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Molecular characterization of cholera autoinducer-1(CAI-1) mimic as a potent cqsS receptor agonist

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ABSTRACT

The binding of the signaling molecule Cholera Auto inducer-1 (CAI-1) to the cognate transmembrane receptor CqsS regulates the behavioural pattern in *Vibrio cholerae*. CAI-1 serves as the liaison between *Vibrio* species thereby, regulating their virulence & pathogenecity. This study highlights on the *in-vitro* characterization of CAI-1 mimic molecules from *Melia dubia* leaf extract. The methanol fraction of the aqueous leaf extract that muffled the Haemolyis & Biofilm formation simultaneously, enhancing the production of extracellular protease and oxidative stress response in a Low Cell Density (LCD) condition was subjected to GC-MS analysis which unmasked an array of 19 compounds. Molecular Docking was chosen as a tool to predict the compound that had a greater binding affinity to CqsS receptor than the native CAI-1. Consequently, through *in-silico* analysis it was found out that 4-Ethyl Resorcinol in the methanol fraction had the maximum binding affinity. Further experiments *in-vitro*, validated the prediction that 4-Ethyl Resorcinol in *Melia dubia* leaves was a potent CAI-1 functional mimic molecule i.e. CqsS receptor agonist.
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KEYWORDS

Cholera Auto inducer-1;
CqsS;
Melia dubia;
Vibrio cholerae;
4-Ethyl Resorcinol.

INTRODUCTION

Poor sanitation and hygiene are the two reasons that have bolstered the incidence of intestinal infections in many underdeveloped and developing countries. Cholera is one such disease that is caused by the water borne pathogen *Vibrio cholerae*. Ample numbers of Cholera cases are gauged every year on a global scale. According to one of the reports, about 37,783 cholera cases (84 Deaths) were incident in the India between 1997-2006 but only very less numbers were given for WHO Records[1]. Conventional therapy employs the administration of antibiotics to slacken the worse conditions. Antibiotics like Furazolidone, Erythromycin, Trimethoprim-Sulphamethoxazole, Chloramphenicol, Azithromycin, Ciprofloxacin are preferred for adult administration[2-4] whereas, Erythromycin and Azithromycin are widely preferred for children and pregnant women[4,5]. The foremost limitation with the conventional therapy is the development of MDR strains of *Vibrio cholerae* make this therapy abortive[2]. Therefore, an Anti-Virulent rather than an Anti-Bacterial drug is vital to counteract Cholera.

The discrete behavioral pattern of bacteria to the

diverse milieu they face is an outcome of the communication between them called Quorum Sensing (QS). This is facilitated through chemical molecules called autoinducers which bind to specific receptors on the bacterial cell. While QS up regulates virulence in most of the pathogens like *Pseudomonas aeruginosa* and Uropathogenic *Escherichia coli*[6-8], paradoxically QS down regulates virulence in *Vibrio cholerae*. CAI-1 and Autoinducer-2 (AI-2) serve as the cell signaling molecules in *Vibrio cholerae* for inter-*Vibrio* and inter species communication[9,10]. CAI-1 is synthesized by the enzyme CqsA[9,11]. Under the conditions of Low Cell Density (LCD), Lower concentration of CAI-1 results in the kinase activity of its cognate transmembrane receptor CqsS. CqsS transfers the phosphate group to a protein called LuxU and this, further transfers it to the regulator protein LuxO. LuxO is the point of convergence of both the system I (CAI-1) & system II (AI-2)[12-15]. System III (signal remains unknown) converges with SystemI & SystemII at LuxO protein through activation of VarS/A pathway but the exact mechanism of the series of steps that occur to activate LuxO is unknown[13,16]. Phosphorylated LuxO along with σ^{54} is in turn, a transcriptional activator of 4 Quorum regulatory RNAs (Qrr1-4). Qrr1-4 bound to the RNA chaperone Hfq, binds to mRNA transcript of HapR (the global virulence repressor protein in *Vibrio cholerae*). This binding renders the translation of HapR impossible[17].

HapR is a transcriptional repressor of VpsT, the latter is a transcriptional activator of genes required for bio film formation[11,18]. HapR also represses the production of AphA, a protein needed for activation of Cholera Toxin & Toxin co regulated pilin (the main virulence factors of Vibrio *cholerae*)[19]. HapR represses Haemolysin, the major virulence factor of *Vibrio cholerae* Biotype Eltor both transcriptionally and post-translationally (Amy MT *et al.,* 2010). HapR is also an activator of HapA protease and RNA polymerase sigma factor (RPOS σ^{54}). HapA protease serves as a "detachase" in detaching the *Vibrio cholerae* cells from the human cells, via digestion of GM-1 receptor of human intestinal cells, which serves as a bridge between bacterial and human cells[20]. σ^{54} increases the stress response of the bacterial cells to extreme nutrition and oxidative conditions[21]. Thus, under LCD conditions, virulence factors like Cholera toxin, Toxin co regulated pilus, Haemolysin, Bio film are expressed but factors like HapA protease, σ^{54} are repressed because of the absence of translation of HapR mRNA transcript. At High Cell Density (HCD), the binding of CAI-1 to the cognate receptor switches CqsS from functioning as kinase to phosphatase enzymes. Ultimately, there occurs dephosphorylation of LuxO protein, which brings about repression in qrr1-4 production. Qrr1-4 repression subsequently leads to a successful translation of HapR mRNA transcript. As a result, virulence factors like Haemolysin, Bio film, Cholera toxin, Toxin co regulated pilus are repressed and HapA protease and σ^{54} are expressed at HCD conditions. Hence, it could be inferred that "virulence and auto inducer concentration are inversely proportional" in case of *Vibrio cholerae.* Exploiting the bacterial camaraderie using signal interfering mimic molecules is a way used here to baffle the *Vibrio cholerae* QS system which ultimately aims at suppressing the virulence factors & pathogenecity during the early hours of an infection. The efficacy of *Melia dubia* leaves to serve as CAI-1 mimic source was investigated in this research work.

Melia dubia (Malai vembu) a plant of *Meliaceae* has a wide array of medicinal properties. Various parts of this plant have been used as a folk medicine in the treatment of Urinary Tract Infections (UTIs) in southern parts of Tamil Nadu. Even though the exact biological activity of this plant remains unknown, the faith in traditional folklores has driven the usage of this plant as a traditional medicine. *Melia dubia* has a wide range of quorum quenching molecules that target the SdiA protein of Uropathogenic *Escherichia coli*[8]. Thus, this plant was taken into consideration for evaluating its dexterity against Cholera.

MATERIALS AND METHODS

Extraction of plant material

Melia dubia, a species from the town Kumbakonam, nearby Thanjavur, Tamil Nadu was gathered. Dr. M. Jegadeesan identified and authenticated the plant materials. The voucher herbarium (TUH 285) specimens of the plant were deposited in the Department of Environmental and Herbal Science, Tamil University, Thanjavur, Tamil Nadu, India. The leaves of the

plant were dried and powdered. Since, extraction is difficult from fresh leaves, dried leaves were used. Cold Percolation method described by[8] was employed. In this method, to about 100 g of dried leaves, 900mL of solvent (water) was added. After 72 hours of percolation, the supernatant was recovered by filtration and the solvent in the supernatant was evaporated. Finally, the aqueous extract was lyophilized and stored at -80ºC in amber colored bottles.

Biofilm assay

This assay was done as described by Sabu Thomas *et al.*[22]. In this assay, 10 µL of the *Vibrio cholerae* culture purchased from Microbial Type Culture Collection (MTCC), Chandigarh (MTCC 3905), was added to a 96-well microtitre plate having 100 µL of LB medium and 100 µL of the testing component (Solvent fraction/Candidate drug). This was incubated in room temperature. Planktonic cells were washed off. This step was followed by washing the wells twice with de-ionized water.210 µL of 0.1% Crystal Violet (w/v) was added to the wells and a short incubation time of 10 minutes was given. Dye was discarded and the wells were again washed twice with de-ionized water. Finally, the crystal violet adhering to the bio film was solubilised in 210 µL of Dimethyl Sulphoxide (DMSO). Optical Density (OD) was measured at 595 nm in Bio Rad i-Mark microplate reader.

A graph of OD at 595 nm vs. concentration of the fraction/candidate drug was plotted and the Minimal Biofilm Eradication Concentration (MBEC) was found out. Three sets of experiments were carried out one without any drug which was aimed at comprehending the biofilm pattern at regular time intervals and the other two were done with the fractions and the predicted compound in the fraction (candidate drug).

Haemolysis assay

This procedure was a modification of the one done by Amy *et al.*[23] & Annette *et al.*[24]. 10 mL of sheep blood was centrifuged at 2400rpm for 5 minutes. The pellet obtained was washed twice with 10 mL Phosphate Buffer Saline (PBS). 10 µL of this erythrocyte suspension was incubated with the cell supernatant (that was previously obtained by centrifuging the cell culture sample at 12400rpm/10 minutes /20 º C) for 1 hour at 37ºC. Finally, this incubated sample was centrifuged at 2400 rpm/5 minutes. Optical density of the supernatant obtained was read at 540 nm using 1 % Triton X as the positive control. Water along with the erythrocyte suspension was considered to be the blank. % Haemolysis was calculated using the following formula:

$$\%Haemolysis = \frac{Absorbance(Sample) - Absorbance(Blank)}{Absorbance(Positive\ Control)} \quad (1)$$

This experiment was done in the following manner:

- Every hour the supernatant was subjected to Haemolysis assay, to understand the expression pattern of Haemolysin produced by *Vibrio cholerae* Biotype Eltor
- The culture grown was grown along with the solvent fraction that had the best anti-biofilm effect, for 7 hours (LCD) and the culture supernatant was tested for haemolytic activity
- Similarly, culture was grown with the candidate drug (Predicted cell signaling mimic in the fraction) for 7 hours and the culture supernatant was tested upon for haemolytic activity.

Protease assay

Azocasein assay was employed for quantitative estimation of protease. This method was described by Hiroaki *et al.*[25]. Cell supernatant was obtained by centrifuging the culture tubes at 2600 rpm/10 minutes/4ºC. 200µL of supernatant was incubated with 800 µL of Azocasein for 30 minutes at 37ºC. To this volume, 1200 µL of 1% Tri Chloro Acetic Acid (TCA) was added to arrest the enzymatic reaction. Ice incubation for 30 minutes was done and it was centrifuged at 13000rpm/5 minutes. To 1600 µL of this supernatant, 400 µL of 1.8N NaOH was added. Optical density was measured at 420 nm against the blank (Azocasein + TCA + NaOH). Proteolytic unit was calculated using the following formula:

$$Proteolytic\ Unit = \frac{Absorbance\ at\ 420nm}{Absorbance\ at\ 600nm} \quad (2)$$

This experiment was again carried out in the following manner:

- Every hour the supernatant was subjected to Azocasein protease assay, to understand the expression pattern of HapA protease produced by

Vibrio cholerae Biotype Eltor

- The culture grown was grown along with the solvent fraction that had the best anti-biofilm effect for 7 hours (LCD) and the culture supernatant was tested for Proteolytic activity
- Similarly, culture was grown with the candidate drug (Predicted cell signaling mimic in the fraction) for 7 hours and the culture supernatant was tested upon for haemolytic activity.

Oxidative stress response assay:

This assay was based on stress response of the *Vibrio cholerae* to H_2O_2[21]. 15mM H_2O_2 was incubated with culture for various time intervals (30 minutes & 60 minutes). The % survival counts were calculated based on time zero and indicated time points plate counts. The experiment was repeated for LCD (7th hour sample), HCD (15th hour sample), crude CAI-1 treated, Methanol fraction treated and Candidate drug treated *Vibrio cholerae* Biotype Eltor strain.

The stress response profile was evaluated based on plotting a graph of % survival vs. culture samples treated with various moieties. The increased stress response corresponds to the production of RPOS (RNA Polymerase Sigma Factor – σ^{54}) which in turn is activated by HapR. This assay was done in order to confirm the binding of CAI-1 mimic molecule which activates the production of HapR. Thus, to confirm that the cell signaling mimic acts through the QS pathway, this assay was performed.

Antibacterial assay

Antibacterial assay was done as per the protocol described by Niaz R *et al.*[26]. Sterile discs impregnated with the methanol fraction of *Melia dubia* aqeous leaf extract were placed on Mueller Hinton Agar medium, lawn cultured with *Vibrio cholerae* Biotype Eltor strain. The plates were incubated overnight in an incubator at 37ºC. Plates were observed the very next day for zone of lysis. The experiment was repeated to check for the Anti-bacterial effect of the Candidate drug.

GC-MS Analysis

The methanol fraction of the aqueous leaf extract of *Melia dubia* that showed the best anti virulent activity was subjected to GC-MS analysis for prediction of compounds using a PerkinElmer Clarus 500 GC-MS system. The oven program was kept at the temperature 60ºC at 7ºC/ min and ramped at 150ºC (2 minutes) at 8ºC/minute to 250ºC (10 minutes). Helium (1 ml/min) was used as carrier gas. The injector temperature was adjusted to 280ºC and the mass range was set at 40-600 amu. 2 µL of sample dissolved in methanol was injected into the system. The compounds were identified by the comparison of their spectra with those in the NIST (National Institute of Standard and Technology) mass spectral library.

COMPUTATIONAL STUDIES

Homology modeling of *vibrio cholerae* cqss protein

The amino acid sequence of the transmembrane CAI-1 receptor protein, CqsS of *Vibrio cholerae* Eltor (Uniprot Accession Number: Q9KKM66) was loaded into modeler 9v8 and searched for homology using BLAST Homology search option in Schrodinger Maestro. The protein that had the maximum sequence similarity to CqsS receptor was chosen as the template and about 10 models were generated independently in the modeler. The model with the least Root Mean Square Deviation (RMSD) value was selected and was saved in mol2 and pdb formats for docking studies.

Ligand and protein preparation

The 19 compounds reported by GC-MS of the methanol fraction of *Melia dubia* aqueous leaf extract were drawn using ACD Chemsketch. The energy minimized 3D ligand file was prepared for docking using Schrödinger LigPrep software. The modeled CqsS was prepared for docking by using protein preparation wizard of Schrödinger Maestro. Receptor grid was generated encompassing the sites with 0.375Å spacing.

Molecular docking

Docking studies were conducted using the Glide application of Schrödinger Maestro. The ligands and the prepared protein files were imported and docked using XP precision. The ligands that have docked pose with G Score lower than that of the natural ligand CAI-1 were identified and deemed the potential leads. The compound having the least G score (Candidate Drug) was further tested upon *in-vitro* for CqsS receptor

agonism and anti-virulent properties.

Cytotoxicity – MTT assay

MTT Assay[27] was employed to assess the cytotoxic effects of the candidate drug (4-Ethyl Resorcinol). HEp-2 cells were purchased from NCCS, Pune. In a 96 well microtitre plate, 100µL of DMEM medium was added to 100 µL of HEp-2 cells (10^5cells/mL) along with growth and medium control. This was subjected to 5% CO_2 incubation for 24 hours. 100 µL of candidate drug (4-Ethyl Resorcinol) [0, 10 & 25 folds concentration] were added, again an incubation time of 24 hours in a 5% CO_2 was allowed. Then, 10µL of (10 mg/mL) MTT was added & 5 % CO_2 incubation was done (3 hours). Finally, spent medium was discarded & replaced with 310µL of Isopropanol. Optical density was read at 590 nm and the % Cell viability was calculated using the following formula:

$$\%\text{Cell Viability} = \frac{\text{O.D.(Drug treated sample)} - \text{O.D.(Medium Control)}}{\text{O.D.(Growth Control)} - \text{O.D.(Medium Control)}} \quad (3)$$

Through this assay the effect of the candidate drug on % cell viability were assessed.

RESULTS

Comprehending the virulence gene expression pattern

Results indicate that virulence factors were expressed in a time dependent manner. It could be observed that factors like biofilm, haemolysin levels were inflated during the initial hours of growth (7th hour) and these levels gradually were ebbed away during the later phases of growth (Figure 1 & Figure 2). Contrarily, factors like protease and stress response levels were lower during the early phases and their levels gradually increased along the growth cycle, with the maximum at 15th hour (Figure 3 & Figure 4).

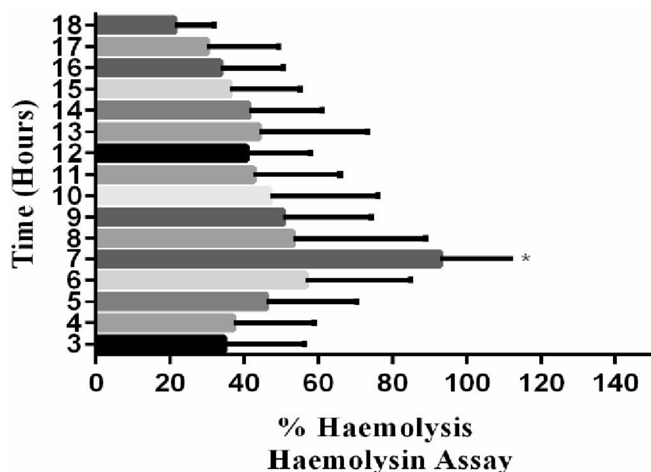

Figure 1 : Time dependent decrease in Haemolytic activity. Error bars indicate the standard errors of the mean of three independent trials

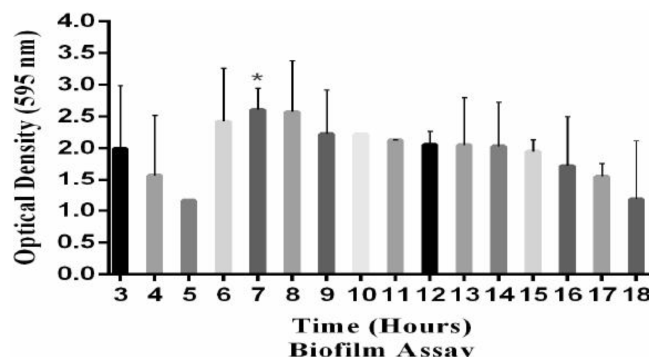

Figure 2 : Time dependent decrease in Haemolytic activity.Error bars indicate the standard errors of the mean of three independent trials

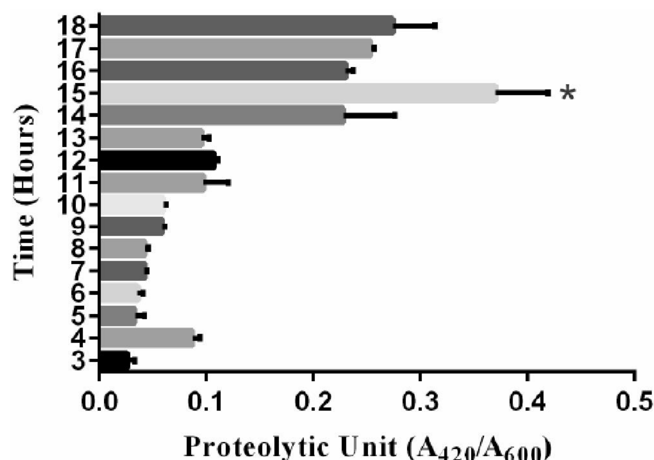

Figure 3 : Time dependent increase in Protease Activity (Quantitative Assay). Error bars indicate the standard errors of the mean of three independent trials

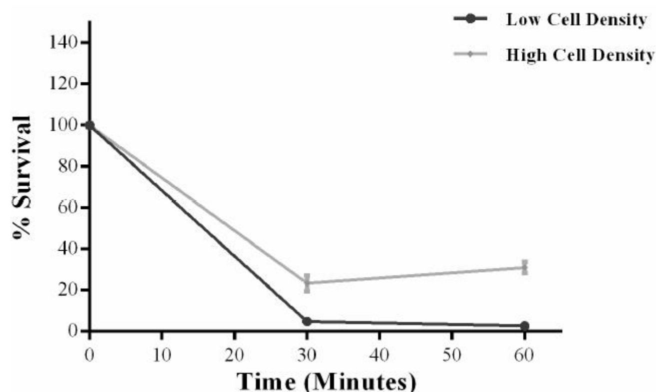

Figure 4 : % Survival versus Time of Exposure

Screening of *Vibrio cholerae* cell signaling molecules from *Melia dubia* leaves

Initially, biofilm assay with the crude fraction of *Melia dubia* aqueous leaf extract showed a 73.78% reduction in biofilm formation at a concentration of 1 µg/mL (Figure 5). Various solvent fractions of the crude leaf extract were made using column chromatography. Solvent fractions of solute were made using Ethyl Acetate and Methanol as the solvents (increasing the relative polarity). These solvent fractions were further tested for anti-virulence. It was observed that the Methanol fraction had considerable anti-biofilm activity than the Ethyl acetate fraction (Figure 6). This fraction was further tested upon for anti-virulent activity.

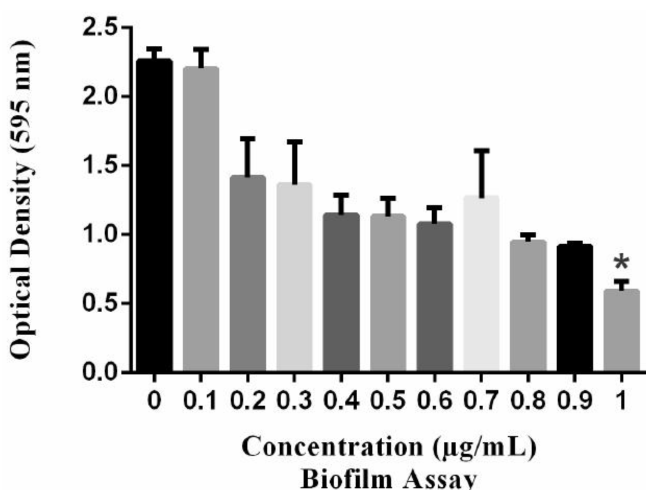

Figure 5 : Anti-Biofilm effect of the crude leaf extract (aqueous) of *Melia dubia*. Error bars indicate the standard errors of the mean of three independent trials

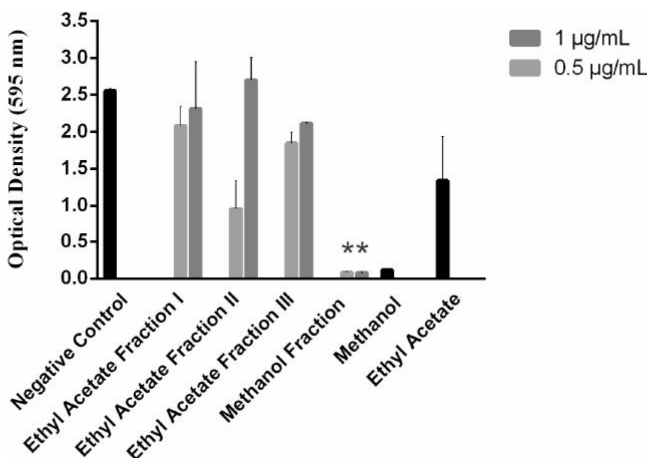

Figure 6 : Anti-Biofilm activities of Ethyl Acetate & Methanol fractions at two different concentrations. Error bars indicate the standard errors of the mean of three independent trials

Haemolysis, Protease, Stress response assays were done in order to confirm the presence of a cell signaling mimic molecule in the methanol fraction (1µg/mL). Results indicated that the compound in methanol fraction was a powerful cell signaling (CAI-1) mimic, since, at LCD biofilm, haemolysis were suppressed and simultaneously there was an upsurge in protease expression and stress response,

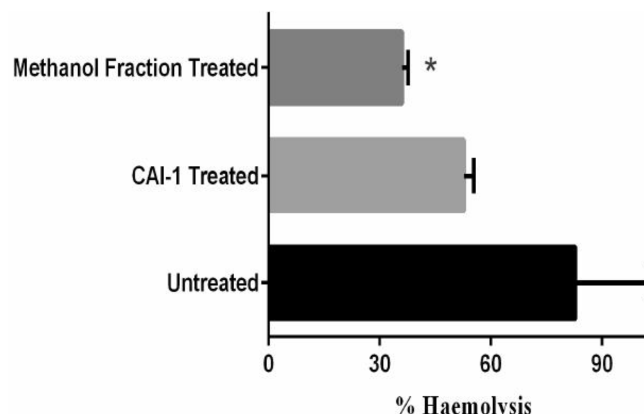

Figure 7 : Anti-Haemolytic activity of Methanol fraction. Error bars indicate the standard errors of the mean of three independent trials

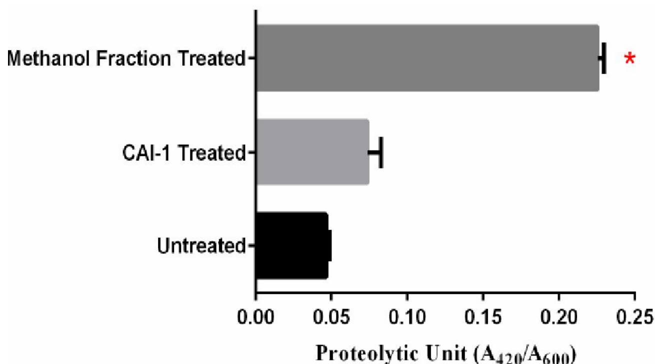

Figure 8 : Increase in Protease production upon Methanol fraction treatment. Error bars indicate the standard errors of the mean of three independent trials

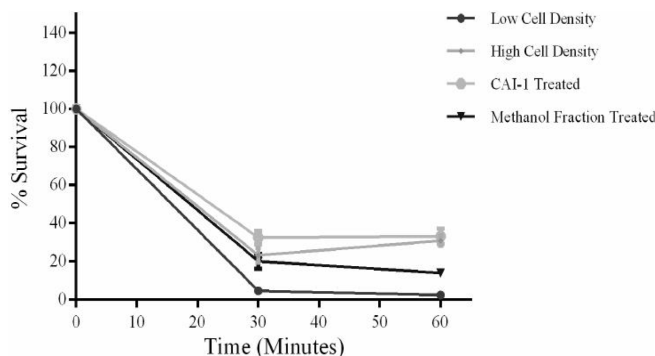

Figure 9 : Increase in Stress response due to HapR production upon Methanol fraction treatment

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which generally happens at HCD (Figure 7- 9).

Crude CAI-1 was from the culture supernatant was used as a positive of aqueous leaf extract had a potent CAI-1 mimic molecule, which had the potential to simulate HCD conditions in LCD. Additionally, antibacterial assay done with the methanol fraction revealed that none of the concentrations ranging from 0.2 µg/mL to 1 µg/mL were antibacterial (Figure 10).

Identification of the most plausible ligand for CqsS receptor

GC-MS analysis of the methanol fraction of *Melia dubia* leaf extract unmasked an array of 19 compounds (TABLE 1). CqsS protein was modeled using homology modeling. The protein PhoP from *Bacillus subtilis* (PDB id: 1MVO_A) was chosen as the template since CqsS shared 34% sequence identity with this protein. Molecular docking studies demonstrated the higher binding affinity (Least G Score = -6.255) of Compound14 (4-Ethyl Resorcinol) to CqsS receptor than the Compound20 (Cholera Autoinducer-1) (G Score = -2.334) (Figure 11). Upon superimposition of Compound_14 and Compound_20 in the CqsS protein, both the ligands bound to the same binding pocket in CqsS (Figure 12). Interaction pattern of these ligands with CqsS receptor showed that both the ligands made similar interactions with His 73 residue (Figure 13, Figure 14). These results suggest that Compound_14 (4-Ethyl Resorcinol) had a stronger binding affinity for CqsS protein than the native CAI-1. Thus, 4-Ethyl Resorci-

TABLE 1 : List of list of ligands obtained from *Melia dubia* using GC-MS analysis

Compound	Peak Name	Retention time
1.	3-Pentanol, 2-methyl-	5.22
2.	Ethanamine, N-ethyl-N-[(1-methylethoxy)methyl]-	5.58
3.	1-Methoxy-2-propyl acetat	5.81
4.	1,4-Cyclohexanediol, trans-	6.15
5.	2(5H)-Furanone, 3-methyl-	6.94
6.	2-Furanone, 2,5-dihydro-3,5-dimethyl	8.96
7.	Phenol, 2-methoxy-	9.1
8.	2-Pyrrolidinone	9.75
9.	4-Ethylbenzoic acid, ethyl ester	10.35
10.	2-Methylcyclohexylamine	11.03
11.	Benzaldehyde, 4-methyl-	12.43
12.	Ethanone, 1-(2-hydroxy-5-methylphenyl)-	13.88
13.	Phenol, 2,6-dimethoxy-	14.74
14.	1,3-Benzenediol, 4-ethyl-	17.88
15.	Hexahydropyrrolizin-3-one	19.57
16.	2-Propionyl-3,4,5,6-tetrahydropyridine	20.32
17.	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	26.13
18.	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a;1',2'-d]pyrazine	26.55
19.	Isoindole-1,3,5-trione, perhydro-2-cyclohexyl-	27.87

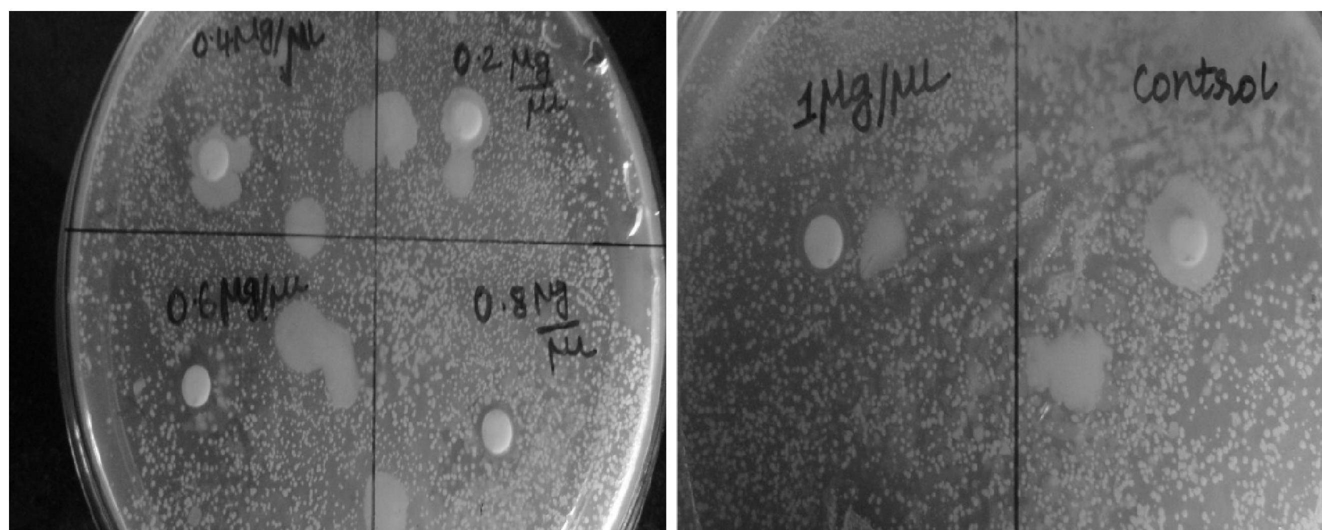

Figure 10 : Antibacterial assay of Methanol fraction showing no zone of bacteriolysis (Methanol was used as the Control)

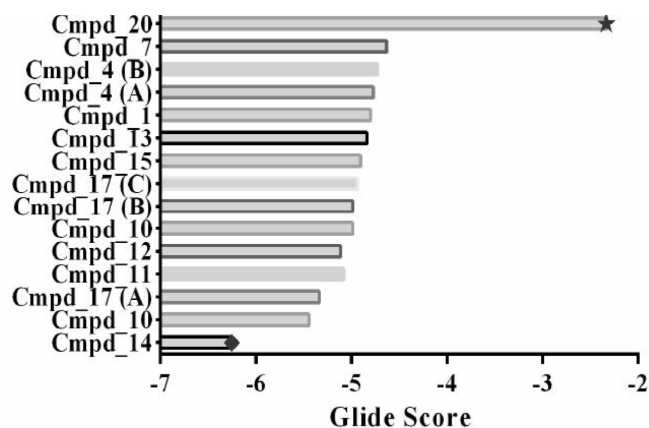

Figure 11 : Docking Studies. (A) Glide Score of the best docked compounds. Error bars indicate the standard errors of the mean of three independent trials

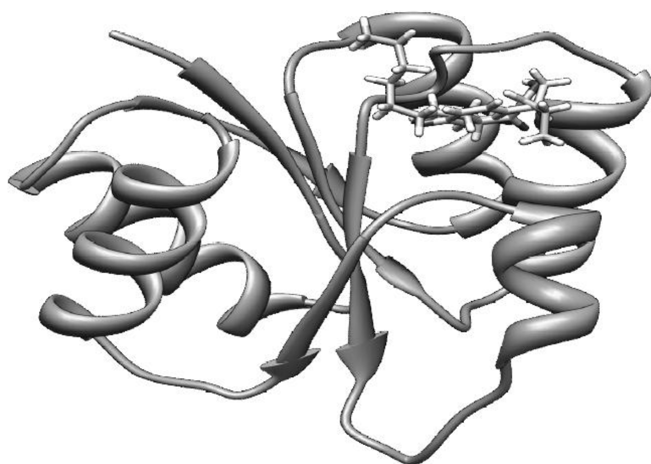

Figure 12 : Interaction pattern of compd_14(4-ethyl resorcinol with molecule CqsS)

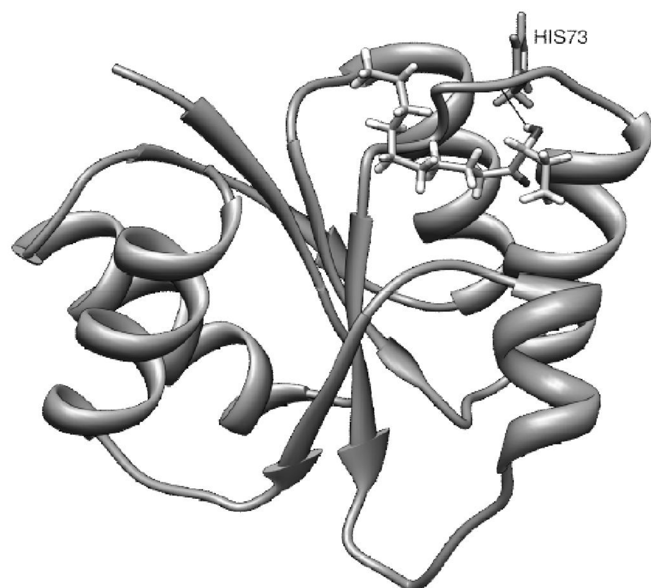

Figure 13 : Interaction pattern of compd_20(CAI-1) with modeled CqsS

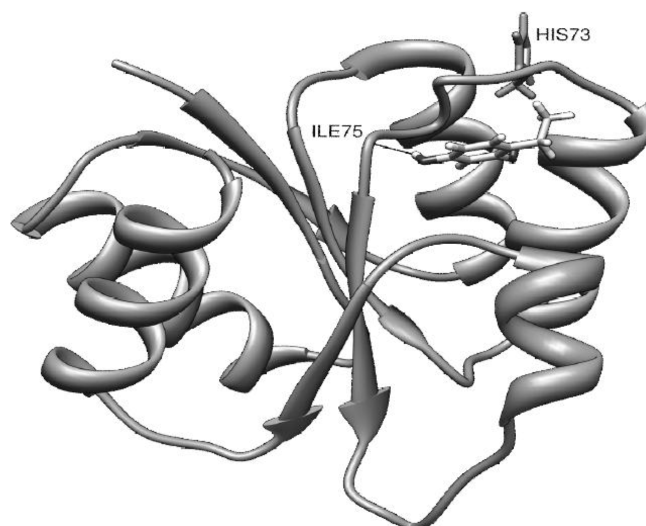

Figure 14 : Interaction pattern of compd_14 and compd_20 trends to be the same.

nol was considered to be the candidate drug.

Testing of receptor response to the candidate drug

4-Ethyl Resorcinol was purchased from Sigma-Aldrich, India. The Minimal Biofilm Eradication Concentration (MBEC) was found out to be 400μg/mL (2.8985mM) (Figure 15).

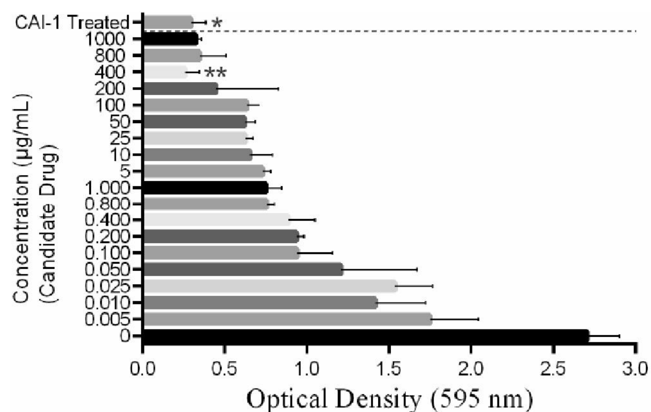

Figure 15 : Minimum Biofilm Eradication Assay (MBEC). Error bars indicate the standard errors of the mean of three independent trials.

This concentration was used as the standpoint in the subsequent anti-virulent assays. In each of the anti-virulence assay, crude CAI-1 (Cell supernatant of an overnight culture) was used as a positive control. MBEC of the Candidate drug disrupted the biofilm formation at 7th hour corresponding to a LCD. Candidate drug treatment also reduced Haemolysis (Figure 16) & increased the Protease production during LCD (Figure 17). Further Candidate drug increased the % Survival

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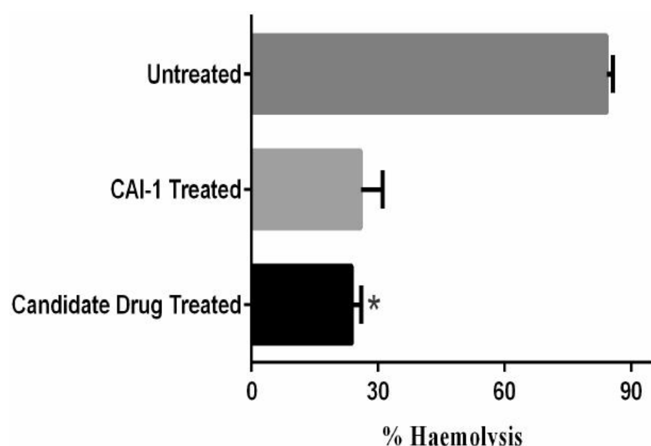

Figure 16 : Anti-Haemolytic Effect of candidate drug. Error bars indicate the standard errors of the mean of three independent trials.

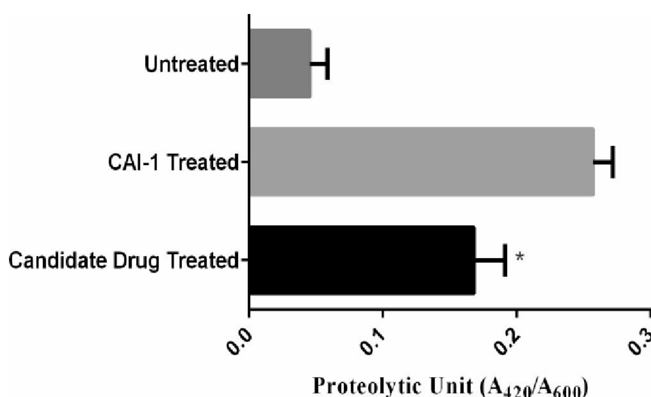

Figure 17 : Protease Assay – Candidate drug treatment. Error bars indicate the standard errors of the mean of three independent trials

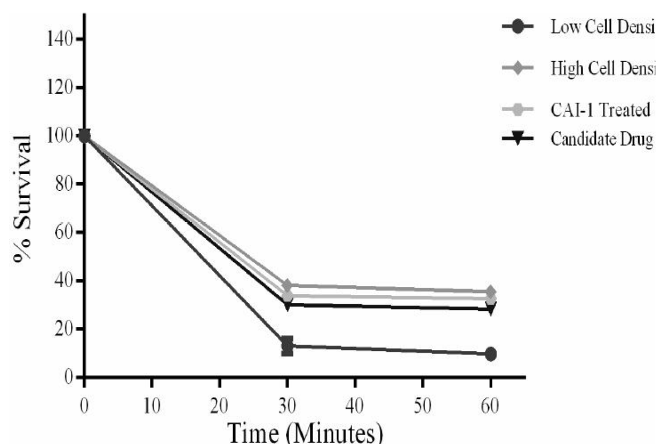

Figure 18 : Stress Responds Assay- Candidate drug treatment

rate after exposure of Hydrogen Peroxide (Figure 18).

Antibacterial assay confirmed that the candidate drug (4-Ethyl Resorcinol) was antivirulent and not antibacterial. Clearly, the results confirmed that none of the concentrations from 5ng/mL to 1000µg/mL had any anti-bacterial effect (Figure 19). In this assay, positive control was an octadisk (Hi-Media) having 8 antibiotics (viz. Penicillin, Azithromycin, Vancomycin, Cefazolin, Clindamycin, Cloxacillin, Erythromycin, Teicoplannin). Zone of bacteriolysis were observed in the plate having the positive control but not the others. These results made the point clear that 4-Ethyl Resorcinol was Anti-Virulent and not Anti-Bacterial.

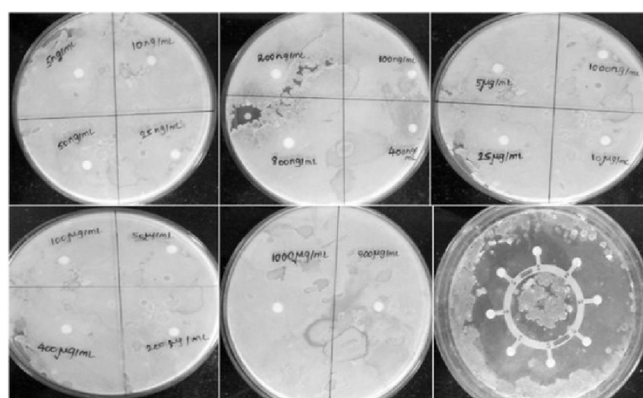

Figure 19 : Antibacterial Assay (Effect of Candidate drug on *Vibrio cholerae* at varying concentrations)

Checking for the effect of candidate drug on HEp-2 cell lines.

Cytotoxicity Assay using MTT showed that the candidate drug was not cytotoxic to the HEp-2 cells at the effective concentration (400µg/mL) and even at 10 folds

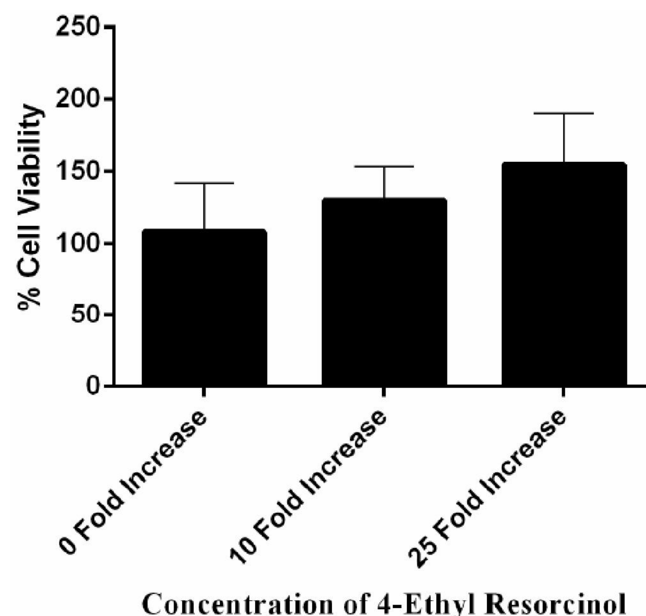

Figure 20 : % Cell Viability Vs Varying Concentrations of Candidate Drug. Error bars indicate the standard errors of the mean of three independent trials

(4000 µg/mL) and 25 folds (10000 µg/mL) increased concentrations (Figure 20). The % Cell viability was found to be increase upon treatment of drug. Thus, it was inferred that 4-Ethyl Resorcinol was not cytotoxic to HEp-2 cells even at higher concentrations.

DISCUSSION

The time dependent expression of virulence factors (viz. biofilm, haemolysin, protease, stress response) in the strain under study *Vibrio cholerae* ELTOR (MTCC 3905) confirms the fact that this strain is QS dependant i.e. it has no natural frameshift mutations in HapR region like *Vibrio cholerae* Eltor C6706[11] and unlike *Vibrio cholerae* Eltor N16961[28]. The time dependant increase in protease activity explains the transient life style of *Vibrio cholerae* in the intestinal environment.

The reduction in virulence (biofilm & haemolysin) and increment in protease production, post treatment of *Vibrio cholerae* with methanol fraction of *Melia dubia* leaf extract at LCD conditions have explained their possible role to serve as source of CAI-1 mimic molecules. Stress response to H_2O_2 has further confirmed the prediction of CAI-1 mimic molecules in the fraction and it is known that Rpos (RNA Polymerase σ Factor) enhances the nutritional and oxidative stress response of *Vibrio cholerae* Biotype Eltor. Rpos gene expression needs HapR for activation[21]. Hence, stress response would be elevated only when HapR is expressed. This indirectly means that HapR is produced upon methanol fraction treatment. Thus, was ascertained that the methanol fraction of the aqueous leaf extract had a potent CAI-1 mimic molecule, which had the potential to simulate HCD conditions in LCD.

Candidate drug (4-Ethyl Resorcinol) treatment resulted in repression of virulence and intensified the protease production at LCD. Increased % survival upon candidate drug treatment reflects on the fact that rpos is produced, indirectly meaning that HapR is expressed. This confirmed that the binding of candidate drug to CqsS receptor elicits an agonist response, which activates the production of HapR, suppressing the virulence and enhancing protease and rpos production. Thus, 4-Ethyl Resorcinol is a potent functional CAI-1 mimic molecule as it shares no structural homology with CAI-1 and is a CqsS receptor agonist.

The hike in % cell viability of HEp-2 cells upon treatment with candidate drug explains the possible role of 4-Ethyl Resorcinol to serve as a carcinogen or a nutrient. Clearly, the Material Safety Data Sheet (MSDS) from SIGMA –ALDRICH states that 4-Ethyl Resorcinol is not a Carcinogen. Hence, 4-Ethyl Resorcinol is completely non toxic and is a nutrient to HEp-2 cells.

Probing QS is thus an approach to stifle *Vibrio cholerae* pathogenesis. Analogues of CAI-1 that could serve as an agonist of CqsS receptor, would elicit the dephosphorylation of LuxO protein. This would in turn derepress the HapR production. Thus, employing CAI-1 mimic molecules (either structural/functional mimic), anti-virulent effect can be achieved in a LCD state along with the production of HapA protease. The production of HapA protease in a LCD state would detach the organism from human cells, thereby, blowing the organism away from human intestine. By this approach, virulence is suppressed & bacteria are ejected from the human system by HapA protease that serves like a typhoon. Hence, an anti-virulent rather than an antbacterial effect is achieved. This study has also highlighted on the existence of Prokaryotic cell signaling "like" molecules in Eukaryotes. Further studies into derivatization of 4-Ethyl Resorcinol could bring about even more efficacious CqsS receptor agonists that could serve as effective drugs against Cholera.

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