperine, *trans*-anethole, 4-allyl-anisole from natural spices like red chili, sweet fennel, white pepper are significantly effective against CT production in *Vibrio cholerae* as prophylactic measure/or to stop cholera. Natural resources always have less side effects. However, in this cited patent, resistance pattern of these compounds was not studied for their long run use as anti-cholera drug.

Reference may be made to the paper entitled 'Effect of fatty acids and cholesterol present in bile on the expression of virulence factors & motility of *Vibrio cholerae*. Infect. Immun. 2007,75, which discloses that heterogenous bile components like unsaturated fatty acids; oleic acid, linoleic acid, and arachidonic acid have been shown to inhibit virulence factors in *Vibrio cholerae*. However, no formulation was developed/used in the cited study which showed inhibitory effect on virulence factors. Further, in the study author has used O1 classical *Vibrio cholerae* strains, responsible for early epidemics in the world. Whereas, in the present invention

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study has been done on the currently emerged variant of *Vibrio cholerae* strains, which is responsible for recent cholera epidemics in different parts of the world.

Reference may be made to the paper entitled 'Red bayberry extract inhibits growth and virulence gene expression of the human pathogen *Vibrio cholerae*, J. Antimicrob. Chemother, 2008, 61, which discloses that extract from *Myrica rubra* (red bayberry) is able to inhibit virulence expression in *Vibrio cholerae* and administration of extracts, 12 hr after also reduced the recovery of *Vibrio cholerae* by over 1000-fold relative to bacteria in infant mice. However, the active compounds from *Myrica rubra* are not yet identified.

Reference may be made to the patent, US8940740B2, which discloses small molecule inhibitors of bacterial motility and a high throughput screening assay for their identification such as quinazoline-2, 4-diamino analogs completely suppressing the motility of *Vibrio cholerae*, so it can indirectly diminish production of cholera toxin and other major virulence factors. For this study, from ~8000 synthetic compounds were screened through high-throughput screening and ten possible compounds were screened against motility. However, it does not mention anything about direct inhibition of cholera toxin production.

Reference may be made to the patent, US 2012/0157529 A1, for composition and method for prevention, mitigation or treatment of an enteropathogenic bacterial infection. Extract containing palmitoleic acid from macademia nuts was used for treating of enteropathogenic infection. They have followed expression of A/X regulatory protein to get inhibited by palmitoleic acid in *Vibrio cholerae*. However this study is done to study the expression of ToxT and ligand responsible for alteration in ToxT function and no direct study on the inhibition in cholera toxin production has been done.

Reference may be made to the patent, US2015/0361045 A1, reciting composition and method for prevention, mitigation or treatment of an enteropathogenic bacterial infection. For this study oleic acid was used for decreasing the expression of A/X regulatory protein of bacterial virulence factors thereby preventing, mitigating, or treating bacterial infection. However, the active

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compound has been shown as oleic acid and the source is synthetic. They also targeted *tox*T function for reducing virulence and only one fatty acid source has been used. Whereas, in the present invention a defined combination of fatty acids is used to inhibit the virulence in *Vibrio*.

Reference may be made to the patent, US2012/0142682 A1, reciting anti-virulence compounds inhibiting bacterial MONO-ADP-ribosyltransferase toxins. Bacterial virulence factors are from the maRT family of enzymes and found in CT, DT, pertussis toxin, heat-labile enterotoxin, C3-like exoenzyme, ExoA and other bacterial toxins. For this study, method was developed to check anti-virulence compound against cholix toxin. However, the target for this is very specific and not done on cholera toxin production inhibition as in the present case.

The aim of this study was thus inhibition of toxin production as a target without affecting the growth of the pathogenic *Vibrio cholerae* strains. The anti-virulence formulation does not lead to the development of resistant strains and thus helps in overcoming the drawbacks associated with MDR. Natural resources like microalgae, which contain high amount of protein, minerals, fatty acids, etc. are interesting candidate to study the anti-virulent capacity. Any formulation if developed from the microalgae, can act as drug/prophylactic with fewer side effects in human than synthetic antimicrobials. In the present study, microalgae was chosen to isolate the anti-virulence factors which also includes variety of beneficial compounds for human health as well as anti-virulent compounds to inhibit the exotoxin production in *Vibrio cholerae* for diarrhea affected people.

OBJECTIVES OF THE INVENTION

The main objective of the present invention is therefore to develop an anti-virulent formulation from edible microalgae which is active against enteropathogenic bacteria specifically against *Vibrio cholerae* infections in human.

Another object of the present invention is to develop a formulation that inhibits toxin production in toxigenic bacteria especially enteropathogenic bacteria such as *Vibrio cholerae*.

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Still another object of the present invention is to screen microalgae not functioning as antibiotic/antimicrobial agents; either bacteriostatic or bactericidal.

Yet another object of the present invention is to develop anti-virulent formulation against the newly evolved variant *Vibrio cholerae* strains producing high cholera toxin and being more virulent and which is recently reported to cause cholera epidemics.

Still another object of the present invention is to develop anti-virulent formulation against multi drug resistant *Vibrio cholerae* strains.

Yet another object of the present invention is to use total lipid from the selected microalgae against virulence factors like toxin production.

Still another object of the present invention is to study the induction of resistance development in the presence of total lipid extract of *Chlorella variabilis* against the cholera toxin production in *Vibrio cholerae*.

Yet another object of the present invention is to identify the composition of fatty acids in total lipid extract of *Chlorella variabilis*.

Still another objective of the present invention is to identify saturated and unsaturated fatty acids such as linoleic acid, linolenic acid, gamma linolenic acid, dihomo-gamma-linolenic acid, oleic acid, palmitic acid, methyl palmitate etc. in the total lipid extract of *Chlorella variabilis*. Amongst them unsaturated fatty acids showed potential as anti-virulent components.

Yet another object of the present invention is the development of a formulation involving mixture of unsaturated fatty acid as anti-virulent agent against enteropathogenic bacteria, specifically against *Vibrio cholerae*.

SUMMARY OF THE INVENTION

The present invention relates to the utilization of isolated species of microalgae *Chlorella variabilis* ATCC No PTA-12198 for use in the development of anti-virulent compounds. The total lipid extract of *Chlorella variabilis* essentially consisting of saturated fatty acids,

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unsaturated fatty acids and phytol. Mixtures of unsaturated fatty acids of *Chlorella variabilis* are responsible factor to reduce cholera toxin production.

O1 El Tor variant *Vibrio cholerae* SRK-19 strain is a multi-drug resistant strain which produces high amount of cholera toxin. Total lipid extract has shown no resistance development in generation study against the cholera toxin production in standard/classical *Vibrio cholerae* strains. Standard unsaturated fatty acids and combination of unsaturated fatty acids are able to inhibit the cholera toxin production significantly in *Vibrio cholerae* strain.

The present invention relates to a formulation comprising 9-*cis*,12-*cis*-Linoleic acid (linoleic acid), 9,12 Octadecadienoic acid (linolenic acid, LA), *cis,cis,cis*-6,9,12-Octadecatrienoic acid (γ-linolenic acid, GLA), *cis*-8,11,14-Eicosatrienoic acid (dihomo-gamma-linolenic acid, DGLA), cis-9-Octadecenoic acid (oleic acid) from *Chlorella variabilis* which exhibits anti-virulence activity against *Vibrio cholerae*. Presence of unsaturated and saturated fatty acids in the total lipid extract of *Chlorella variabilis* were calculated from the percent area detected by GC-MS data. Percent area were calculated as linoleic acid 4.64%, linolenic acid 0.15%, gamma-linolenic acid 2.14%, dihomo-gamma-linolenic acid 0.19%, oleic acid 2.6%, phytol 0.15%, palmitic acid 0.5% and methyl palmitate 1.67%. Experiments waere done separately and in combination for all standard fatty acids.

Formulation (C1) was prepared with linoleic acid + linolenic acid + gamma-linolenic acid + dihomo-gamma -linolenic acid + oleic acid. All five unsaturated fatty acids in a single dose showed ~90-94% inhibition in cholera toxin production in variant *Vibrio cholerae* SRK-19. All five unsaturated fatty acids were combined (C1) according to the present percent area showed in GC-MS and result showed ~94% of inhibition in cholera toxin production. When the dose of combined standard unsaturated fatty acids were increased to double (C3), it showed 100% of inhibition in the cholera toxin production, whereas half dose of combined unsaturated fatty acids (C2) showed inhibition rate of around 76%.

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In this invention another major component of total lipid extract of *Chlorella variabilis* are palmitic acid, methyl palmitate and phytol. Experiment showed very less inhibition percentage in cholera toxin production with palmitic acid, methyl palmitate and phytol. Further, combination C1 was added with phytol, palmitic and methyl palmitate separately to check any kind of changes in result, there was no change in the percent of inhibition in the result.

Total lipid extract of *Chlorella variabilis* (TLCV) @ 0.1% concentration showed around 93% of inhibition in cholera toxin production in O1 El Tor variant *Vibrio cholerae* SRK-19 strain. Further different combination of standard fatty acids C1 were added, *viz.* normal percent, half of the normal percent and double of the normal percent for each unsaturated fatty acid (different formulations). In each experiment among five unsaturated fatty acid, percent of one fatty acid was changed and other four were remain same. Inventor found that in each and every combination inhibition in cholera toxin production was around 92-97%.

In an embodiment of the present invention, the concentration of total lipid extract from *Chlorella variabilis* is optimized which will maintain the viability of the bacterial cells.

In another embodiment of the present invention, the effect of total lipid extract from *Chlorella variabilis* on another virulence factor, toxin-co-regulated pili (*tcp*), responsible for colonization during infection in the shrimp *Artemia salina* model was studied.

In yet another embodiment of the present invention, the cell cytotoxicity was studied in the presence of O1 El Tor *Vibrio cholerae* variant strain and also the reduction of cell cytotoxicity in the presence of total lipid extract of *Chlorella variabilis* in human colorectal adenocarcinoma cells was evaluated.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Fig. 1 shows the cholera toxin production in different serogroups of *Vibrio cholerae* strains in the absence (WOTLCV) and presence of total lipid extract from *Chlorella variabilis* (WTLCV, 0.1%). *Vibrio cholerae* strains including O1 El tor, O1 classical and non-O1/non-O139. After 8 hr (4h static and 4h in shaking) of incubation cell free supernatant was collected by

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centrifugation of a bacterial culture at 10,000 rpm for 10 min. at 4°C, followed by filtration through a 0.22μm filter and used for the production of cholera toxin quantification by GM1 ELISA. The amount of cholera toxin production is represented by mean ± SD.

Fig. 2 shows the dose dependent assay with total lipid extract from *Chlorella variabilis* (%WTLCV) on the cholera toxin production and growth in O1 El Tor *Vibrio cholerae* SRK-19 strain. Black and white bars indicate cholera toxin production without (WOTLCV) and with total lipid extract (WTLCV), respectively. Secondary axis indicated *Vibrio chloerae*, SRK-19 growth (CFU/ml) in the absence and presence of total lipid extract of *Chlorella variabilis*. The amount of cholera toxin production and CFU/ml are represented by mean ±SD.

Fig. 3. Generation study was done from single colony culture of *Vibrio cholerae* SRK-19 strain and continued upto 10th generation in the absence (WOTLCV) and presence of lipid extract of *Chlorella variabilis* (WTLCV). This starter culture was added with 0.1% of lipid extract of *Chlorella variabilis* and another set of experiment was without addition of sample.

Fig. 4 shows fluorescent dye DAPI labelled *Vibrio cholerae* SRK-19 colonization in the gut of *Artemia salina* nauplii of 24 hr in the absence and presence of total lipid extract of *Chlorella variabilis*. Less colonization is visible in the presence of total lipid extract of *Chlorella variabilis* in compare to control. Bacterial colonization is visible in blue in color and total lipid extract of *Chlorella variabilis* is red in color.

Fig. 5 shows the cytotoxicity in the Human colorectal adenocarcinoma cells, HT-29 when treated alone with *Vibrio cholerae* SRK-19 strain. In the presence of total lipid extract of *Chlorella variabilis* in 0.4 and 0.5%, no cell cytotoxicity was observed.

Fig. 6 shows the effect of total lipid extract of *Chlorella variabilis* (WTLCV), standard phytol (0.15%), methyl palmitate (1.67%), palmitic acid (0.5%), linoleic acid (4.64%), γ-linolenic acid (2.14%), dihomo-gamma-linolenic acid (0.19%), and oleic acid (2.6%) on the inhibition in cholera toxin production of *Vibrio cholerae* SRK-19 strain and compared with control (SRK-19) where no standard fatty acids were added. The amount of cholera toxin production is represented by mean ±SD.

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Fig. 7 shows the effect of total lipid extract of *Chlorella variabilis* (WTLCV), C1= {(linoleic acid (0.5%) + linolenic acid (4.64%) + γ-linolenic acid (2.14%) + dihomo-gamma-linolenic acid (0.19%) + oleic acid (2.6%)}, half dose of C1 i.e. C2 half of all the percent mentioned in C1, double of C1 i.e. C3 means all the percent were doubled in the experiment. C1+ phytol (0.15%), C1+ methyl palmitate (1.67%), C1+ palmitic acid (0.5%) on the inhibition in cholera toxin production of *Vibrio cholerae* SRK-19 strain and compared with control (SRK-19) where no standard fatty acids were added. The amount of cholera toxin production is represented by mean ±SD.

Fig. 8 shows the effect of total lipid extract of *Chlorella variabilis* (WTLCV), **C1**= {(linoleic acid (0.5%) + linolenic acid (4.64%) + γ-linolenic acid (2.14%) + dihomo-gamma linolenic acid (0.19%) + oleic acid (2.6%)}, **C4**= {(linoleic acid (0.5%) + linolenic acid (4.64%) + γ-linolenic acid (2.14%) + dihomo-gamma-linolenic acid (0.19%) + oleic acid (**1.3%**)}, **C5**= {(linoleic acid (0.5%) + linolenic acid (4.64%) + γ-linolenic acid (2.14%) + dihomo-gamma linolenic acid (0.19%) + oleic acid (**5.2%**)}, **C6**= {(linoleic acid (0.5%) + linolenic acid (4.64%) + γ-linolenic acid (2.14%) + dihomo-gamma-linolenic acid (**0.095%**) + oleic acid (2.6%)}, **C7**={(linoleic acid (0.5%) + linolenic acid (4.64%) + γ-linolenic acid (2.14%) + dihomo-gamma linolenic acid (**0.38%**) + oleic acid (2.6%)}, **C8**={(linoleic acid (0.5%) + linolenic acid (4.64%) + γ-linolenic acid (**1.07%**) + dihomo-gamma-linolenic acid (0.19%) + oleic acid (2.6%)}, **C9**= {(linoleic acid (0.5%) + linolenic acid (4.64%) + γ-linolenic acid (**4.24%**) + dihomo-gamma-linolenic acid (0.19%) + oleic acid (2.6%)}, **C10**= {(linoleic acid (0.5%) + linolenic acid (**2.32%**) + γ-linolenic acid (2.14%) + dihomo-gamma-linolenic acid (0.19%) + oleic acid (2.6%)}, **C11**= {(linoleic acid (0.5%) + linolenic acid (**9.28%**) + γ-linolenic acid (2.14%) + dihomo-gamma-linolenic acid (0.19%) + oleic acid (2.6%)}, **C12**= {(linoleic acid (**0.25%**) + linolenic acid (4.64%) + γ-linolenic acid (2.14%) + dihomo-gamma-linolenic acid (0.19%) + oleic acid (2.6%)}, **C13**= {(linoleic acid (**1.0%**) + linoleic acid (4.64%) + γ-linolenic acid (2.14%) + dihomo-gamma-linolenic acid (0.19%) + oleic acid (2.6%)}. Effect of combination of all fatty acids on the cholera toxin production of *Vibrio cholerae* SRK-19 strain. Here control is SRK-19 without adding any fatty acids. The amount of cholera toxin production is represented by mean ±SD.

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Table 1 Different composition of unsaturated fatty acids standards and percent inhibition in cholera toxin production in *Vibrio cholerae*

SL no	Formulation	Composition of formulation	% inhibition in Cholera Toxin production
1		Linoleic Acid (4.64%)	92.93±0.005
2		Linolenic Acid (0.15%)	91.29±0.010
3		Y-Linolenic Acid (2.14%)	94.05±0.010
4		D-Homo-gamma-Linolenic Acid (0.19%)	90.47±0.008
5		Oleic Acid (2.60%)	92.31±0.011
6	**C1**	C1 (Linoleic Acid 4.64%+Linolenic Acid 0.15%+Gamma-Linolenic Acid 2.14%+Dihomo-Gamma-Linolenic Acid 0.19%+Oleic acid 2.60%)	94.16 ± 0.01
7	**C2**	C1 half (Linoleic Acid 2.32%+ Linolenic Acid 0.075%+Gamma-Linolenic Acid 1.07%+ Dihomo–Gamma-Linolenic Acid 0.095%+Oleic acid 1.3%) half amount of C1	75.87 ± 0.01
8	**C3**	C1 double (Linoleic Acid 9.28%+Linolenic Acid 0.3%+Gamma-Linolenic Acid 4.28%+ Dihomo – Gamma-Linolenic Acid 0.38%+Oleic acid 5.2%) double amount of C1	100 ± 0.001
9	**C4**	C2 (Linoleic Acid 4.64%+Linolenic Acid 0.15%+Gamma-Linolenic Acid 2.14%+ Dihomo-Gamma-Linolenic Acid 0.19%+Oleic acid 1.3%) Oleic acid in half amount	91.90 ± 0.005
10	**C5**	C3 (Linoleic Acid 4.64%+Linolenic Acid 0.15%+Gamma-Linolenic Acid 2.14%+ Dihomo-Gamma-Linolenic Acid 0.19%+Oleic acid 5.2%) Oleic acid in double amount	94.46 ± 0.01

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11	**C6**	C4 (Linoleic Acid 4.64%+Linolenic Acid 0.15%+Gamma-Linolenic Acid 2.14%+ Dihomo-Gamma-Linolenic Acid 0.095%+Oleic acid 2.60%) DiHomo-Gamma- Linolenic acid in half amount	92.21± 0.006
12	**C7**	C5 (Linoleic Acid 4.64%+LA 0.15%+GLA 2.14%+ D-GLA 0.38%+Oleic acid 2.60%) DiHomo-Gamma-Linolenic acid in double amount	97.43± 0.004
13	**C8**	C6 (Linoleic Acid 4.64%+Linolenic Acid 0.15%+Gamma-Linolenic Acid 1.07%+ Dihomo-Gamma-Linolenic Acid 0.19%+Oleic acid 2.60%) Gamma-Linolenic acid in half amount	93.85± 0.006
14	**C9**	C7 (Linoleic Acid 4.64%+Linolenic Acid 0.15%+Gamma-Linolenic Acid 4.28%+ Dihomo-Gamma-Linolenic Acid 0.19%+Oleic acid 2.60%) Gamma-Linolenic acid in double amount	94.15±0.001
15	**C10**	C8 (Linoleic Acid 4.64%+Linolenic Acid 0.075%+Gamma-Linolenic Acid 2.14%+ Dihomo-Gamma-Linolenic Acid 0.19%+Oleic acid 2.60%) Linolenic acid in half amount	93.95±0.013
16	**C11**	C9 (Linoleic Acid 4.64%+Linolenic Acid 0.3%+Gamma-Linolenic Acid 2.14%+ Dihomo-Gamma-Linolenic Acid 0.19%+Oleic acid 2.60%) Linolenic acid in double amount	96.51±0.003
17	**C12**	C10 (Linoleic Acid 2.32%+Linolenic Acid 0.15%+Gamma-Linolenic Acid 2.14%+ Dihomo-Gamma-Linolenic Acid 0.19%+Oleic acid 2.60%) Linoleic acid in half amount	94.77± 0.003
18	**C13**	C11 (Linoleic Acid 9.28%+Linolenic Acid 0.15%+Gamma-Linolenic Acid 2.14%+ Dihomo-Gamma-Linolenic Acid 0.19%+Oleic acid 2.60%)	96.41± 0.006

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		Linoleic acid in double amount	

DETAILS OF BIOLOGICAL RESOURCES USED IN THE INVENTION

1. Microalgae *Chlorella variabilis*. The microalga was isolated from Jalandhar Beach, Diu with geo co-ordinates 20°42'33.0"N 70°59'08.6"E; Union Territory of Daman and Diu, India. The isolated strain has been deposited with the ATCC, USA under the Budapest Treaty on 26/10/2011 vide accession number ATCC PTA-12198.

2. The brine shrimp, *Artemia salina* cysts was obtained from O.S.I. PRO 80, USA, from the trader Aquarium world, Shop no 31, GST Road, Guindy, Chennai-600032, Tamil Nadu, India.

3. Bacterial strain of *Vibrio cholerae* SRK-19 strain used to test the applicability of the claimed formulation was obtained from ICMR-National Institute of cholera and endemic diseases culture collection repository, Kolkata. It was a clinical strain.

DETAILED DESCRIPTION OF THE INVENTION

In the present invention virulence factor inhibitors from microalgal sp. against the bacterium causing devastating cholera disease are disclosed, wherein mixture of agents or individual extract or formulation as an active component from microalgae sp. was the means for treating and/or preventing cholera like disease. Bacterial virulence is the ability of a pathogen to cause disease by bacterial factors like toxins or by mechanisms that actively cause damage to host tissues.

In accordance with an aspect to develop anti-virulence therapies, the present invention aims at 'disarming' the pathogen by inhibiting virulence factors that can cause direct harm to the host. Herein the virulence factor, cholera toxin belongs to the superfamily AB5 toxin. Both cholera toxin and the *Escherichia coli* heat-labile enterotoxins LT and LT-II are structurally related.

In an embodiment of the present invention, different serogroups of *Vibrio cholerae*, all clinical

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strains, produce different amount of cholera toxin. All clinical *Vibrio cholerae* strains were collected from NICED, Kolkata. Highest amount of cholera toxin was produced by the variant *Vibrio cholerae* O1 El Tor strains [SRK-19].

Exemplary embodiments of the present invention are described herein with reference to the accompanying figures. **Fig. 1** is schematic presentation of production of cholera toxin by different serogroups of *Vibrio cholerae*. In the said mechanism production of cholera toxin is suppressed. Similar structure toxin like heat labile toxin and other bacterial toxin from same group also can be suppressed. The terms "reducing", "suppressing and "inhibiting have their commonly understood meaning of lessening or decreasing. In view of the above, it is understandable that toxins which are structurally and/or functionally similar to cholera toxin will be suppressed by full extent or some, by the anti-virulence compounds identified in this invention. Competitive GM1-ELISA-tests were performed wherein the total lipid extract from microalgal sp. was evaluated for the ability to inhibit the cholera toxin production in *Vibrio cholerae*.

In another embodiment, production of anti-virulent agents from the chlorophyceae family of microalgae, *Chlorella variablis* was evaluated. **Fig. 1** is the schematic presentation of suppression of production of cholera toxin in the presence of total lipid extract from *Chlorella variabilis* in sub-bactericidal concentration. Specifically, the present invention includes an extract of total lipid as an active ingredient, and agents for inhibiting the production of toxin by bacteria. Here, toxin producing bacteria can be *Vibrio cholerae* or other enteropathogens. Anti-virulent compounds may be produced from other algae than chlorophyta including phylum like cyanobacteria, ochrophyta etc., which may need further study. Different microalgal species from mentioned phylum *viz. Chlorella vulgaris, Chlorococcum* sp., *Nostoc* sp., *Synechocystis* sp., *Spirullina platensis, Graesiella emersonii, Chroococcus sp.*, *Lyngbya* sp., *Phormidium* sp., *Nanochloropsis sp.*, etc. may have potential anti-virulence property against *Vibrio cholerae* or other enteropathogenic bacteria. However, detailed study is required.

Another aspect of the invention involves extracting total lipid from harvested microalgae with a

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solvent (chloroform: methanol). The chain lengths of the fatty acids in naturally occurring triglycerides vary, but most contain 16, 18, or 20 carbon atoms. In another embodiment, GC-MS data confirms the presence of different unsaturated fatty acids, and saturated fatty acids in the total lipid extract of *Chlorella variabilis*.

In another embodiment, the anti-virulent compounds of the present application demonstrated inhibitory effect against cholera toxin production and it was observed that the compounds from *Chlorella variabilis* are mainly unsaturated fatty acids: linoleic acid, linolenic acid, γ-linolenic acid, dihomo-gamma-linolenic acid (polyunsaturated fatty acids) and oleic acid (monounsaturated fatty acids). These compounds were used to prepare formulation[s] to inhibit bacterial virulence factors. Another embodiment of the present invention involves application/use of compounds in the preparation of a formulation useful as prodrugs/prophylactic thereof, to inhibit bacterial virulence factors and use of one or more compounds selected from a formula to inhibit bacterial virulence factors.

Candidate compounds can be obtained from wide natural sources. Suitable compound formulations according to the present invention include powder, sachets, sol, gel, pills, capsule, tablets, liquid drops, liquid, injectable and the like. Also considered are the food items, beverage products, powdered, granular foods, dairy products, chocolates, snacks, cookies, desserts, and liquid comestibles, like soft drink, health drink, herbal water, juice, milk-shakes, curd, lassi, buttermilk, soups etc. product containing the composition of the present invention.

Fig. 2 is the schematic representation of total lipid extract which was screened in dose response. Administration of formulation of the invention, e.g. therapy or treatment could continue over a period of days, weeks, months or year, until infection is treated. *Chlorella variabilis* is used as a super food as it contains lot of essential proteins, vitamins, minerals, dietary fiber, and macro as well micro nutrients. It may have very less side effects on the natural flora of the gut.

In another embodiment of the invention, it was observed that the anti-virulent formulation from *Chlorella variabilis* does not results in the induction of resistance against cholera toxin

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production in O1 El Tor variant *Vibrio cholerae* **(Fig. 3)**. No anti-virulence resistance was observed in *Vibrio cholerae* on the continuous use of total lipid extract of *Chlorella variabilis*.

In another embodiment of the invention, reduction in colonization of *Vibrio cholerae* bacterial cells in the presence of total lipid extract of *Chlorella variabilis* in the brine shrimp *Artemia salina* nauplii. No toxicity was observed in the concentration (CFU/mL) of *Vibrio cholerae* used, which can cause infection in the human body. *Artemia* nauplii were alive in the presence of 10^3 CFU/ml of *Vibrio cholerae* and colonized in the gut of animal in the absence of total lipid extract of *Chlorella variabilis* **(Fig. 4)**.

In yet another embodiment of the invention, no cell cytotoxicity was observed in human colorectal adenocarcinoma in the presence of total lipid extract of *Chlorella variabilis* due to cholera toxin in human colorectal adenocarcinoma cells **(Fig. 5)**.

Fig. 6 and Fig. 7 are the schematic representation of use of linoleic acid, linolenic acid, γ-linolenic acid, dihomo-gamma-linolenic acid and oleic acid in single dose or when used in combination of different percentage of compounds to inhibit the virulence factors in *Vibrio cholerae*. According to the GC-MS data, percent area of the single compounds present in the total lipid extract of *Chlorella varaibilis* are: linoleic acid (4.64%), linolenic acid (0.15%), γ-linolenic acid (2.14%), dihomo-gamma-linolenic acid (0.19%) and oleic acid (2.6%). Less inhibition on the virulence factor was observed in the presence of methyl palmitate, palmitic acid and phytol **(Fig. 6)**.

According to another embodiment of the invention, a subject in need of prevention, mitigation or treatment is administered an effective amount of a composition containing unsaturated fatty acids or similar derivatives, mimetic, or extract containing the same, to decrease the expression of *Vibrio cholerae* virulence factors thereby preventing, mitigating, or treating bacterial infection. With different combinations of unsaturated fatty acids one may fulfill the need to prepare formulations as an anti-virulent agent **(Fig. 8)**.

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To this effect, the efficacy of the anti-virulence formulation can be further assessed in animal challenge studies. More specifically, the efficacy of formulation to inhibit cholera toxin production and colonization in the host cell can be further assessed in mice/rabbit using assays known in the art. Although a preferred embodiment of the present application has been described in detail herein and illustrated in the accompanying drawings, it is to be understood that the application is not limited to these embodiments and that various changes and modifications could be made in the formulation without departing from the scope and spirit of the present application.

EXAMPLES

The following examples are given by way of illustration only and therefore should not be construed to limit the scope of the present invention in any manner.

Example 1: Cholera toxin production in different *Vibrio cholerae* strains

Vibrio cholerae strains were grown at 37°C in AKI medium comprising gms/l, peptone 15.00, yeast extract 4.0, NaCl 5.0, and NaHCO3, pH 7.4±0.2. Broth cultures grow best with aeration such as AKI medium, the overnight cultures reached densities of $>10^{12}$ colony forming unit (CFU/ml). Production of cholera toxin in different serogroups of *Vibrio cholerae* strains, O1 El Tor strains; SRK-19, SRK-25, SRK-28, SC-32, AM-168, AM-352, CRC-220, VC-0709, N16961, O1classical strain-O395 and non-O1, non-O139 strain-I-8437 were observed in AKI media culture. Overnight grown *Vibrio cholerae* strains culture were diluted freshly to achieve an OD600 nm of 1.0 with AKI media, so that bacterial cell concentration were same. One of the culture set were added with 0.2% of methanol and all culture sets including control were kept under static condition followed by shaking condition at 150 rpm at 37°C for 4 hr each. After incubation, cell-free supernatant (CFS) was prepared by centrifugation of bacterial culture at 12,000 g for 10 min, followed by filtration of supernatant through a 0.22 μm filter (Pall filters). The CFS was diluted 10 and 100 times with sterile phosphate-buffered saline (pH 7.0). Standard of cholera toxin from Sigma-Aldrich, was diluted with phosphate-buffered saline of known concentrations and were used to estimate the amount of cholera toxin production in different *Vibrio cholerae* strains by a Monosialoganglioside GM1 enzyme-linked immunosorbent assays (GM1-ELISA). Cholera toxin produced in the culture with addition of methanol was 100%, as

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control. Among all *Vibrio cholerae* strains O1 El Tor SRK-19 strain produced highest amount of cholera toxin (**Fig.1**). In O1 El Tor SRK-19, SRK-25, SRK-28, SC-32, AM168, AM-352, CRC-220 strains, the *ctx*B gene alleles were determined for classical type and in VC-0709, N16961 *ctx*B gene alleles of El Tor type, by a mismatch amplification mutation-PCR (MAMA-PCR) assay as described by Morita et al. (2008). MAMA-PCR was done by using boiled template of O1 El Tor and O1 classical *Vibrio cholerae* strains. SRK-19, SRK-25, SRK-28, SC-32, AM168, AM-352, CRC-220 were determined to be variant O1 El Tor *Vibrio cholerae* strains. Further *Vibrio cholerae* SRK-19 was found as multidrug resistant strain in antibiotic assay.

Example 2: Effect of total lipid extract of *Chlorella variablis* on the growth and quantification of cholera toxin production

Vibrio cholerae SRK-19 strain was cultured in nutrient broth overnight at 37°C at 180 rpm. Overnight culture was diluted with fresh AKI media. Total lipid extract (diluted in methanol) of *Chlorella variabilis* (ATCC 12198) was added in 0.1% concentration to *Vibrio cholerae* SRK-19 strains culture and kept under static condition followed by shaking condition at 180 rpm at 37°C for 4 hrs each. As mentioned biomass was dissolved in methanol, so methanol alone was also added to *Vibrio cholerae* culture to check the effect on growth and cholera toxin production. As mentioned above quantification of cholera toxin in the absence and presence of total lipid extracts of microalgae was done by comparing with standard cholera toxin by GM1 ELISA assay. Before cell free supernatant preparation, each culture set with or without total lipid extracts were serially diluted with 0.9% saline and spread on nutrient agar plates. After overnight incubation next day colony formation unit was counted. No growth inhibition was observed in the presence of total lipid extract of *Chlorella variabilis* with 0.1%-0.5% of concentration.

All the results for cholera toxin suppression were compared with microalgae extract-free control culture (only bacterial culture). For all *Vibrio cholerae* strains in spite of different biotypes, 0.1% of total lipid extract of *Chlorella variabilis* was able to supress cholera toxin production in different *Vibrio cholerae* strains (**Fig.1**). There was no inhibitory effect of methanol on the cholera toxin production.

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Further, total lipid extract of *Chlorella variabilis* was used for dose dependent experiment with different concentrations. With gradual increase of extract (0.001%, 0.01%, 0.025%, 0.05%, 0.1% & 0.2%) inhibition in cholera toxin production also increased (**Fig. 2**). Total lipid extract from *Chlorella variabilis* upto higher concentration from 0.01 to 0.5% didn't show any bactericidal effect on the *Vibrio cholerae* growth.

Example 3: Serial passage experiments for induction of resistance

Serial passage experiment was done to check the induction of resistance for total lipid from *Chlorella variabilis* against cholera toxin production. Generation study was done from single colony culture of *Vibrio cholerae* SRK-19 strain and continued up to 10th generation in the absence and presence of total lipid extract of *Chlorella variabilis*. Initially, overnight culture of SRK-19 strain was diluted with fresh AKI media and OD 600 nm was adjusted up to 1.0. This starter culture was added with 0.1% of total lipid extract of *Chlorella variabilis* (no effect on the growth of bacteria was observed) and another set of experiment was done without addition of test sample. Cultures were placed in shaking condition for 24 hr. Cell free supernatant was collected for GM1 ELISA and plating was done by serial dilution with sterile phosphate buffer saline from remaining culture, OD600 nm was adjusted to 1.0 with fresh AKI media and this procedure for adding lipid extract was followed for up to 10 days. No growth inhibition was found after repeated exposure with total lipid extract for 10 days. No different effect was observed on the inhibition rate in cholera toxin production for all 10 days (**Fig. 3**). On first day, more than 91% inhibition was observed, consecutively for all another 9 days inhibition rate was more than 91%. Inhibition rate remained the same in the presence of total lipid extract of *Chlorella variabilis* for 10 generations study series. This total lipid extract of *Chlorella variabilis* proved as anti-virulent as no resistance was induced.

Example 4: Colonization in *Artemia salina* nauplii

The colonization potential of *Vibrio cholerae* SRK-19 strain was labelled with fluorescent dye DAPI and used for the challenge study in *Artemia* model in the absence and presence of total lipid extract of *Chlorella variabilis*. Hatched *Artemia salina* nauplii of 24 hr was challenged with fluorescent labelled SRK-19 strain (CFU/ml 10^3), and after 8 hr of culture, colonization of *Vibrio cholerae* was observed in the intestine to rectum of *Artemia salina* (**Fig. 4**). Fluorescent labelled

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SRK-19 strain was cultured with total lipid of *Chlorella variabilis* for 4 hr and then culture was challenged with *Artemia* and kept for more 8 hr after incubation. It was found that the colonization of SRK-19 strain in the presence of total lipid was less as compared to control experiment **(Fig. 4)**.

Example 5: Cytotoxicity study with *Chlorella variabilis*

Human colorectal adenocarcinoma cells, HT-29 were grown and maintained in Dulbecco's modified Eagle's medium containing high glucose with 10% FBS, 100 U/ml Penicillin G and 100 µg/ml Streptomycin sulphate. All cells were maintained in presence of 5% CO_2 at 37°C. HT-29 cells were treated with the filter sterilized culture supernatant after 8 h (4h static and 4h in shaking) of growth of *Vibrio cholerae* SRK-19 at 37°C grown in absence or presence of the total lipid extract of *Chlorella variabilis* of 0.5%. All supernatants of *Vibrio cholerae* either treated or untreated with total lipid sample were used with 500 µl and cells were incubated for different time periods **(Fig. 5)**. Cells were observed under phase contrast microscope (Olympus, Japan) at magnification 40X. It was observed that 0.5% concentration of total lipid extract of *Chlorella variabilis* didn't show any growth restriction or bacteriostatic action in *Vibrio cholerae* cells **(Fig. 5)**. No cytotoxicity in HT-29 was observed in the presence of total lipid extract of *Chlorella variabilis*.

Example 6: Unsaturated fatty acids as anti-virulent compounds

Fatty acid profiling for total lipid extract of *Chlorella variabilis* was done in GC-MS and it was revealed that mixtures of fatty acids are present. Specifically unsaturated fatty acids; 9-*cis*,12-*cis*-Linoleic acid (ω−6, C18:2, linoleic acid), 9,12 Octadecadienoic acid (ω−3, C18:3, linolenic acid), *cis,cis,cis*-6,9,12-Octadecatrienoic acid (ω−6, C18:3, γ-linolenic acid), *cis*-8,11,14-Eicosatrienoic acid (ω−6, C20:3, dihomo-gamma-linolenic acid), cis-9-Octadecenoic acid (ω−9, C18:1, oleic acid) and saturated fatty acids- palmitic acid (C16:0), methyl palmitate, an acyclic diterpene alcohol- phytol were documented in the total lipid extract of *Chlorella variabilis*. Formulations of these unsaturated fatty acids are effective to inhibit cholera toxin production without affecting the viability of *Vibrio cholerae* cells at sub-bactericidal concentrations **(Fig. 6)**.

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Standard fatty acids singly and in combination of linoleic acid, linolenic acid, γ-linolenic acid, dihomo-gamma-linolenic acid & oleic acid showed inhibition in cholera toxin production **(Fig. 6, 7)**. When actual concentration {linoleic acid, linolenic acid, γ-linolenic acid, dihomo-gamma-linolenic acid & oleic acid of combination C1 formulation was added with *Vibrio cholerae* SRK-19 strain, in its actual percent, the inhibition rate was 97%, and in case of half of the C1 dose i.e. C2 inhibition rate came down to 75% and when percent of C1 was doubled i.e. C3 the inhibition rate was 100%. Whereas, palmitic acid, methyl palmitate and phytol shown less inhibitory effect on the cholera toxin production in *Vibrio cholerae* SRK-19 strain **(Fig. 6)**.

Different combinations of formulation (C4 to C14) showed 92-97% of inhibition rate in cholera toxin production **(Fig. 8)**. The different percent of inhibition in cholera toxin production is given in **Table 1**. It shows the C3 formulation have highest inhibition rate. Other formulations are also significant as anti-virulent agent except C2. Very less difference in inhibition rate of cholera toxin was observed in prepared formulations. The formulation of the present invention is an effective agent for the treatment and/or prevention of cholera and diarrheal diseases.

The present invention thus provides:

1. A formulation for inhibition of cholera toxin essentially consisting of linoleic acid, linolenic acid, γ-linolenic acid, dihomo-gamma-linolenic acid, oleic acid, wherein the ration of linoleic acid, linolenic acid, γ-linolenic acid, dihomo-gamma linolenic acid, oleic acid is in the range of from 30:1:13:1:15 to 32:1:16:1.5:19.
2. The formulation, wherein the ratio of linoleic acid, linolenic acid, γ-linolenic acid, dihomo-gamma-linolenic acid, oleic acid, wherein the ration of linoleic acid, linolenic acid, γ-linolenic acid, dihomo-gamma-linolenic acid, oleic acid is 4.64:0.15:2.14:0.19:2.60.
3. A process in the preparation of the aforesaid formulation consisting/ comprising the following steps:

   [a] total lipid is extracted from dried biomass of microalgae *Chlorella variabilis* ATCC 12198 with mixture of chloroform and methanol;

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[b] the solution containing *Chlorella variabilis* ATCC 12198 obtained in step [a] is transmethylated to perform GC-MS to identify active components;

[c] after identification of the active components in step [b] linoleic acid, linolenic acid, γ-linolenic acid, dihomo-gamma-linolenic acid, oleic acid are mixed in the ratio ranging from 30:1:13:1:15 to 32:1:16:1.5:19 to get the anti-virulent formulation.

4. The process, wherein chloroform and methanol are mixed in 2:1 *v/v* ratio.
5. The formulation wherein it is used against enteropathogenic bacteria.
6. The formulation wherein it is used against *Vibrio cholerae.*
7. The process, wherein in the presence of total lipid from *Chlorella variabilis* shows no cell cytotoxicity caused by *Vibrio cholerae.*

ADVANTAGES OF THE INVENTION

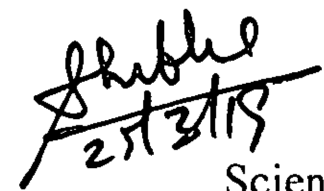
- The developed formulation works against recently emerged variant of *Vibrio cholerae* strains causing severe diarrhea.

- The novel approach is to target virulence regulation instead of viability of the bacteria. Anti-virulence strategies have advantage to preserve the host endogenous microbiome, less side effects and exerting less selective pressure, which may result in decreased resistance.

- *Chlorella variabilis* also contains many micro and macro nutrients, which have health promoting effects.

- The present invention relates to the utilization of edible microalgae for the purposes of reducing infections caused by enteropathogens specifically *Vibrio cholerae.*

Dated this 25th day of March 2019.



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ABSTRACT

ANTI-VIRULENCE FORMULATION FROM MICROALGAL LIPIDS AGAINST VIBRIO CHOLERAE AND PROCESS FOR THE PREPARATION THEREOF

The present invention relates to a formulation essentially consisting of linoleic acid, linolenic acid, γ-linolenic acid, dihomo-gamma-linolenic acid, oleic acid, wherein the ration of linoleic acid, linolenic acid, γ-linolenic acid, dihomo-gamma linolenic acid, oleic acid of *Chlorella variabilis* and method of preparation thereof. The formulation exhibits anti-virulence activity against enteropathogenic bacteria, more specifically against *Vibrio cholerae* strain causing severe diarrhea.

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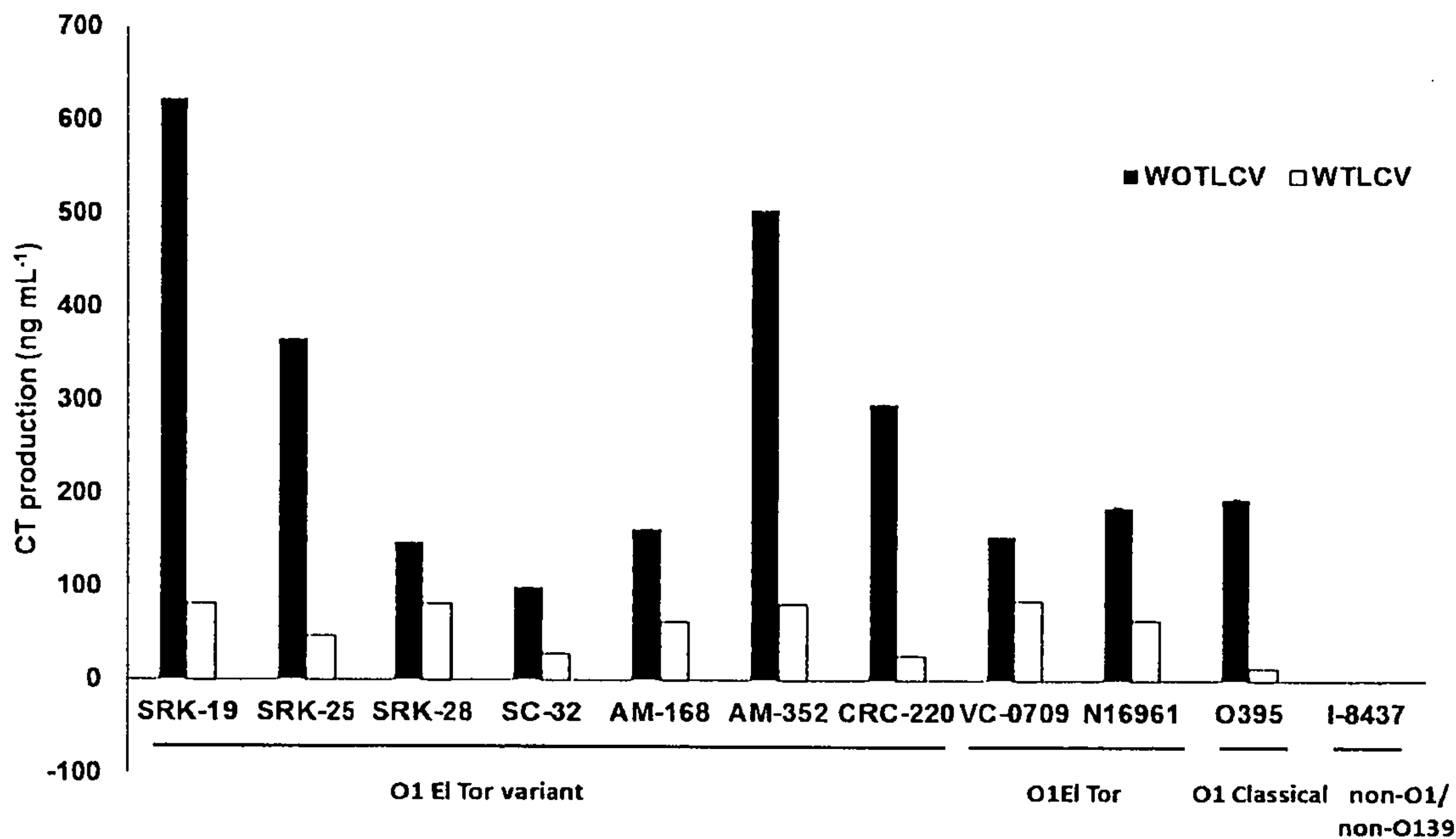

Figure 1

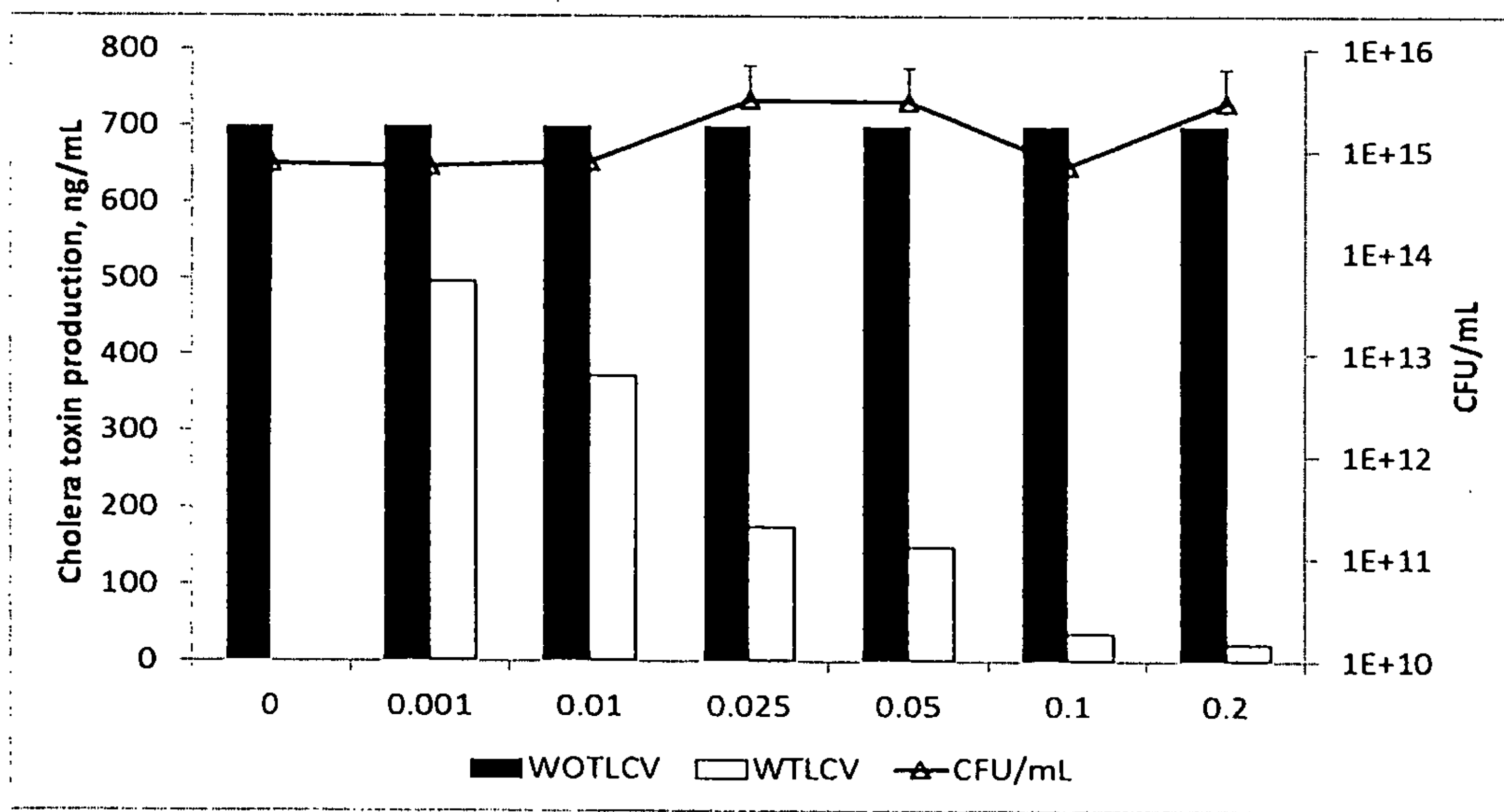

Figure 2

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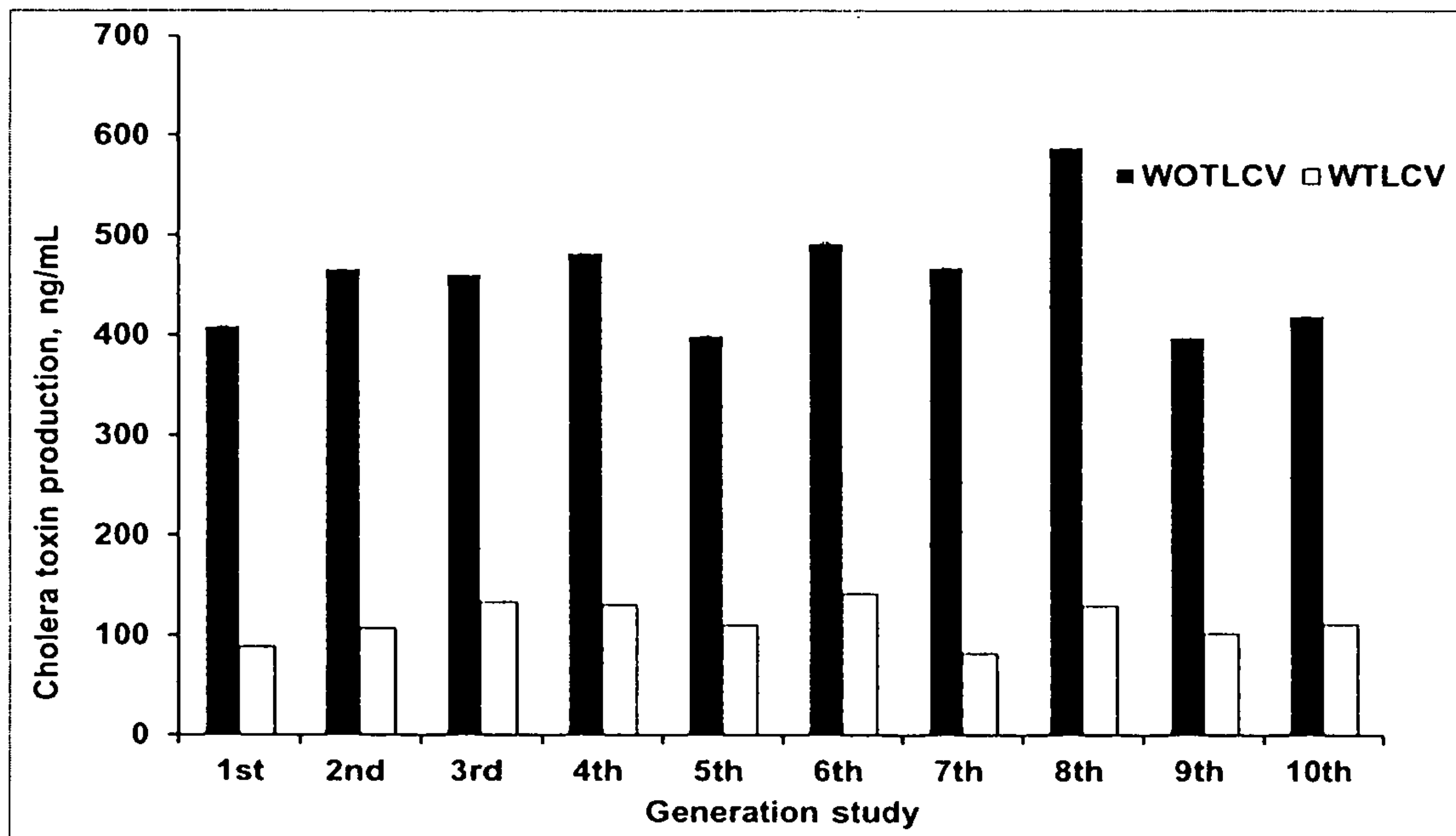

Figure 3

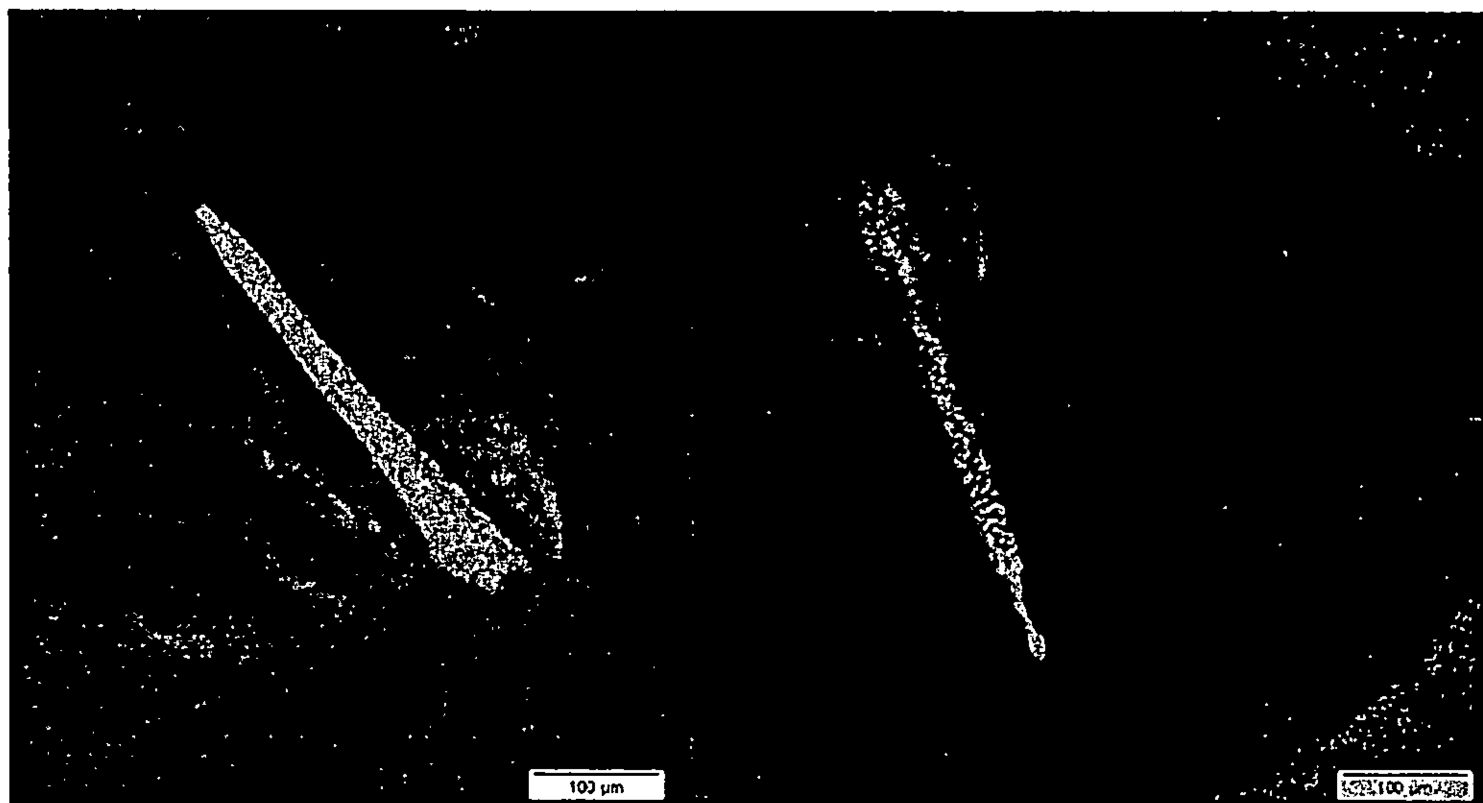

Figure 4

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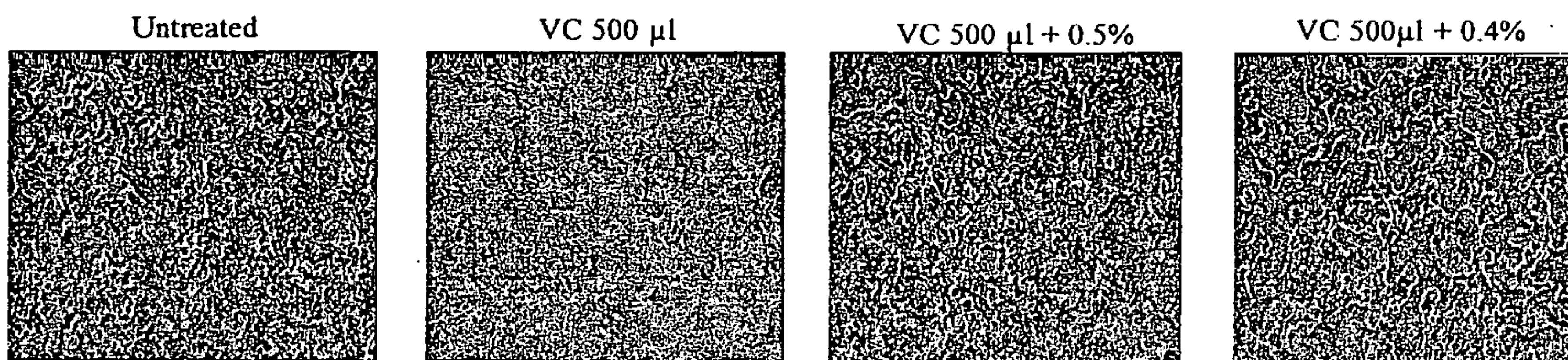

Figure 5

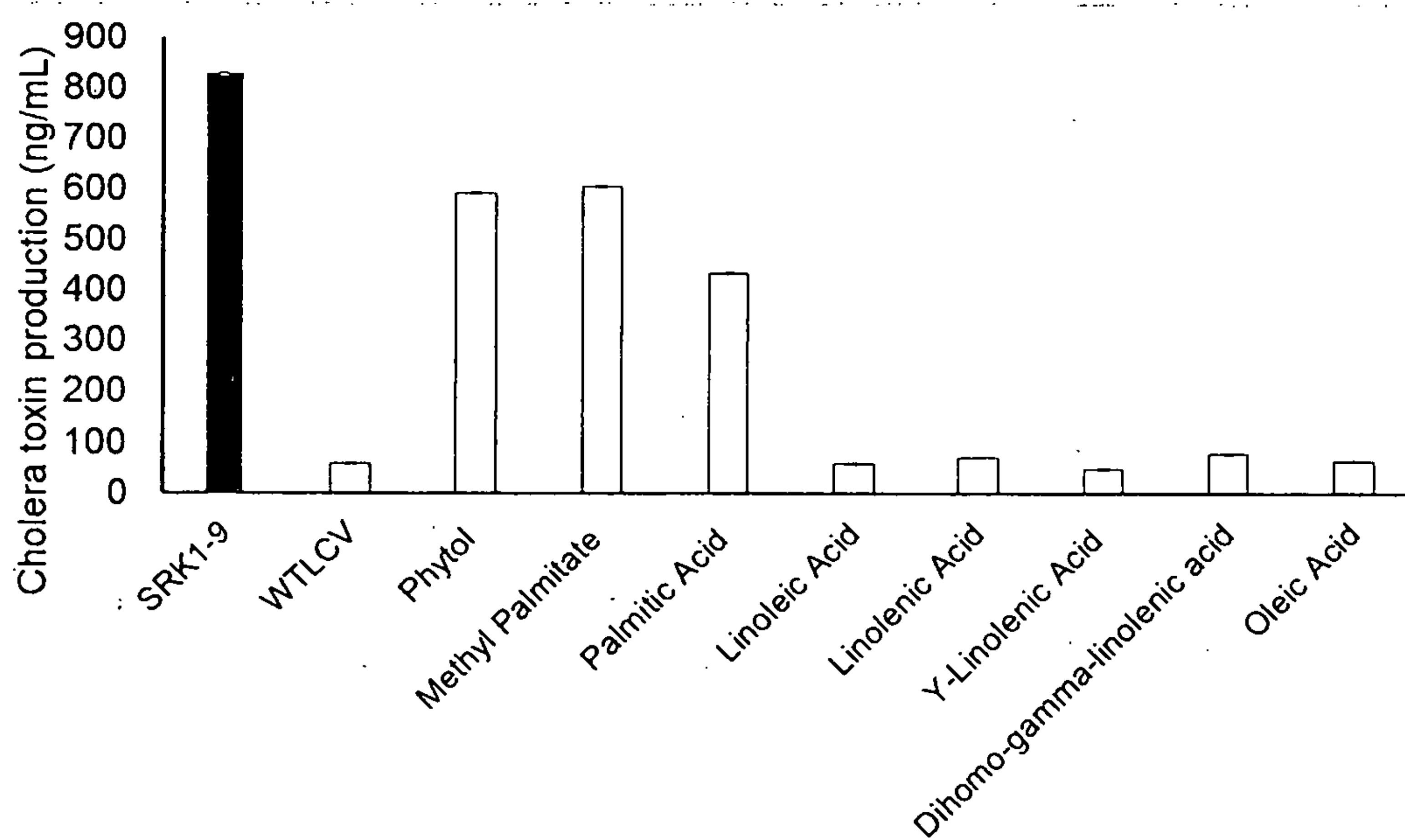

Figure 6

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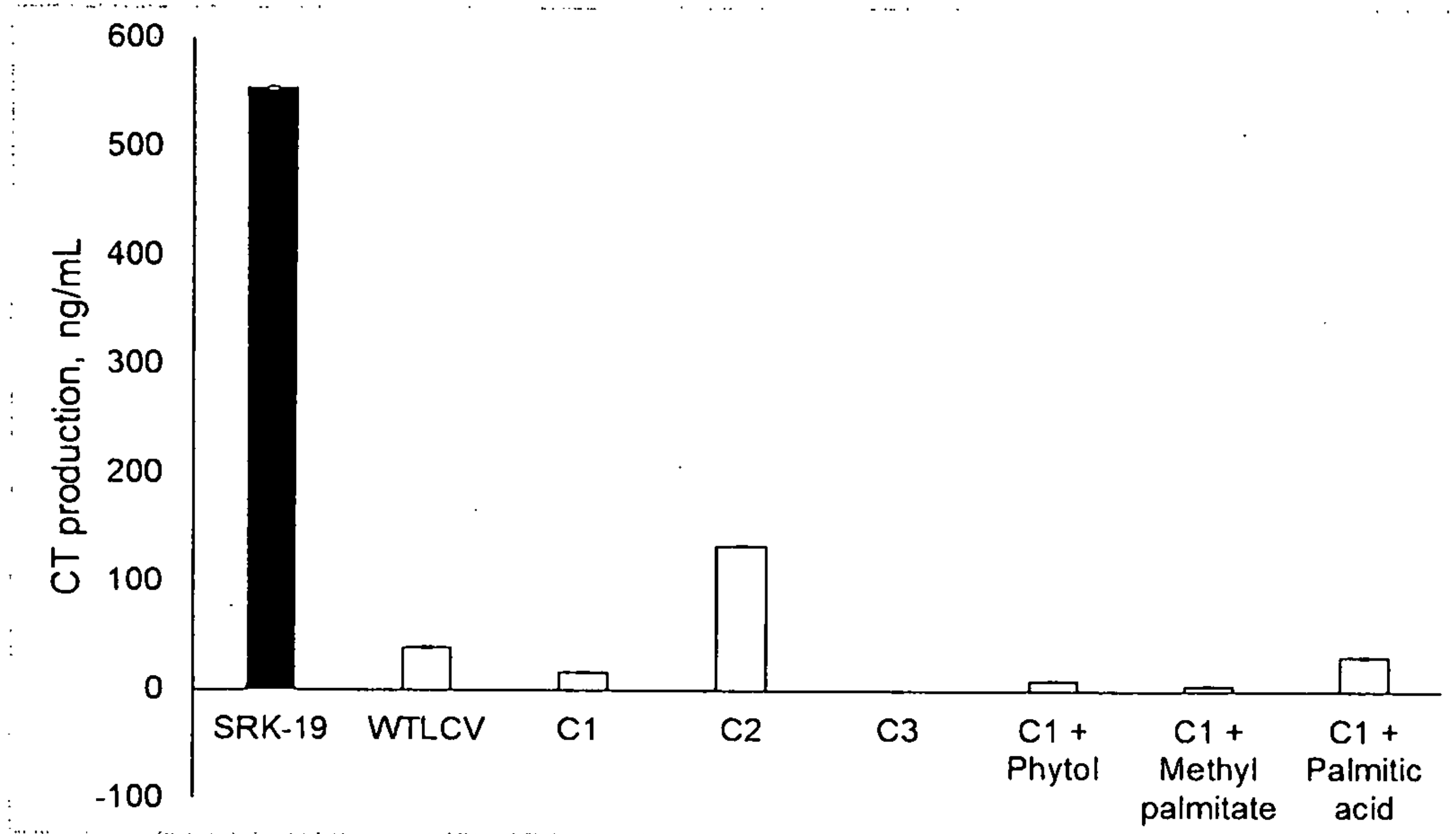

Figure 7

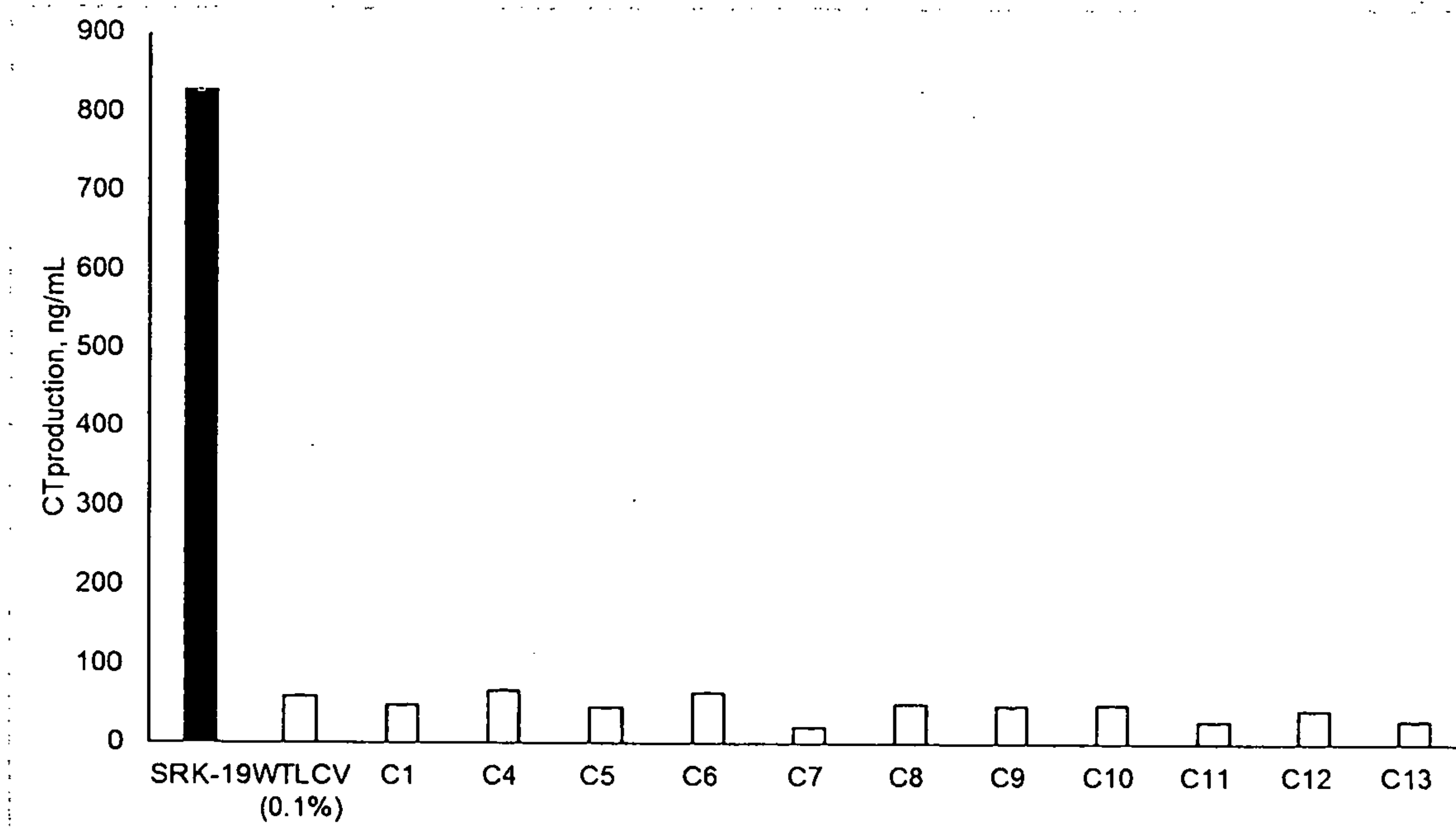

Figure 8

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