

Exploration of Targeting Mechanisms of Cinnamon Essential Oil Against Water Borne Multiple Drug Resistant Isolate *Aeromonas Hydrophila* C4

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Abstract

Introduction:

Cinnamon is dietary part of almost every Indian, hence there is no harm to consume it or apply it as antibacterial agent. Herein, we have observed that 0.5, 0.6 and 0.7 % concentrations cinnamon essential oil sufficient to control *A. hydrophila* within 5hrs of exposure. Cinnamaldehyde of is major component of used essential oil which has targeted the Flgn, Fij and Flja protein of flagella flowed by inhibition of swimming motility. Cinnamaldehyde was associated with damaging of cell membrane and leakage of electrolytes in extracellular environment.

Aim of the study:

In the current study cinnamon essential oil has been used to control the growth of multiple drug resistant *A. hydrophila*. *A. hydrophila* was isolated from well water system, and it was imipenem resistant bacteria as well.

Materials and Methods:

Cinnamon essential oil was characterized by using HPLC and FTIR. Antibacterial killing mechanism was determined by using electrical conductivity test, cytotoxic test by MTT assay, cell wall integrity test and motility test.

Results:

Cinnamon essential oil has potential to control the growth of *A. hydrophila*, and complete inhibition of cells was observed within 5hrs of exposure to 0.7% concentration of essential oil. By motility test and molecular docking it has been confirmed that for killing of bacterial cells, cinnamaldehyde of cinnamon essential oil targeting the cell membrane proteins as well as flagellar proteins Flgn, Fij and Flja.

Introduction

Aeromonas hydrophila are water borne pathogens, they can be isolated from fresh water, marine water as well as from chlorinated water (Janda and Abbott 2020). They are categorized in to two groups, motile and nonmotile; motile generally causes infections in human, while non motile are associated with fish and amphibian infections. They have diverse host range such as fishes, amphibians, reptiles and mammals including humans. *A. hydrophila* is an opportunistic pathogen and it has made one of the fish outbreak motile Aeromonad septicemia (MAS) in 19892 in china and next outbreak in Southeastern US in 2009 (Zhang et al. 2014). Consequence of this, both countries faced a big economy loss (Pang et al. 2015). *A. hydrophila* have various virulence factors such as adhesions, toxins, hemolysin and lipase (Aslani et al. 2004). Hence, while colonization in human or animals they secrete virulence factors and cause infection such as meningitis, soft tissue infections, septicemia, gastroenteritis, pneumonia, endocarditis and etc (Ko et al. 2000). But irregular use of antibiotics against *A. hydrophila* has posed a

pressure to evolve antibiotic resistance traits and currently they acquired resistant to most commonly prescribing antibiotics (Igbiosa et al. 2012; Stratev and Odeyemi 2016). Most of *A. hydrophila* have acquired resistance against second and third generation of antibiotics as well (Stratev and Odeyemi 2016; Rao and Gan 2014). Cephalosporins are last line of drug against gram negative bacteria, if bacteria are evolving resistance to last line of drug than they hard to control with currently available antibiotics. Emergence of antibiotic resistance and lack of discovery of new antibiotic have diverted the attention of research to other alternative such as essential oil therapy.

Essential oils have been practicing since the ancient time for the cure of several ailments. Therefore, they have been in used for the control of several health issues. Recently, interest towards the essential has been increased, since they have broad efficacy. Cinnamon essential is most common spice that is accompanied with various properties and more interestingly it is a part of daily diet of most of Indians. It is exhibited antibacterial, antiviral, anti-inflammatory, anti-diabetic, anti-malarial properties (Rao and Gan 2014). The important constituent of cinnamon essential oil is cinnamaldehyde, which is associated with microbicidal activity (Friedman 2017). Cinnamaldehyde exhibit broad antimicrobial activity such antibacterial, nematocidal activity, antifungal, antiviral, anti helminthes (Yossa et al. 2012; Visvalingam et al. 2013; Kwon et al. 2003). Hence, in the current study we had explored three oils eucalyptus, peppermint and cinnamon essential oils against imipenem resistant *A. hydrophila* that was isolated from fresh water. Different concentrations 0.1 to 0.7 % of essential oils were used and we found that cinnamon essential oil worked well as compare to eucalyptus and peppermint in controlling *A. hydrophila*. Motility experiment depicted that gradually increasing the concentration of cinnamon essential oil, the motility of *A. hydrophila* was also affected and at high concentration 0.7% there was no motility. To explore the mechanism of cinnamon essential on flagella which is associated with swimming motility, molecular docking was performed, by docking of cinnamaldehyde with FlgN, FliJ and FliA, and it was observed that cinnamaldehyde has high affinity for FlgN protein. Moreover, cinnamon essential oil is also targeting cell wall which was confirmed by cell integrity test and electrical conductivity test. The current finding has attributed that cinnamon essential has broad killing efficacy in a short duration against Multiple drug resistant *A. hydrophila*. Cinnamon essential can significantly control of spread of microbial infections without any side effect and risk of emergence of resistant bacteria.

Method And Materials

Bacteria and chemicals:

Aeromonas hydrophila was isolated from well water, Kozhikode, Kerala. After isolation, isolate was maintained in 50% glycerol stock at - 20°C and was cultured at 37° C.

For the analysis, analytical grade chemicals obtained from HiMedia and Merck, which have been used in the current study. Eucalyptus, Cinnamon and Peppermint essential oils were procured from the Nerma Science (India). Essential were diluted in methanol for the further study and diluted solutions were stored

at 4° C. Cinnamaldehyde (Sigma Aldrich), was used as standard for the determination of presence of cinnamaldehyde in cinnamon essential oil.

Sampling, isolation and identification of bacteria:

Well water sample was serial diluted and spread on the chromo cult media (HiMedia). Furthermore, the blue color colonies were identified and chosen for further study. Genus level characterization was done as per Holt et al.1994, method with some modification. Molecular identification of isolated bacteria was carried out by using 16s rRNA sequencing method. Briefly, bacterial DNA was isolated and amplified by using 16s rRNA Primer and followed by sequencing of amplified primer sequence. Sequenced data was Blast by using NCBI and corresponding phylogenetic tree was constructed by using neighbor joining method.

Well diffusion assay for detection of antibacterial efficacy of essential oils:

Well diffusion assay was performed as per the protocol of Dahiya and Purkayastha, 2012 with some modifications. In brief, over night culture of *A. hydrophila* with OD 0.1 was spread on the Mueller Hinton agar plates. Moreover, the wells were created on spread plate with the help of borer and further different concentrations (0.1 to 0.7%) of essential oils were added into the well. Plates were incubated at 37° C for 24 hrs, after incubation the zone size was measured.

Vapor phase assay:

Vapor phase agar diffusion test was performed to know the antimicrobial efficacy of vapor of essentials; it was performed as per the protocol of Lopez et al. 2005, with some modifications. To perform the vapor phase agar diffusion assay, Mueller Hinton agar plates spread with *A. hydrophila* was taken and further essential oils were placed on the center of filter paper and then filter paper was placed on the lead of petri dish. Petri dishes were covered with essential oil added filter paper and sealed with the parafilm. Petri dishes were incubated at 37° C for 24 hrs. After incubation inhibition zone was measured.

FTIR of cinnamon essential oil:

FTIR analysis was done to determine the presence of functional group of cinnamon essential oil by using PerkinElmer FTIR spectrophotometer. Analysis was carried out by diluting essential oil in methanol followed by measuring the absorbance, ranged from 4000-400cm⁻¹

HPLC of Cinnamon essential oil:

For the determination of presence of cinnamaldehyde in procured cinnamon essential oil, HPLC was performed. The separation was carried out by using Shimadzu class VP V 6.13 SPI HPLC equipped with C18, 50 × 4.6 mm column. Analysis was carried out by maintaining the oven temperature of column at 40°C. Mobile phase was prepared by using 35% of acetonitrile in 0.1 % acetic acid. For dilution of samples HPLC grade methanol was used. The flow rate of sample was 1.0 ml /min Chromatogram was observed at A285nm after running of sample for 25 min, analysis was carried out by injecting 20 µl sample. Data analysis was performed by using class VP software.

Antibiotic susceptibility test:

Antibiotic susceptibility test was performed as per the Kirby Bauer disk diffusion method according to CLSI, 2013 guideline. Briefly the overnight culture with 0.1 OD was taken and spread on the MHA media (Mueller Hinton Agar) poured plate, further the antibiotics disks cefoxitin(CXT), erythromycin(ERT), Teicoplanin(TEI), Clindamycin(CLD), Linzolid(LZD), Azythromycin(AZM), cefpirome(CFP), ceftazidime(CAZ), imipenem(IPM), ceftriaxone(CTR) piperacillin (PIT), cefetaxime(CTX), colistin(CLS), and cefepime(CPM) were placed on plate and incubated at 37°C for 18hr. After incubation the diameter around the disk was measured.

Blood Agar test:

Blood agar test was performed as per the protocol of Buxton, 2005 with some modification. 5.0% sheep blood was added in Trypton Soy Agar media after autoclaving. Prepared media was poured into sterilized petridish and then isolated bacteria *A. hydrophila* was streak on the media. Further, the plate with spread culture was incubated at 37°C for 24 h. After incubation the clear zone was observed for the beta hemolytic activity.

Congo red binding assay:

Congo red binding assay was performed as per the protocol of chen et al. 2003 with some modification. Herein, 0.3% congo red dye was added in BHI supplemented with 1% starch. After autoclaving media, plates were prepared by using cong red agar media and streak with *A. hydrophila* for 24 h at 37°C. After incubation dark red to blackish red colony was observed for determination of amyloid fibers in bacteria.

Time dependent killing analysis:

Time dependent killing experiment was performed as per the protocol of Singh and Katoch, 2020, to determine the killing of cells by using cinnamon essential oil for 5 hrs at every one hr time interval. Overnight log cell were centrifuged and dissolved in PBS. Bacterial suspension was treated with three concentrations (0.5, 0.6 and 0.7 %) of cinnamon essential oil and incubated for 5hrs and then CFU was determined at every 1h for next 5 hrs.

Cell wall integrity test (measuring the leaked cell's DNA or RNA by damage of cell wall by essential oil):

Cell wall integrity test was determined by measuring the cell constituent in supernatant treated with cinnamon essential oil. The experiment was performed as per the protocol of Du et al. 2012 with some modifications, briefly, the overnight cell were washed and treated with three concentration of (0.4, 0.5 and 0.6 %) of cinnamon essential then cells were incubated for 5hrs and every 1hr cells were centrifuged and supernatant absorbance was taken at 260nm, the procedure was repeated for next 4hrs.

Electric conductivity test:

Electrical conductivity assay was done to determine the cell membrane permeability. The experiment was done as per the protocol of Kong et al. 2008, with some modification, Briefly, the overnight cells were washed with PBS and then centrifuged further cells were dissolved in 5.0% of glucose solution (5.0%DMSO v/v) and centrifuge until the electrical conductivity of cells were near to that of 5.0% of glucose. The conductivity of 0.7 % cinnamon essential oil added with 5.0% glucose were measured and recorded as L1. Essential oil treated bacterial cells conductivity measured per hour recorded as L2. The conductivity of bacteria treated with boiling water for 5 min with 5.0% glucose recorded as L0. Cell membrane electrical conductivity was calculated by using formula-

Relative electric conductivity (%) = $(L2 - L1) / L0 \times 100\%$

Role of essential on motility of bacteria:

Swimming motility test was performed by using 0.3 % agar in trypton soy broth. Motility agar plates were prepared and inoculated with culture and further kept it for incubation at 37° C for 18hrs. After incubation zone size was checked.

In addition to this, swimming motility was performed with essential to check the effect of cinnamon essential on motility. The test performed on the slides, sterilized slide was taken and then 1 ml of motility agar media was spread on the slide followed by incubation of culture. Cinnamon essential oil infused disk was placed on each slid near to inoculum. Then after, slides were placed in petridished and kept it for the incubation after incubation the zone size of motility was examined.

Cytotoxic and DNA-damaging effects of essential oils by using MTT dye:

Cytotoxic effect of cinnamon essential oil was determined by MTT assay. MTT assay was performed as per the Abate et al. 1998, protocol with some modifications. Briefly, 100 µl over night log culture of *A. hydrophila* with OD0.1 was taken and mix with cinnamon essential to maintain the concentration 0.7% in microtiter plate well. Further the microtiter plate was incubated for 24 h at 37 °C. After incubation the cells were treated were incubated with 0.3% MTT dye in PBS for 20 min. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is tetrazolium dye and it is yellow in color. Metabolically cells have tendency turn the yellow color to purple color. After incubation with MTT dye cells were dissolved in dimethylsulphoxide and absorbance were taken A540.

Molecular docking:

Molecular docking was performed by using autodock vina, herein Intermediary steps such as pdbit files of ligand and proteins were generated by using AutoDock Tools (ADT) (Trott and Olson 2010). ADT was used to add the polar hydrogen bonds, Kollman charges and other parameters to proteins. Furthermore, the files were saved in pdbqt for docking. Autogrid was used for the preparation of grid map by using grid box. AutoDock Vina was used for docking with the help of protein ligand information, along with to grid box properties in the configure file. Autotodock/vina employs as a local search global optimizer. During the docking procedure, both the protein and ligands are considered as rigid. The results with the most favorable free energy of binding were recorded. The pose with lowest energy of binding or binding affinity was extracted and aligned with receptor structure for further analysis.

Statistical analysis

The statistical analysis was performed by a one-way analysis of variance (ANOVA). We considered that the difference is significant for $p \leq 0.05$. The experiments were carried out for three replicates and the results are expressed as mean \pm SD.

Results

In the current study *A. hydrophila* was isolated from the well water near to Kozhikode, Kerala. Isolation was followed by biochemical characterization and molecular identification by using 16s rRNA sequencing methods. On the basis of 16s rRNA it had been confirmed that the isolate was *A. hydrophila* and it had shown 97.6% similarity with closely related species (Fig. 1). Hence, aligned sequence similarity with isolate depicted that the isolated bacteria was novel strain and have had 97.6% similarity with *A. hydrophila*. Sequence had been submitted to genbank and submitted sequence no. is MW590617.

Antibiotic resistant pattern and pathogenic characterization of *A. hydrophila*:

Antibiotic susceptibility test was performed to determine the resistant profile of *A. hydrophila* by using cefoxitin(CXT), erythromycin(ERT), Teicoplanin(TEI), Clindamycin(CLD), Linzolid(LZD), Azythromycin(AZM), cefpirome(CFP), ceftazidime(CAZ), imipenem(IPM), ceftriaxone(CTR) piperacillin (PIT), cefetaxime(CTX), colistin(CLS), and cefepime(CPM) antibiotics. *A. hydrophila* was resistant to cefoxitin, tiecoplanin, clindamycin, linzolid, imipenem, ceftriaxone, piperacillin, cefetaxime, and colistine, while sensitive to erythromycin, azythromycin, cefpiron, ceftazidime and cefepime (table.1). The antibiotic resistant profile of bacteria illustrated that *A. hydrophila* was a multiple drug resistant bacteria. Further, their pathogenic characteristics were explored by using congo red binding assay, blood hemolytic test and ability to form biofilm.

Table-1 Characterization of *A. hydrophila* for identification of antibiotic resistance profile and pathogenic characteristics

Name of bacteria	Antibiotic resistance profile	Congo red Binding assay	Blood Agar test	Biofilm formation by CV assay
<i>A. hydrophila</i>	CXT ^R ERT ^S TEI ^R CLD ^R LZD ^R AZM ^S CFP ^S CAZ ^S IPM ^R CTR ^R PIT ^R CTX ^R CLS ^R CPM ^S	+++	+++	± 0.732
Note- R= resistant, S= sensitive, +=positive, - = negative				

Congo red binding assay was performed to determine the presence of curli (amyloid) fiber on the bacteria surface. Herein, the dark red colony was observed that is basically indicating the presence of curli fibers (table.1). Bacteria, generally utilizing this accessory structure for the biofilm formation. Furthermore, blood agar test was performed to determine the presence or absence of beta hemolytic activity in isolated *A. hydrophila*, formation of clear zone around the streak zone on blood agar plate clearly indicated that isolated strain had beta hemolytic activity (table.1). Adding to this, biofilm forming efficiency was examined by using crystal violet assay and it was observed that isolated *A. hydrophila* is a high biofilm forming bacteria. By determining these pathogenic characteristics, it had been confirmed that the isolated *A. hydrophila* was a pathogenic bacteria with multiple drug resistant characteristic.

Minimum inhibitory concentration of eucalyptus, peppermint and cinnamon essential oils against *A. hydrophila*:

To determine the minimum inhibitory concentration (MIC) of essential oils (eucalyptus, peppermint and cinnamon), we had used seven different concentrations (0.1 to 0.7%) against *A. hydrophila* by using well agar diffusion method. After analyzing the inhibition zone of each concentration of essential oils, it had been clear that the MIC of cinnamon essential was 0.3%, while peppermint had shown inhibition activity at 0.5%, and eucalyptus at 0.7% (Fig. 2). Herein, cinnamon essential had shown highest killing activity as compare to peppermint and eucalyptus essential oil. In addition to agar well diffusion test, vapor phase test was also performed with 0.7% concentration only, since essential oils are enriched with volatile substances as well, hence to examine the volatile properties of used essential oils, vapor phase test was

performed. The vapor phase test indicated that cinnamon and peppermint have had given high content of volatile substances as compare to eucalyptus (Fig. 2). On the basis of vapor phase experiment, it had been confirmed that cinnamon and peppermint essential oil were enriched with volatile substances. Hence, further study was conducted only on cinnamon essential oil.

Determination of bioactive functional compound in cinnamon essential oil:

FTIR spectra of Fig. 3 depicted about the presence of functional group of cinnamon essential oil. Graph had shown two major peaks in between $300-1200\text{ cm}^{-1}$ and $1700-1600\text{ cm}^{-1}$. As per Li et al. 2016, absorbance at 1666.90 cm^{-1} depicting about stretching of aldehyde group that indicating the presence of cinnamaldehyde. However, the absorbance peak at 1271 cm^{-1} corresponds to presence of phenol group which is indicating about the presence of eugenol.

For further identification of main bioactive component of cinnamon essential oil, HPLC was performed by using cinnamaldehyde as a standard. In HPLC of cinnamon essential oil, there was a peak observed in chromatograph that was corresponding to cinnamaldehyde at same retention time of standard cinnamaldehyde (Fig. 4). It confirmed about the presence of cinnamaldehyde in cinnamon essential oil.

Exploration of killing mechanism of cinnamaldehyde for A. hydrophila:

Bactericidal effect of cinnamon essential oil was performed by determining the colony forming unit after the treatment of bacteria with 0.5, 0.6 and 0.7% of cinnamon essential for 5 hrs. At every 1 hr, total viable count was performed for 5 hrs. There was $4.6\text{ log}_{10}\text{ cfu/ml}$ was observed after 1 hr with 0.5 % treatment of cinnamon essential oil. Similarly, there was significant reduction in cfu observed on increasing the concentration of cinnamon essential oil along with increasing the exposure time (Fig. 5). There was no colony observed at 5hrs, hence, the results indicated that cinnamon essential oil has potential to kill the cells within 5hrs.

Cell wall integrity test:

Effect of cinnamon essential oil on cell wall integrity of bacteria was determined by measuring the concentration of nucleic acid in cell suspension. In Fig. 6 the graph depicted about the graduation increase in concentration of DNA in supernatant on increasing the incubation time with cinnamon essential oil. Herein, we had used 0.7% of cinnamon essential oil. The result indicated about the leakage of cell on exposure to cinnamon essential oil.

Electrical conductivity test:

Electrical conductivity test was performed to measure the concentrations of electrolytes leaked from the cell membrane. Herein, we had used 0.7% essential oil treatment for 5hrs and we observed that on gradually increasing the incubation time the electrical conductivity was also increased (Fig. 6). The result indicated that cinnamon essential targeting the cell membrane.

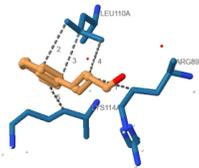
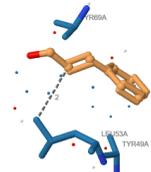
Effect of essential oil on motility of *A. hydrophila* (By examine motility on slide: A new concept):

Herein, the effect of cinnamon essential was examined against *A. aeromonas* motility (swimming). Here, concentration dependent inhibition activity of cinnamon essential was examined against the swimming motility of *A. hydrophila*. We had designed the experiment to performed on slide, despite of using petridishes, it is new concept as well(Fig. 7). After observing the motility results, it was cleared that swimming motility was affected by the cinnamon oil; however at 0.7% the motility was completely inhibited. In addition to this, twitching motility was also performed (result has not shown), and there was no such significant effect was observed. Furthermore, the molecular docking was also performed to see the impact at molecular level and it has been found that the cinnamaldehyde has potential to bind with flagellar gene (flgH), which is responsible for the motility in *A. hydrophila* (table.2). Cinnamaldehyde interacted with ARG and LEU amino acid of flagellar protein. Herein, we had performed molecular docking by using cinnamaldehyde against flgn, flj and flha. These are the gene responsible for the motility of bacteria.

Cytotoxic test:

In addition to this, the cytotoxicity of cinnamon essential oil was determined by using MTT test. MTT is a redox dye. It is yellow in color, however, the metabolically active cells have tendency turn the color from yellow to purple while metabolically inactive cells it is remain yellow in color. Herein, when 0.5, 0.6 and 0.7% concentrations of cinnamon essential oil applied on the *A. hydrophila*, than it has been observed that cells without cinnamon essential turned yellow color to purple while treated cells were remain yellow, the result indicated that at all the three concentration of cinnamon essential oil; there were no live cells (Fig. 8).

Table.2 Molecular Docking:

Compound	Cinnamaldehyde	Docking Score(DS) (kcal.mol ⁻¹)	Interacted amino acids (≤5Å)	Residue chain	Image of ligand protein interaction
Flgn		-3.7	ARG	89B	
			LEU	110A	
			LEU	110A	
			LEU	110A	
			LYS	114A	
Flij		-4.8	TYR	49A	
			LEU	53A	
			TYR	69A	
Flha		-4.8	PRO	550A	
			PRO	550A	
			ALA	585A	
			GLN	589A	

Discussion

A. hydrophila, is an opportunistic pathogens, it has potential to infect human as well as aquatic animals. Hence, in the current study, *A. hydrophila* was isolated from well water and characterized and identified by using 16s rRNA sequencing method. For the determination of antibiotic resistant pattern, antibiotic susceptibility test was performed; the results indicated that isolate was multiple drug resistant bacteria. Emergence of multiple drug resistant in aquatic microbes, precisely marine bacteria is more common, since every year million tones of antibiotic discarding in the marine habitat (Minguez et al. 2016). Anthropogenic activity, agriculture runoff and dumping of pharmaceutical waste are the most common cause of emergence of antibiotic resistance in aquatic bacteria. Herein, we had isolated multiple drug resistant *A. hydrophila* from fresh water. *A. hydrophila* most commonly a marine bacteria, but mixing of marine water with fresh during flood can be a possible reason for the intrusion of marine bacteria in fresh water system. Though it is a marine bacterium, but isolated from fresh water with multiple drug resistant and pathogenic trait, hence for the control of such superbug essential oil treatment was adopted.

Aromatic plants contain a variety of volatile substance, that have properties to heal the damaged cells and tissue as well as get relives from several type of ailment of body including microbial infections.

Presently, a big population of world is relying on the herbal medicines (Swamy et al. 2016). Essential oils are aromatic compound extracted from the plants, they have been in use in traditional medicine since a long back. They exhibit broad efficacy and are effective in relieving stress, chronic pain, reducing nausea and cancer (Shin et al. 2016; Bikmoradi et al. 2017). Essential oils have broad antimicrobial activity as well; they can control bacterial, virus and fungal infections. Hence in the current study we have used cinnamon, eucalyptus and peppermint essential oil for the control of *A. hydrophila*. There were various concentrations (0.1 to 0.7%) of essential oils used against *A. hydrophila*. Cinnamon essential oil had shown high inhibition activity against *A. hydrophila* as compare to eucalyptus and peppermint, therefore the cinnamon essential explored extensively in the current study.

In the used cinnamon essential oil, cinnamaldehyde was the main active component, identified by HPLC (Fig. 4), and was responsible for the killing of bacterial cells as well (Topa et al., 2018). Cell wall integrity test and electrical conductivity indicated about cell wall as a target site of cinnamon essential oil (Fig. 6). Sikkema et al. 1995, reported about the interaction of lipophilic compound of essential oil with hydrophobic part of proteins of membrane is responsible for disruption of cell membrane. Apart from the disruption membrane, cinnamaldehyde is also involved in hampering the swimming motility of *A. hydrophila* (Fig. 7). Swimming motility clearly indicated about the role of cinnamon essential in inhibition of swimming motility. Molecular docking of cinnamaldehyde and flgn gene attributed that ARG, LEU and LYS amino acids of protein Flgn are interacting with cinnamaldehyde (Table.2). Finding of current study indicating that use of cinnamon essential can be as an ideal therapeutic agent for the control of marine and fresh water borne bacteria.

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Figures

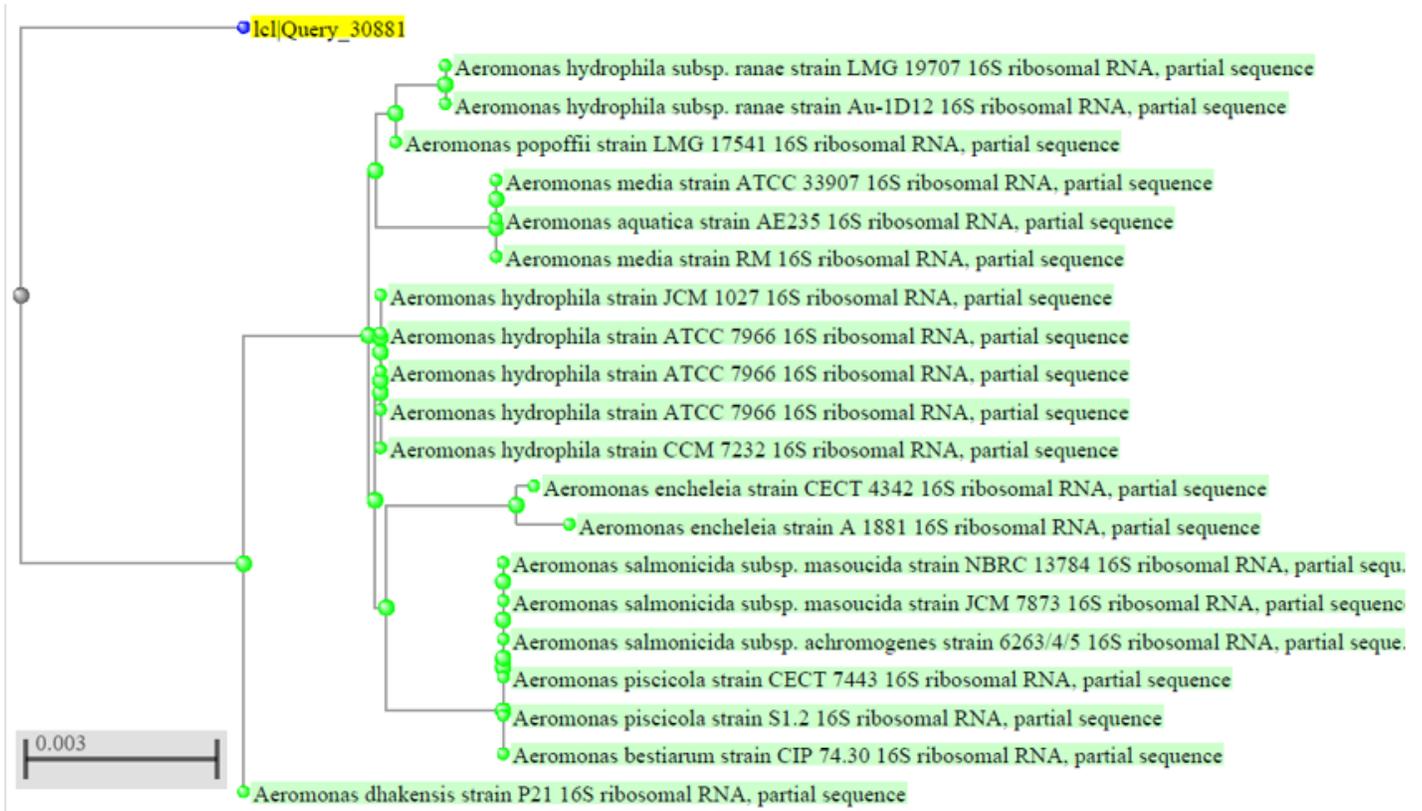


Figure 1

Phylogenetic tree of isolated *A. hydrophila*

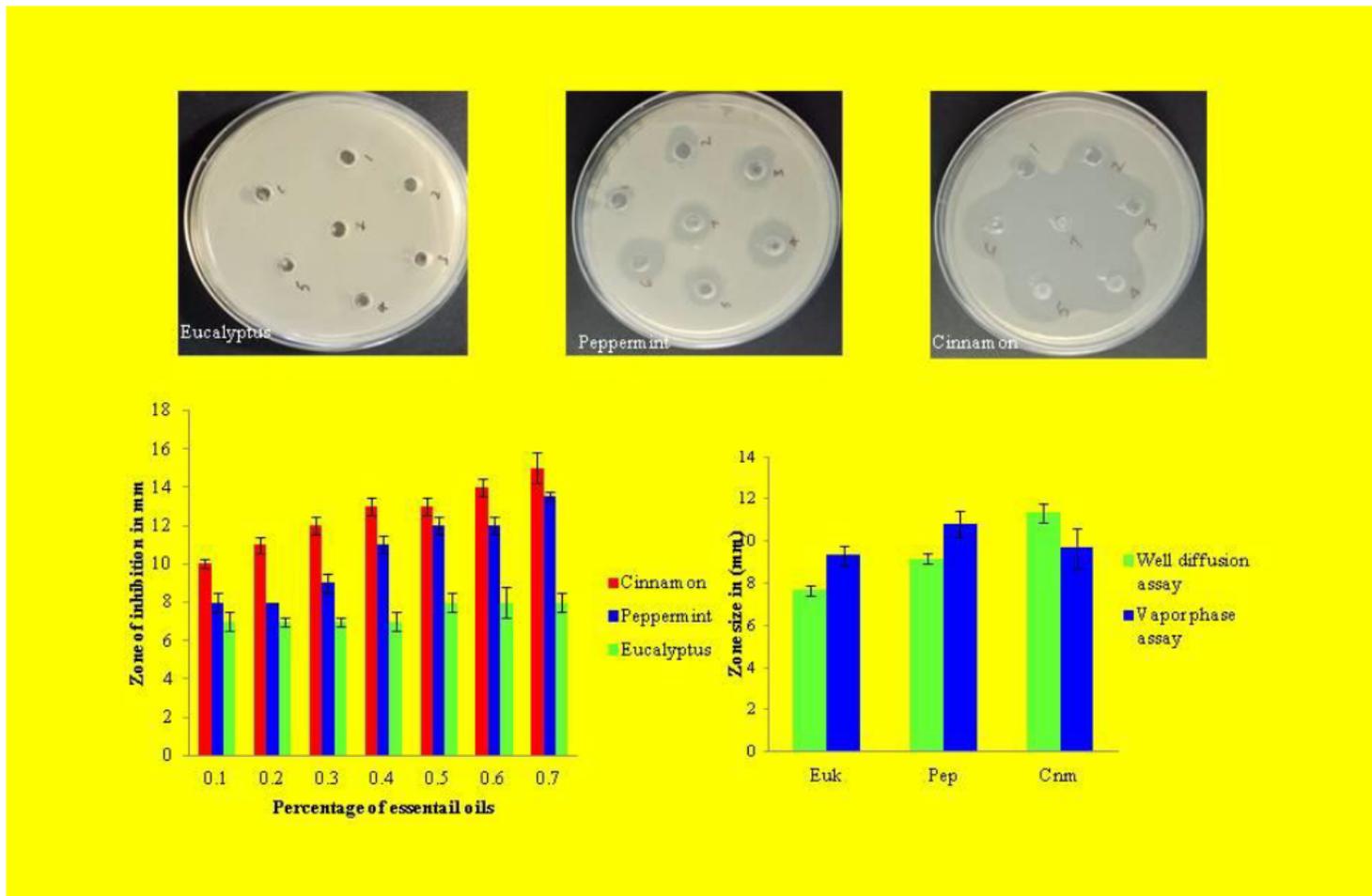


Figure 2

Antimicrobial effect of essential oils (eucalyptus, peppermint and cinnamon) with various concentrations (0.1 to 0.7%) against *A. hydrophila* (plates figure representing the inhibitory zone size) while corresponding graphs of well diffusion assay. In next graph there is comparative analysis of will diffusion assay (green bar) and vapor phase assay (blue bar),

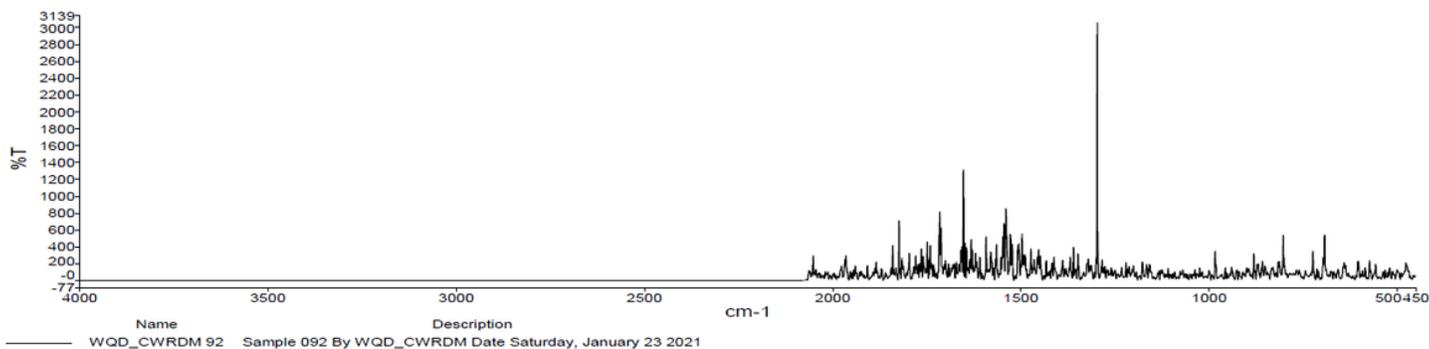


Figure 3

FTIR analysis of cinnamon essential oil

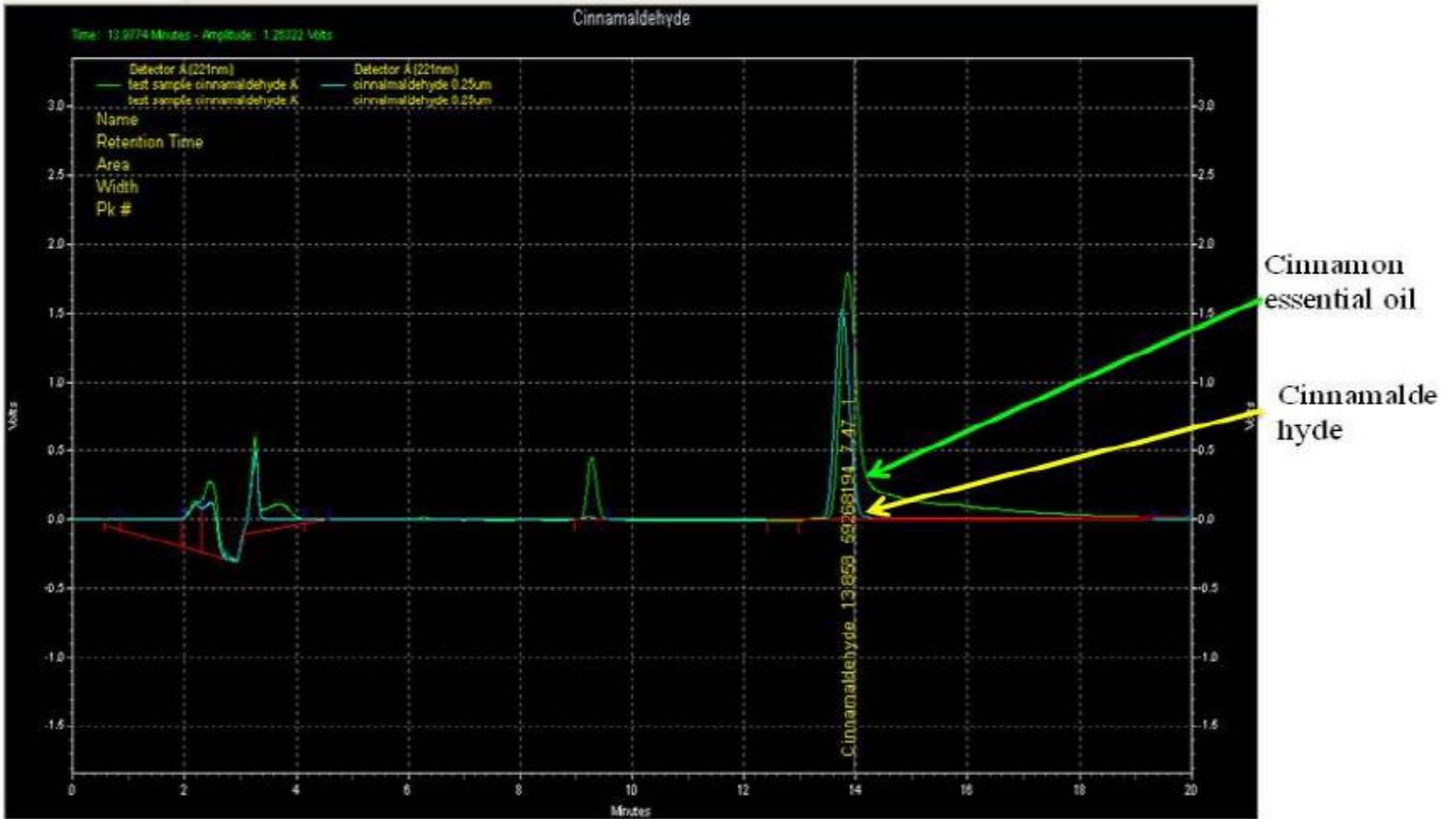


Figure 4

HPLC analysis of cinnamon essential for detection of cinnamaldehyde (green line), here cinnamaldehyde was used as standard (red line of chromatograph),

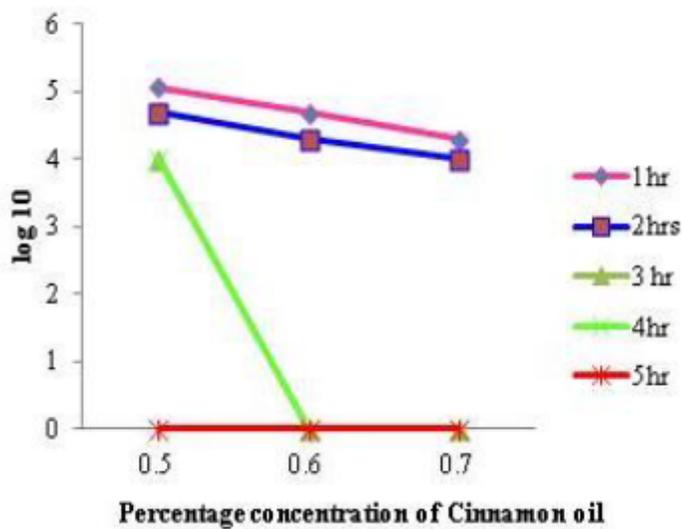
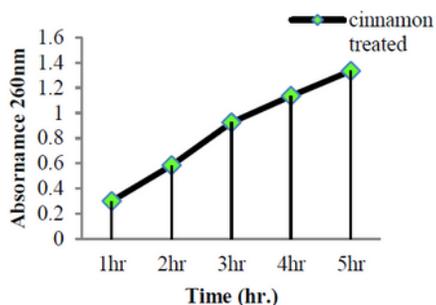


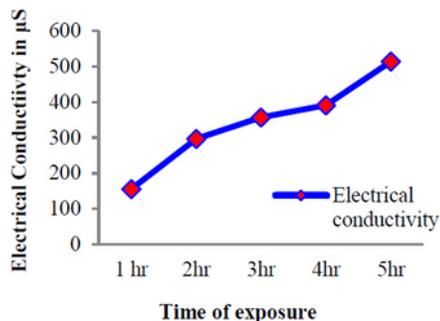
Figure 5

Colony forming unit determination of *A. hydrophila* treated with 0.5, 0.6 and 0.7 % of cinnamon essential oil

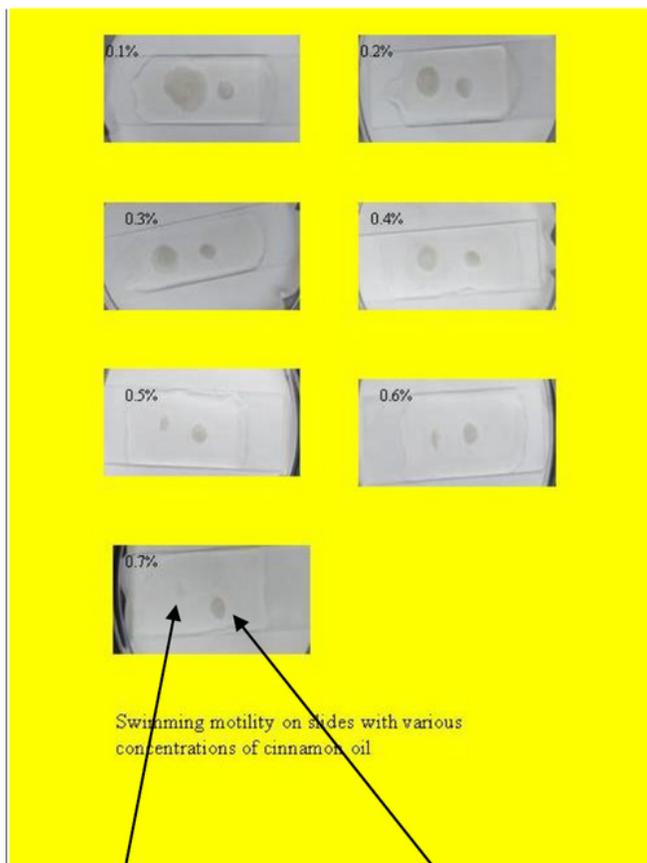
a) Cell membrane integrity



b) Electrical conductivity



c) Role of cinnamon on swimming motility of *A. hydrophila*:



Swimming motility on slides with various concentrations of cinnamon oil

Aeromonas hydrophila Cinnamon essential oil impregnated disc

Figure 6

A. hydrophila was treated with 0.7 % concentration of cinnamon essential oil for 5 hrs and then concentration of nucleic acid in supernatant was measured by spectrophotometer, b) Electrical conductivity of *A. hydrophila* treated with 0.7 % concentration of cinnamon essential oil, c) Effect of cinnamon essential oil on swimming motility of *A. hydrophila*, each slide contain sterile filter paper disk impregnate with various concentrations (0.1 to 0.7%) of cinnamon oil,

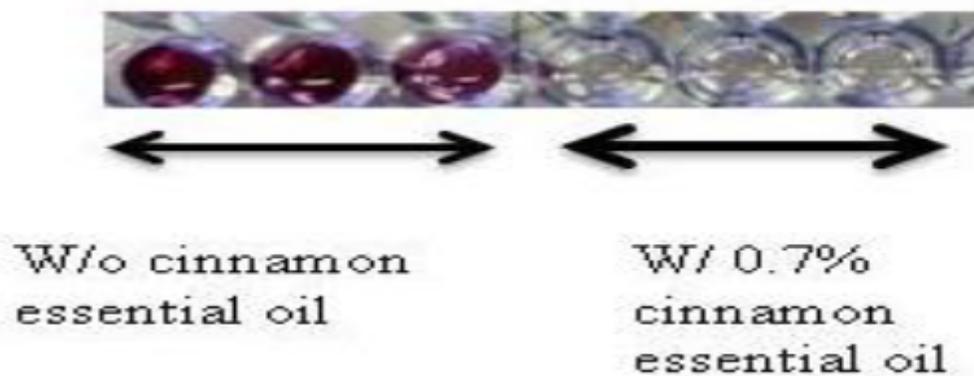


Figure 7

Cytotoxic test by using MTT dye on Microtiter plate, well contain *A. hydrophila* no treated and treated with 0.7 % concentration of cinnamon essential oil.

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Figure 8