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QUORUM QUENCHING ABILITY OF DIETARY SPICE *CINNAMOMUM VERUM* ON PATHOGENIC BACTERIA

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Keywords:

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ABSTRACT: Quorum sensing is a bacterial cell communication mechanism mediated through signal molecules such as AHL's. Many opportunistic pathogens use these quorum sensing circuits to coordinate their virulence gene expression. Wide range of naturally occurring substances particularly plant extracts have been evaluated for their ability to modulate quorum sensing in Gram-negative bacteria. Preliminary screening of popular food spices for QSI activity revealed that *Cinnamomum verum* (Dalchini) had good QSI activity. Recognizing its medicinal value and dietary consumption, an attempt was made to evaluate QSI activity of *Cinnamomum verum* on bacterial pathogens like *Pseudomonas* and *Serratia* and confirmed by using bioindicator organism *Chromobacterium violaceum*. *Cinnamomum verum* extract had more QSI activity than the antibacterial activity in *Serratia*. In *Pseudomonas*, the antibacterial activity was more while QSI activity was comparatively less. Interestingly spice extract could reduce swarming nature in both the organisms, which is one of the important virulent factor regulated by QS. The phytochemical components in the crude extract were analyzed by qualitative methods. MS analysis of the treated samples revealed the fact that AHL molecule is subjected to lysis or degradation. Chemical characterization of bioactive components in the crude extract of *Cinnamomum verum* is under process.

INTRODUCTION: Quorum sensing is a bacterial cell communication mechanism which is mediated through signal molecules such as AHL's. Many pathogenic bacteria especially opportunistic pathogens use these quorum sensing circuits to coordinate their virulence gene expression in accordance with population density. Recent research has revealed that eukaryotes are capable of interfering with bacterial communication by the production of molecular signals that interact with the bacterial QS system.

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Such Quorum quenchers have been intensively investigated as new class of antimicrobial agents. These compounds interfere in bacterial signaling processes[1].

Disruption of quorum sensing (Quorum Quenching) attenuates the pathogenicity in bacteria without imposing resistance. Hence wide range of naturally occurring substances, particularly plants extracts, have been evaluated for their ability to modulate quorum sensing in Gram-negative bacteria[2]. Phytochemicals have enormous structural diversity hence can be explored as quorum quenchers.

Many research groups are working on phytochemicals as quorum quenching agents by testing crude plant extracts. Vattem, D[2] had

initiated the study on the QSI activity of the dietary phytochemicals i.e secondary metabolites of plants against *C. violaceum* and *P. aeruginosa.* Allison L. Adonizio[3] reported inhibition of Quorum sensing controlled virulence factor production in *P. aeruginosa* by South Florida plant extracts. Bryant, S.[4] published that various medicinal plants extracts from the Indian subcontinent had QSI activity against *P. aeruginosa*. Maryam Zahin[5], screened ethanolic extracts of 24 medicinal plants of Indian origin for QSI activity against *Chromobacterium violaceum* CV026 and *Pseudomonas aeruginosa* PAO1.

Sandy Siew -Mian Yeo and Foong -Yee Tham[6] screened twenty Traditional Chinese Medicine (TCM) plants commonly used in South-East Asia for QS inhibitors using two biomonitor strains, *Chromobacterium violaceum* CV026 and *Pseudomonas aeruginosa* PAO1. These research papers proved that phytochemical extracts could effectively inhibit virulence factors regulated by QS.

Food spices are an essential component to a nutritious lifestyle. Research indicates that they contribute to health just as much as fruits. Preliminary screening of popular food spices for QSI activity was carried out in which *Cinnamomum verum* (Dalchini) had good QSI activity. C*innamomum verum* belongs to the family Lauraceae. The dried bark of *Cinnamomum* is used as spice worldwide. It is a very important food spice used in chocolate, apple pie, cinnamon buns, desserts, donuts and candies. It is also used to flavor hot cocoa, liquors and tea. It also comes in handy in flavoring cereals, sweet dishes and many more.

Cinnamon oils have anti-microbial activity. They are effective in preventing the growth of fungi, bacteria and yeast. In fact, Cinnamon essential oils can take the place of traditional food preservative. If added to food as preservative, cinnamon oil does not just act like good preservative, but also help in bringing out the natural taste of food. It was also known to exhibit antioxidant properties. Cinnamon is found to reduce type 2 diabetes in preclinical studies[7]. Recognizing its medicinal value and dietary consumption, an attempt was made to evaluate QSI activity of *Cinnamomum verum* on bacterial pathogens like *Pseudomonas*, *Serratia* and *Chromobacterium violaceum*, a bioindicator organism for studying QSI activity.

MATERIALS AND METHODS:

Microorganisms

***Serratia marcescenes*:** Isolate obtained from rhizosphere soil samples of college campus was characterized by morphological, biochemical methods and by 16s rRNA analysis as *Serratia marcescenes* (16srRNA sequence was deposited in GenBank under Acc.no:JQ21730). It was named as *Serratia* sps *YAJS* and used for QSI activity study.

***Pseudomonas aeruginosa*:** This organism isolated from infected tomato plant was used for QSI activity comparative studies after characterizing by standard microbiological, biochemical, enzymatic and 16s rRNA analysis.

***Chromobacterium violaceum 12472*:** A bio indicator for QSI activity was obtained from Dr. Hammeda bee, Asst. Professor, Department of Microbiology, Osmania University.

QSI activity analysis of bacterial cultures

As the study focused on use of dietary phytochemicals, the aqueous extract of spice *Cinnamomum verum* (Dalchini) was prepared for QSI activity analysis. The spice powder was dissolved in aqueous extracts to get a final concentration of 10mg/ml, filtered using 0.2 micrometer pore size filter and then used to test Quorum sensing inhibitory activity.

Quorum quenching activity on *Serratia* isolate

Serial dilution of the crude extract of food spice was made in nutrient broth and 0.1 ml of overnight grown culture of *Serratia* was added and incubated over night at 30^{o}C. After incubation, growth was monitored by recording absorbance at 600nm. Since the production of protease, prodigiosin were quorum sensing dependent, these parameters were monitored in the treated samples at all dilutions. Prodigiosin assay was done as described by Tomohiro Morohoshi[8]. Protease activity was determined by Mohsen Fathi Najafi [9] method.

Swarming activity was determined by Daniel B. Kearns [10] method. The swarm agar plates were prepared as recommended and 250 µL of spice

extract was seeded with 5 mL of the agar and poured immediately on a 10 mL pre-warmed agar plate as an overlay. Two hundred microliters of the bacterial culture was inoculated at the center of the agar surface and the plate was incubated for 16 hours at 37 °C.

Dnase activity was tested on DNA agar by inoculating the culture as a thick band at the center of the plate. The plate was incubated at 32^{o}C overnight. After incubation the plate was flooded with 1N HCl. Dnase production was identified as a halo zone of clearance (DNA degradation) around the culture streak[11].

Quorum quenching activity on *Pseudomonas*:
Serial dilution of the spice extract was made in nutrient broth and 0.1 ml of overnight grown culture of *Pseudomonas* was added and incubated overnight at 37^{o}C. After incubation, growth was monitored by recording absorbance at 600nm. Since the production of protease and pyocyanin were quorum sensing dependent, these parameters were monitored in the treated samples for all dilutions. Pyocyanin assay was done as described by Li Ying Tan[12]. Protease activity was carried out by Mohsen Fathi Najafi[9].

QSI activity on *Chromobacterium violaceum*:
QSI activity of crude extracts of spice was tested by violacein inhibition assay using *Chromobacterium violaceum* as bioindicator organism[5]. The nutrient agar plates were pre-seeded with overnight grown culture of *Chromobacterium violaceum* strain. Wells were made in the agar plate with a sterile cork borer of 6mm in diameter. The wells were loaded with 50µl of the sample to be tested and incubated overnight at 30^{o}C. The QSI activity was calculated by measuring the diameter of colorless haloes created due to inhibition of violacein pigment but not the growth.

AHL extraction:
5ml of overnight grown culture was used for AHL extraction[8]. Cells were separated by centrifugation and supernatants were extracted twice with equal volumes of ethyl acetate. The extracts were evaporated to dryness. Later they were dissolved in 50-100 µl of ethyl acetate and used for further studies.

Mass spectroscopy:
The AHL samples were extracted as mentioned above and subjected for MS analysis. Samples treated with and without spice extract were subjected to analysis for comparison. Mass spectral analysis was performed at IICT, Hyderabad. Mass spectra were recorded on Finnigan MAT 1020, mass spectrometer operating at 70 eV.

HPLC Analysis:
Aqueous crude extracts of *Cinnamomum verum* were subjected to HPLC analysis at central facilities for research and development (CFRD) at Osmania University, Hyderabad by standard methods[13].

Statistical Analysis:
All experiments were performed in triplicates and standard deviation was calculated to all the samples in MS Excel. The data was also analyzed by ANOVA and one way variance using SPSS package. All the graphs were represented with error bars.

RESULTS:
Quorum quenching activity on *Serratia* isolate:
Serratia is an opportunistic pathogen, which causes a variety of infections. *Serratia marcescens* involved in nosocomial outbreaks in intensive care units. The *Serratia sps YAJS* employed in present study was isolated and characterized as *Serratia marcescens* with 95% similarity by phylogenetic analysis [14].

Serratia produces N-Acetyl Homoserine lactone as quorum sensing signaling molecule which is used for regulation of genes for extracellular virulence factor like prodigiosin, swarming motility, secretion of enzymes like protease and Dnase. These parameters were monitored in the treated samples. From the results represented in **Figure 1**, it was evident that there was difference in growth of *Serratia* isolate before and after treatment with this spice extract. With increase in concentration of extract there was reduction in growth i.e. 8.9% reduction was noticed at 4mg/ml, which further increased to 14.5% at higher concentration. This result was in accordance with the reported antibacterial activity of the spice. Interestingly, spice had quorum quenching ability as it effectively reduced the QS regulated virulent factors like

prodigiosin, protease activity, and swarming nature. Relative prodigiosin values from figure 1 were observed to be influenced as there was 17% reduction at 2mg/ml concentration, which gradually touched to 61.7% at higher concentration. The protease activity of *Serratia* was reduced by 41.1% at 2mg/ml concentration and increased to 54.9% at 8mg/ml. One of the important virulent characters like swarming nature of *Serratia* was also found effected with the treatment as there was 54 % reduction at 8mg/ml concentration in **Figure 2**. From these results it was evident that the phytochemical components of spice aqueous extract were effective in reducing the three virulent factors of *Serratia* tested. However, Dnase activity of *Serratia* was not affected by the treatment.

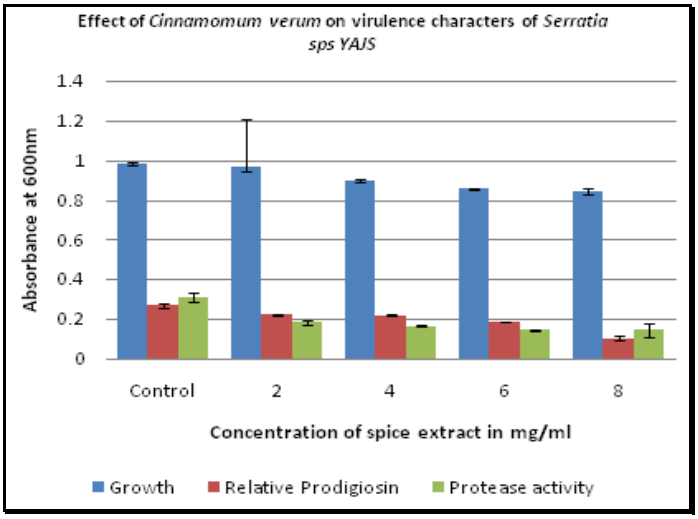


FIG 1: EFFECT OF *CINNAMOMUM VERUM* ON VIRULENCE CHARACTERS OF *SERRATIA SPS YAJS*

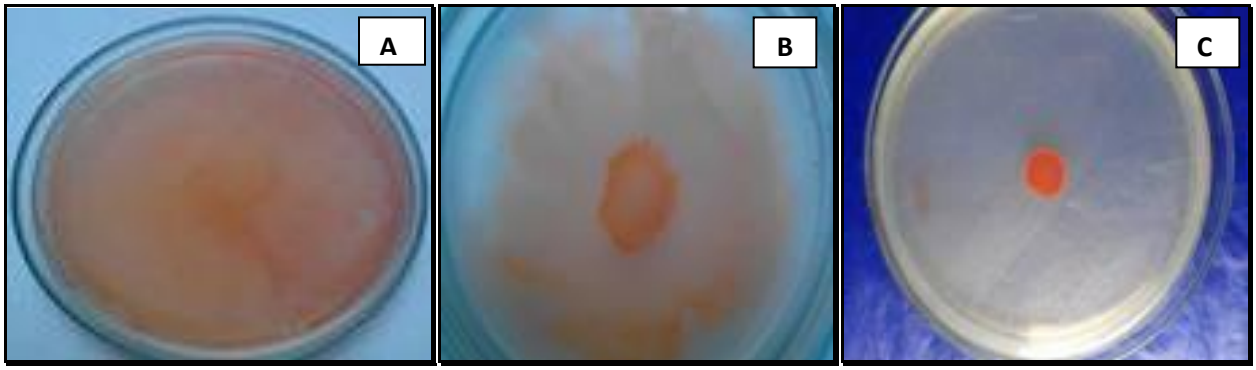


FIG 2: INHIBITION OF SWARMING NATURE BY *CINNAMOMUM VERUM* EXTRACT

Reduction in swarming nature of *Serratia*.
A-Control **B-** 2mg/ml concentration **C-** 8mg/ml concentration

Quorum quenching activity on *Pseudomonas*:

Pseudomonas aeruginosa is the second most prevalent organism in nosocomial infections, third in causing urinary tract infections, fifth in post-surgical infections. This was the major cause of mortality in patients suffering with cystic fibrosis. Continuous and improper use of antibiotics rendered this organism resistant to different antibiotics.

Pseudomonas aeruginosa produces N-Acetyl Homoserine lactone as quorum sensing signaling molecule which is used for regulation of genes for extracellular virulence factors like, phospholipase C, rhamnolipid, superoxide dismutase, HCN, exotoxin A, exoenzyme, pyocyanin, pyoverdin, LasA protease and LasB elastase. Many of these factors cause reactions in the host leading to cell death and tissue necrosis.

An attempt was made to test QSI activity of this food spice against *Pseudomonas* as most of the research was reported against this organism. The effect of spice extract on pyocyanin production, protease activity, swarming nature and growth were assayed in the treated samples. *Cinnamomum verum* extract was observed from **Figure 3a** to have a concentration dependent reduction in growth. Maximum reduction was noticed at 8mg/ml by 44.61% proving its antibacterial activity.

Pyocyanin is produced by strains of *Pseudomonas aeruginosa* as a water soluble blue-green pigment, which belongs to the Phenazine family. Pyocyanin enhances oxidative metabolism, which increases the formation of intracellular reactive oxygen species (ROS), which is advantageous for bacterial survival and thus the infection sustains. Chemicals which could reduce the pyocyanin production will

definitely control the virulence behavior of the pathogen. *Cinnamomum verum* extract recorded least pyocyanin production i.e. 11.64% and 6.86% respectively as showed in **Figure 3b**.

P. aeruginosa proteases are believed to play a major role in pathogenesis via host tissue degradation. In the present study effect of crude spice extract was tested on protease activity in *P. aeruginosa.* At 2mg/ml concentration, *Cinnamomum verum* extract had shown 25.7% reduction, and at higher concentration 45 % reduction was recorded in **Figure 3c**.

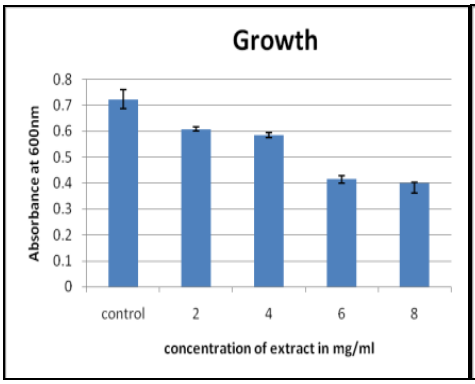


FIGURE 3A

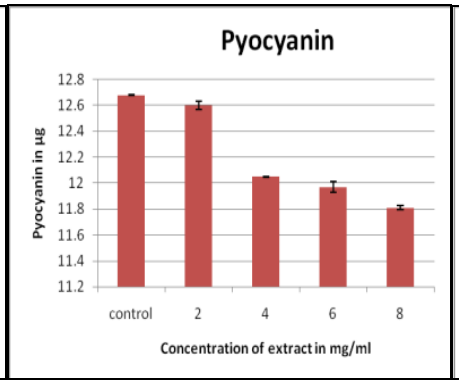


FIGURE 3B

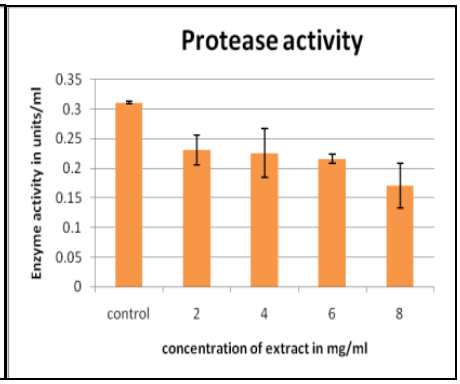
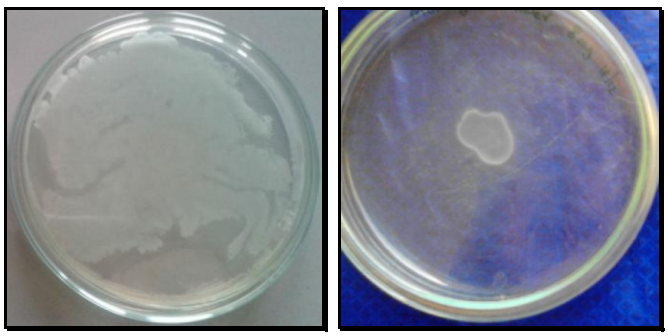


FIGURE 3C

*Error bars depicts standard deviation

Swarming motility is a quorum sensing regulated character which has been characterized as type of flagella-dependent movement on a semi-solid agar surface. In *P. aeruginosa, the* colonies display the formation of complex dendritic, fractal-like patterns. Overhage[15] and coworkers reported that swarming cells exhibited up-regulation of genes associated with virulence character expression. In present study *Cinnamomum verum* extract could influence swarming nature of *Psuedomonas sps* as depicted in **Figure 4**. As *Pseudomonas sps* do not produce extracellular Dnases, this factor could not be tested.

In Pseudomonas, Cinnamomum verum extract could reduce only the protease and swarming nature but could not influence pyocyanin production.



A. BEFORE TREATMENT **B. AFTER TREATMENT**

FIG 4: SWARMING NATURE OF *PSEUDOMONAS*

QSI activity on *Chromobacterium violaceum*

As most of the research papers to date on quorum quenching activity of plant extracts were tested against the bio-indicator organism *Chromobacterium violaceum,* present study was also extended to test spice extract on *Chromobacterium violaceum (ATCC 12472).* This was performed by studying violacein inhibition assay, a plate based method.

Crude aqueous extract was loaded on to Luria Bertani (LB) plates spread with *C. violaceum.* Plates were incubated overnight at 30 °C, and QS inhibition was detected by a ring of colorless, but viable, cells around the well. Loss of purple pigment in *C. violaceum* is indicative of QS inhibition by spice aqueous extract. Strong anti-QS activity was observed with aqueous extracts against distilled water as negative control.

The halos produced on lawns of the biomonitor strain could be the result of either (i) inhibition of cell growth or (ii) quenching of QS signals. Growth inhibition would produce a clear halo versus a turbid halo for the latter. To differentiate, the halo was examined under a higher magnification. The halo-effect is created by pigment less (QS interrupted) cells adjacent to the wells. Clear halos

as seen in **Figure 5** against violet background around the sample wells were measured.

FIG 5. COLORLESS HALOES INDICATE THE QSI ACTIVITY

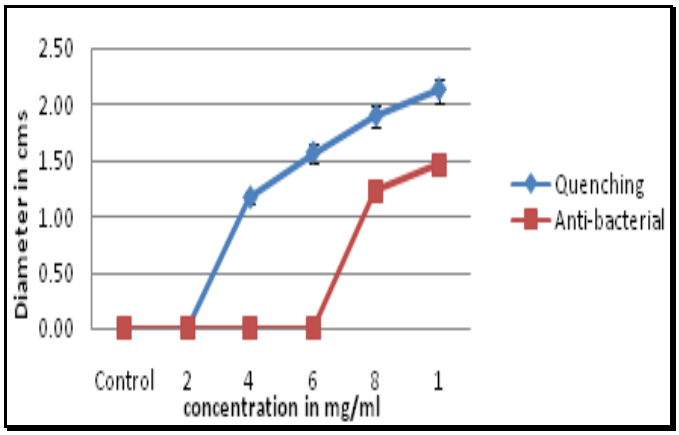


FIG 6: EFFECT OF *CINAMOMMUM VERUM* EXTRACT ON *CHROMOBACTERIUM VIOLACEUM*

Interesting observations were made in this assay as both antibacterial activities were found associated with quorum quenching activity. **Figure 6** showed antibacterial activity of *Cinnamomum verum* extract at 8mg/ml. However quorum quenching activity i.e. violacein inhibition activity was observed even at low levels of concentrations tested. These results indicate the presence of strong quorum quenching compounds in the aqueous crude extract of *Cinnamomum verum*.

To know the fate of AHLs for hypothesizing probable mechanism of quenching activity the treated samples were subjected to Mass spectroscopy. MS analysis revealed the cleavage of C_6AHL at the acyl side chain in **Figure 7** which was evident from the fragmented peaks observed in MS analysis report at m/z 102. A peak at m/z 102 indicates the characteristic Homoserine lactone ring alone and the fragmented acyl side chain were indicated by small peaks. Dilara [16] reported the fragmentation of AHL molecule by Mass spectroscopy which stands as support to the present data obtained. MS analysis reveals the fact that AHL molecule is subjected to lysis or degradation. The inactivation of AHL could be by lysing the molecule or separating their lactone ring from the side chain.

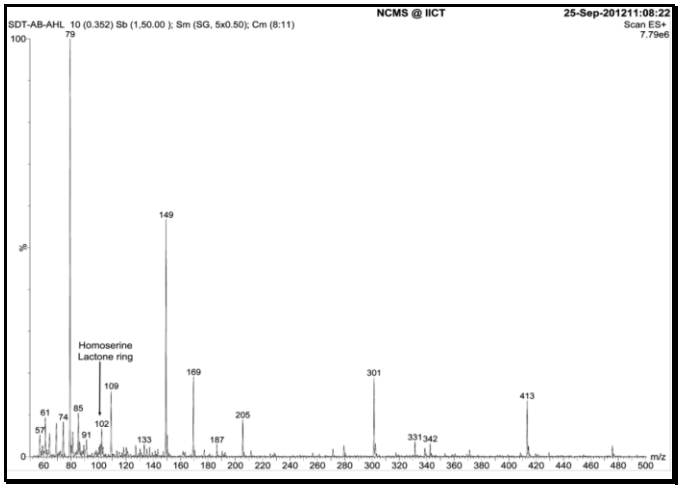
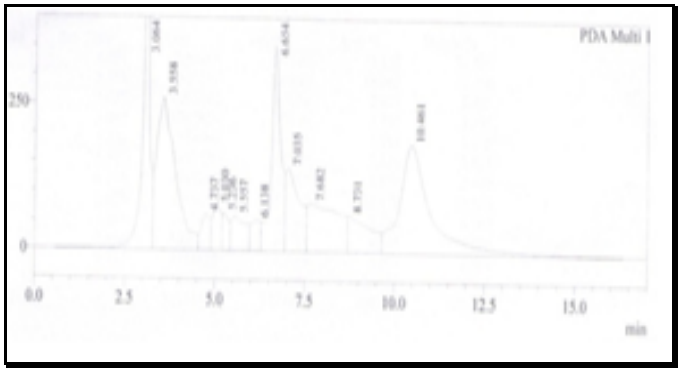
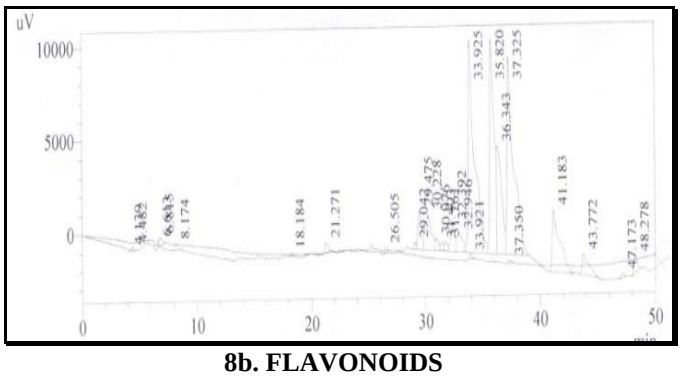


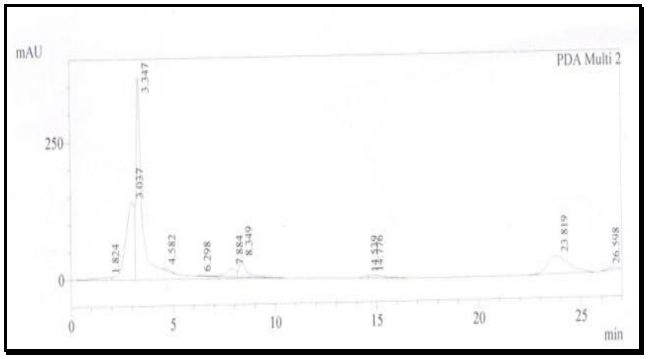
FIG 7: SPECTRAL ANALYSIS OF AHL IN *CINNAMOMUM VERUM* (8mgl/ml) TREATED SAMPLE



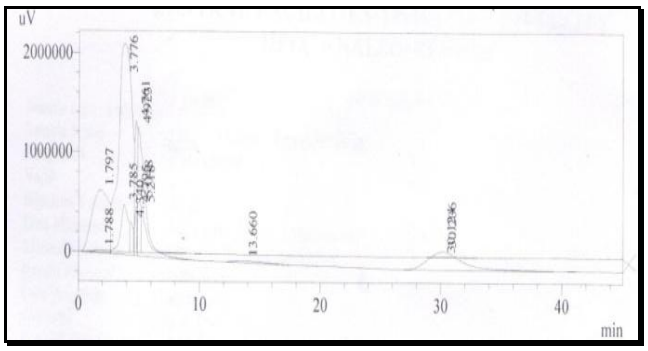
8a. ALKALOIDS



8b. FLAVONOIDS



8c.PHENOLS



8d. TERPENOIDS

DISCUSSIONS: *Cinnamomum verum* showed a significant decrease in expression of QS regulated virulence factors tested. QSI activity of *Cinnamomum verum* extract had not been reported so far against virulence character expression in *Serratia sps.* In present study, spice aqueous extract could reduce pigment production by 61.7% and protease by 54.9% while it could affect growth only by 14.5%. These results suggest that *Cinnamomum verum* extract had more QSI activity than the antibacterial activity in *Serratia.* In *Pseudomonas* the antibacterial activity was more (44.61% reduction in growth) while QSI activity was comparatively less i.e. (6.86% in pigment production and 45 % reduction in protease activity). Interestingly spice extract could reduce swarming nature, one of the important virulent factors regulated by QS in both the organisms.

Antibacterial activity of *Cinnamomum verum* was observed at high concentration (8 and 10mg/ml) in *Chromobacterium violaceum*. However quorum quenching activity i.e. violacein inhibition activity was observed from 4 mg/ml concentration onwards. These results indicate the presence of strong quorum quenching compounds in the aqueous crude extract of *Cinnamomum verum*. MS analysis of treated samples revealed the fact that AHL molecule is subjected to lysis or degradation. The inactivation of AHL could be by lysing the molecule or separating their lactone ring from the side chain. As the crude extract has mixture of phytochemicals, it could be difficult to pinpoint one component responsible for the AHL inactivation.

In order to figure out the bioactive component, qualitative analysis of phytochemicals in the crude extract was performed by Standard methods[13]. Figure 8a-d depicts HPLC analysis of aqueous extracts in which Flavonoids (8b), Phenolics, (8c) Alkaloids (8a) and Terpenoids (8d) were identified.

Cinnamaldehyde, a phenolic compound is a potent QS inhibitor. Studies by Niu[17] reported that Cinnamaldehyde inhibited bioluminescence in two different reporter strains of *Vibrio harveyi* which are able to respond to both AHL and AI-2. This inhibition was mediated by modulation of Lux R activity. Present study also is in agreement with the research findings that it could reduce the QS mediated gene expression. The same group have subsequently reported that Cinnamaldehyde is a potent inhibitor of 3 oxo C_6 HSL (OHHL) quorum sensing systems in reporter strain *E. coli* and was also found to inhibit QS in *Vibrio harveyi* by inhibiting the QS signaling molecule 3-hydroxy C_4 HSL at sub inhibitory concentrations.

The research findings in the above paper stated that cinnamaldehyde was able to bind to cognate receptors and interferes with binding of 3 hydroxy C4 HSL and 3 oxo C6 HSL and inhibit the signaling circuits, but could not inhibit the LasR promoter activity induced by odDHL in an *E. coli* strain containing the Las R biosensor system. Studies by Brackmann[18] proved that cinnamaldehyde was able to inhibit biofilm formation in *Vibrio harveyi* mediated by AI-2 system and reduce pigment and protease production in *Vibrio anguillarum*.

CONCLUSIONS: Aqueous extracts of dietary spice *Cinnamomum verum* had shown a strong Quorum Quenching activity against the three tested bacterial pathogens. The exact mode of action at molecular level has to be further explored with purified bioactive component from spice extract. Recognition of bioactive components in the crude extract of *Cinnamomum verum* is under process. The observation that dietary phytochemicals from

Cinnamomum verum can inhibit QS related processes opens up new scope for antimicrobial chemotherapy.

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