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Research Article

ANTIBIOFILM AND ANTIQUORUM SENSING POTENTIAL OF *CUMINUM CYMINUM* AGAINST *AEROMONAS VERONII*

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ABSTRACT

Bacteria have the capacity to communicate with their population densities through a mechanism called Quorum sensing. They can interfere with bacterial virulence and can produce signaling molecules called Auto inducers (AI). The high amount of signaling molecules induced pathogenicity and can cause biofilm formation. *Aeromonas veronii* can produce natural AHL (N-Acyl homoserine lactones) and can lead to quorum sensing functions. Cumin, a traditionally used medicine containing compounds like tannins, terpenoids, phenols, saponins, steroids, flavonoids, carbohydrates, proteins and amino acid acted as anti-quorum sensing inhibitor. Biofilm formation were assayed by methods like tube assay, Congo red agar and microtiter plate method and they have shown strong biofilm formation and also *Aeromonas veronii* exhibit exopolysaccharide production. Test organism when treated with Cumin extract resulted 3.12mg/mL as the lowest MIC value and 6.25mg/mL as the MBC value. The highest anti-quorum sensing value of cumin ranged at 25mg/mL and 50mg/mL concentrations. Higher concentration of seed extract exhibited low biofilm formation. The results of DPPH assay showed cumin has 99% of antioxidant activity. The present study revealed that the methanolic extract of cumin inhibited quorum sensing activity by interfering AHL production and thus inhibited biofilm formation of organism.

Keywords: Quorum sensing (QS), Biofilm formation, N-Acyl homoserine lactones (AHL), Minimum inhibitory concentration, Auto inducer

1. INTRODUCTION

For various microbial infections and inflammations, plant food sources are widely used from ancient times. Mainly extracts from plants are considered as effective agents against numerous pathogenic infections. A number of microorganisms have antibacterial and antimicrobial properties because of their anti-quorum sensing properties of plants. Quorum sensing is a kind of cell to cell communication of bacteria, which trigger gene transcription. Bacterial communication results production of signal molecules called Auto inducers [1]. Biofilm is a cluster of microorganisms in which they stick to each other and attached to a surface. These adherent cells are embedded in Exopolysaccharide substances (EPS).

Biofilm producing bacteria are responsible for many serious infections and difficult to eradicate. High number of antimicrobial agents is needed to inhibit the growth of pathogenic organisms. Bacteria can attach to any surface. First step in biofilm formation is the attachment of cells and then they aggregate to form a chain like structure. Cells then sporulate and detached from the surface. Biofilm have been a major cause of infections because of their rapid growth. They can survive for long time in various sources like water, soil and plants etc. Because of their fast growth they have been considered as a major issue in food industry. Biofilm can result in Quorum sensing (QS).

QS produce signaling molecules in Gram negative bacteria called N-Acyl homoserine lactone and Gram-positive bacteria can produce oligopeptides as signaling molecules. The binding of auto inducer to a specific receptor protein can affect the pathogenicity of microorganisms. These can lead to biofilm formation. Biofilm and quorum sensing are directly proportional [2].

Cuminum cyminum is also known as cumin or Jeera(seed), belonging to a family of *Apiaceae,* which is a natural spice, contains antimicrobial activity. The fruit Jeera is used in variety of food recepies, mainly in Asian countries [3]. Cumin is a small, boat shaped brownish color seed that has pungent flavor. In this study, we have isolated *Aeromonas veronii* from fish sample. *Aeromonas veronii* is a

Gram-negative bacterium, widely found in fish, meats, dairy products and even in drinking water. The genus *Aeromonas* is an important disease-causing bacterium and also responsible for various pathogenic diseases like gastro enteritis, diarrhoea. The route of infection is via oral consumption of contaminated food or water [4]. We used *Chromobacterium violaceum*, a mini Tn5 mutant (CV026) strain and a purple pigmented wild type organism called *Chromobacterium violaceum* (MCC 2290) for QS activity, purchased from Microbial culture collection, NCCS, Pune, Maharashtra, India.

This work describes an overview of Quorum sensing production, anti-quorum sensing activity and biofilm formation of *Aeromonas veronii* and also, we identified different functional compounds from cumin extract. This work provided us with various properties of quorum sensing for the screening of gene level studies. Hence, the present results aimed to study the effect of Quorum sensing inhibition activity of plant extract.

2. MATERIAL AND METHODS

2.1. Processing of samples

Samples of fish were collected from local market in Coimbatore. Fish were finely chopped using sterile scalpel and blade, and grounded using buffered peptone water. The crude extract was filtered using a muslin cloth or Whatman filter paper and the filtrate was used for serial dilution.

2.2. Media composition

Nutrient medium (Peptone-10g/L, NaCl-5g/L, Beef extract-10g/L, Agar-12g/L final pH 7.3±0.1) and Luria Bertani medium (Casein enzymatic hydrolysate-10g/L, Yeast extract-5g/L, NaCl-10g/L,Agar-15g/L, final p^H 7.5±0.2) were used for bacterial sub culturing.

2.3. Microorganism

16s rRNA primers were ordered from Integrated DNA Technologies and PCR conditions were optimized. The amplified product was then sequenced and was identified using BLAST analysis. Based on microbiological and physiological tests and 16s rRNA phylogeny, the strain was identified as *Aeromonas veronii* with 85% similarities and finally submitted in the NCBI, Gene bank (Accession Number is KX355802).

2.4. Plant material and preparation of extract

Seeds of *Cuminum cyminum* were collected from spices board, Cochin, Kerala, India. Cumin seeds were washed with sterile distilled water, shade dried and pulverized to a fine powder in mixer grinder. Ten (10) g of plant powder was soaked in 100mL of methanol for five days of intermittent shaking. Using Whatman filter No.1, the extract was collected and air dried in a laminar hood. The solvent dried extract was reconstituted in DMSO and stored at 4°C for further use [5].

2.5. Detection of quorum sensing activity of bacterial strain with biosensor strain CV026

Cross streaking of *Aeromonas veronii* with biosensor strain *Chromobacterium violaceum* CV026 was performed in Luria Bertani agar plate and incubated at 30°C for 18 hours [6].

2.6. Quorum sensing production of bacterial strain using *Chromobacterium violaceum* (MCC 2290)

Aeromonas was streaked in LB agar plate and incubated at 30°C for 18 hours. After incubation, 5mL of soft agar with 200µL of violacein pigmented (*Chromobacterium violaceum*) organism was poured and again incubated overnight at 30°C to check the violacein pigment inhibition [7].

2.7. Isolation of autoinducer from *Aeromonas veronii*

Bacterial culture was inoculated in LB broth and incubated overnight for 18 hours at 30°C. After incubation the broth was sterilized using membrane filter (0.22µm) and extracted with acidified ethyl acetate in the ratio of 7:3. The extract was air dried in laminar hood and diluted in LB broth and stored at 4°C for future use [1].

2.8. AHL Detection using Disc diffusion method

After the extraction of natural autoinducer, a test was carried out to confirm the presence of AHL. Luria Bertani agar plates were spread with 100µL of biosensor strain CV026 and sterile discs impregnated with 10µL, 20µL and 30µL of isolated AHL and incubated overnight at 30°C to check the violacein production [7].

2.9. Quorum sensing inhibition activity of plant extract against *Chromobacterium violaceum* (MCC2290) and CV026

Luria Bertani plates were spread with 100µL of violet color strain *Chromobacterium violaceum* (MCC 2290) and placed sterile discs with different concentrations of cumin extracts and incubated at 30°C for 18-24 hours to check the inhibition of pigment production.

Chromobacterium violaceum, CV026 was used to check the violacein productivity of *Aeromonas veronii*. LB agar plates were spread with 0.1mL of CV026 and 8mm diameter wells were cut. Varying amount of cumin extracts, dissolved in DMSO, were loaded along with natural AHL isolated from *Aeromonas veronii*, incubated at 30°C for 18-24 hours to check the zone of inhibition around the well [8].

2.10. Minimum inhibitory concentration of cumin extract

A stock solution of seed extract was prepared in methanol, using 100 mg plant extract in 1 mL of 10% DMSO solution. Each stock solution was diluted to obtain final concentration of 50mg/mL, 25mg/mL, 12.5mg/mL, 6.25mg/mL, 3.125mg/mL 1.56mg/mL, 0.78mg/mL and 0.36mg/mL. *Shewanella putrefaciens, Listeria monocytogenes*, *Yersinia enterocolitica, Escherichia coli, and Staphylococcus aureus* were used as reference strains along with *Aeromonas veronii*. An aliquot of 80µL of each dilution of plant extract, 100µL of LB broth and 20µL of bacterial inocula (10^9colony forming units/ml) and 5µL of 0.5% 2,3,5-Triphenyltetrazolium chloride (TTC) was released into a well on a 96-well (12x8) microtiter plate. The plate was incubated at 37°C for 18 hours to observe the pink coloration. After incubation, bacteria from each well of the plate were sub cultured on a nutrient agar. The level of dilution where no growth on the nutrient agar, indicated MBC (Minimum bactericidal concentration) values [9].

2.11. Phytochemical tests of Cumin extract

The phytochemical analysis for tannins, saponins, terpenoids, steroids, phenol, reducing sugar, Flavonoids, proteins and alkaloid were carried out by following standards procedures [10-12].

2.12. GAS Chromatography-Mass spectrometry analysis

The cumin extract was subjected to GC-MS in order to identify the constituents. The GC conditions were used as follows:

Instrument model was Thermo GC- trace ultra ver 5.0, Thermo MS DSQ II with DB 35- MS capillary standard non-polar column (30m x 0.25mmx0.25µm). Carrier gas used was Helium at 1.0ml/min. TEMP PROG: oven temperature 70°C rose to 260°C at 6°C/min and the injector volume was 1µL. The solvent used for the dilution was methanol.

2.13. Biofilm formation assays

2.13.1. Tube method

Culture (100µL) was inoculated overnight in 10mL LB broth and incubated at 37°C for 24 hours. After incubation, tubes were washed with phosphate buffer saline (pH 7) and dried. Tubes were then stained with crystal violet (0.1%) and the excess stain was washed with deionized water. Tubes were then dried in an inverted position [13].

2.13.2. Congo red agar method

The test organism was streaked in Congo red agar medium and incubated at 30°C for 24 hours [13].

2.13.3. Microtiter Plate Method

Twenty (20) µL of overnight kept culture and 230µL of LB broth media were added to 96 well plate and incubated aerobically. After overnight incubation the contents were discarded and washed thrice with distilled water and stained with 1% crystal violet for 5 minutes. Excess stain was removed and resolubilized the wells with 250µL of 33% glacial acetic acid. OD was read at 490nm [14].

2.13.4. Exopolysaccharide detection

The experiment was used to detect the exopolysaccharide production of isolate via their mucoid or ropy appearance. The Indian ink stain was prepared by wet mount technique. After overnight growth on Congo red agar, examined under light microscope [14].

2.13.5. Biofilm formation of Aeromonas veronii using plant extracts

Biofilm formation of *Aeromonas* using plant extract was evaluated with crystal violet staining technique. The 96 well plate containing 100µL of LB broth, 20µL of bacterial culture, 80µL of plant extract and control well containing LB broth were incubated for 24 hours and the OD was read at 600nm. The wells were washed with PBS. The biofilm layer formed on the wells were fixed with 200µL of acidified methanol and stained with 200µL of crystal violet (1%) for 10 minutes. The excess dye was removed by washing the wells again with PBS and the OD was measured at 595nm [15].

2.14. DPPH Assay

1,1-diphenyl-2-picrylhydazyl (DPPH) was used to estimate the free radical scavenging activity of plant extract. 0.1mM solution of DPPH in methanol was prepared. 500µL of the solution was added to 300µL of extract in methanol at serially diluted concentrations

(50mg/mL, 25mg/mL, 12.5mg/mL, 6.25mg/mL). The mixture was shaken vigorously and kept at room temperature for half an hour finally the absorbance was read at 517 nm by using spectrophotometer. Butylated hydroxy toluene (BHT) was taken as a reference compound. DPPH scavenging activity was calculated using the following formula [16].

DPPH scavenging effect (%) or percentage of inhibition $= (1-A_1/A_0) \times 100$

Where A_1 = Absorbance of the extract or standard and A_0 = Absorbance of the control.

3. RESULTS AND DISCUSSION

Chromobacterium violaceum, CV026, is a mutant strain derived from wild type *C. violaceum* CV 3152 and is unable to produce its own autoinducer, but can produce quorum sensing by the addition of exogenous AHL. Cross streaking of *veronii* with CV026 was observed to trigger violaceum production (Fig.1). Violet color strain *called C.violaceum* MCC 2290, purchased from microbial culture collection, Pune, Maharashtra, India, was used to detect the QS inhibition. The darker pigmentation of the indicator strain was reduced because of the natural AHL production in *A.veronii* contributes pigmentation inhibition (Fig. 2).

Fig. 1: The cross streaking of *Aeromonas veronii* with indicator strain

Fig. 2: Inhibition of darker pigmentation

For the detection of AHL from *Aeromonas veronii*, disc diffusion method was performed. Violacein production was observed around the disc (Fig. 3). Production of violacein color depends on the concentration of AHL added. 30µL of AHL showed maximum production. For disc diffusion method, different concentration of plant extracts (5µL, 15µL, and 35µL) was used to check the anti-quorum sensing activity (Fig. 4). For this method, MCC 2290 was used as indicator strain.

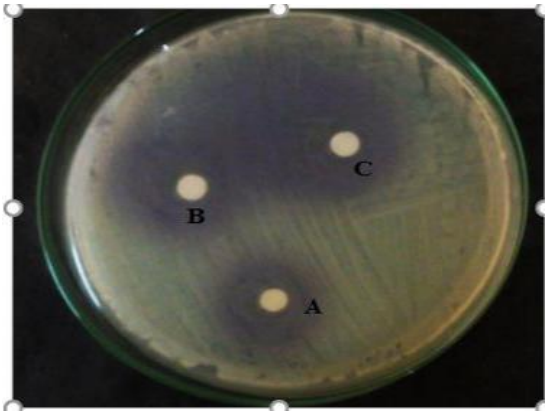


Fig. 3: Production of natural AHL from *Aeromonas veronii*

Anti-quorum sensing activity of cumin extract using *chromobacterium violaceum* CV026 was assayed by agar well diffusion method in the presence of AHL. Inhibition of violacein production was observed in the presence 100µL of natural AHL at different concentration of plant extracts (Fig. 5). By the addition of 100µL of AHL with 10µL of cumin extract showed maximum violacein inhibition.

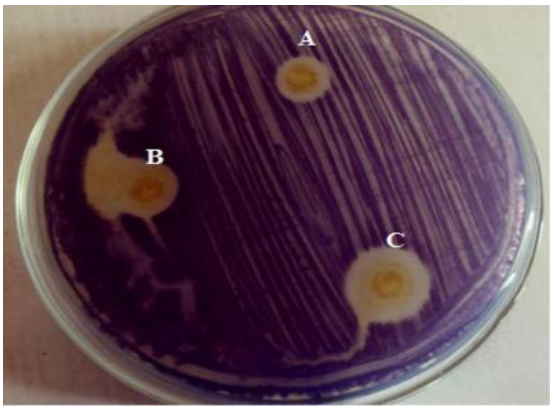


Fig. 4: Anti-quorum sensing activity of Cumin extract using *Chromobacterium violaceum* MCC 2290

The MIC and MBC values of methanolic extracts of cumin were evaluated. *Cuminum cyminum* had

3.12mg/mL as the lowest MIC value and 6.25mg/mL as the lowest MBC value. *Shewanella puitrefaciens*, *Yersinia enterocolitica, Listeria monocytogenes, Staphylococcus* aureus were used as reference strains. *Shewanella putrefaciens* showed 6.25mg/mL as the MIC value and 12.5mg/mL as the MBC value. 20mg/mL as the MIC value in *Yersinia enterocolitica* and *Listeria monocytogenes* and 50mg/mL as the MBC value. *E. coli* had 12.5mg/mL as the lowest MIC value and 25mg/mL as the MBC values.

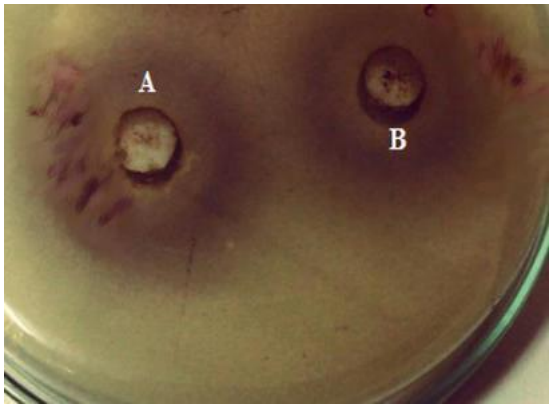


Fig. 5: Inhibition of violacein production in presence of 100µL AHL at different concentration of plant extract [A-2µL (15mm diameter), B-10µL (10mm)]

Table 1: MBC values of six different bacterial strains treated with cumin extract

Bacteria	MIC (mg/mL)	MBC (mg/mL)
Aeromonas veronii	3.12	6.25
Shewanella putrefaciens	6.25	12.5
Listeria monocytogenes	25	50
Yersinia enterocolitica	25	50
Escherichia coli	12.5	25
Staphylococcus aureus	6.25	12.5

The effect of cumin extract on *Aeromonas veronii* was found to be in concentration dependent manner. Higher concentrations of plant extract (50mg/mL, 25mg/mL, 12.5mg/mL, 6.25mg/mL 3.12mg/mL) showed inhibition of bacterial growth. There was no inhibition at 0.78mg/mL and 0.36mg/mL concentrations.

Phytochemical analysis showed presence of tannins, phenols, saponins, terpanoids, sterols, reducing sugars, carbohydrates, proteins, amino acids and flavonoids in methanolic extract of *cuminum cyminum* (Table 2). The GC-MS analysis of cumin extract was performed using DB 35-MS capillary non-polar column (Fig.7).

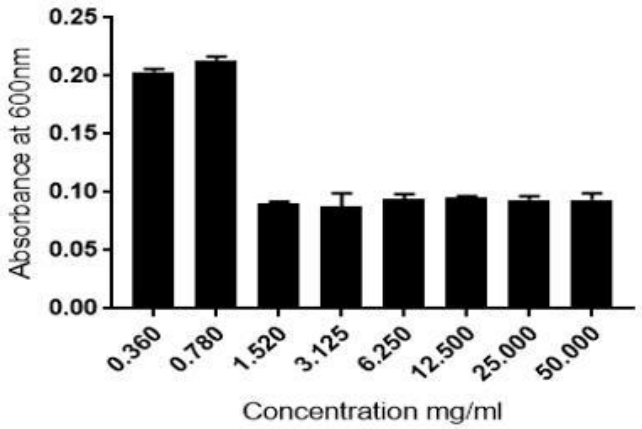


Fig. 6: Minimum inhibitory values of *Aeromonas veronii* in presence of cumin extract

Table 2: Phytochemical constituents of cumin

Phytochemical Tests	Methanolic extracts of *Cuminum Cyminum* (seeds)
Tannin	+
Saponin	+
Terpenoids	+
Libermann-Burchard Test for steroids	+
Phenols	+
Fehling's test for reducing sugars	+
Benedict's test for carbohydrate	+
Ninhydrin test for protein and amino acid	+
Lead acetate test for phenols and tannins	-
Mayer's test for alkaloids	-
Shinoda test for flavanoids	+

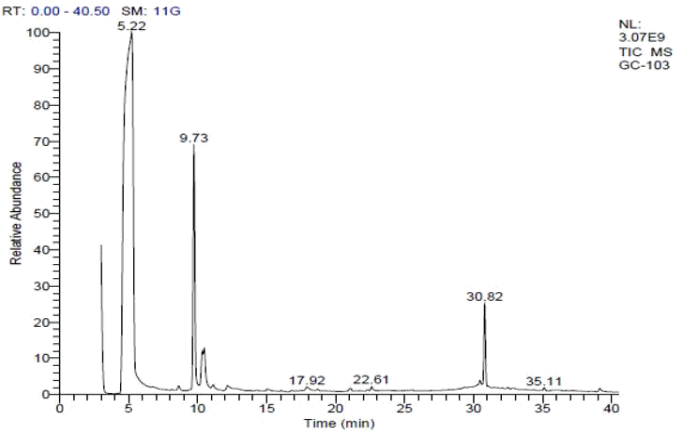
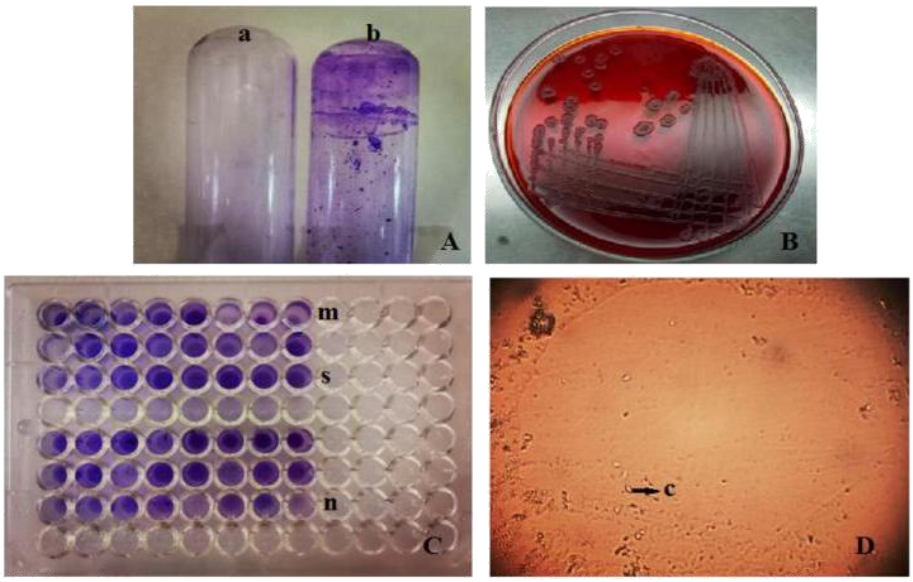


Fig. 7: GC-MS chromatogram of compounds identified in methanolic extract of cumin

At different retention times, the GC-MS results showed few groups of molecules. Compounds like Quarcetin-7-3,4'-trimethoxy, Retinyl palmitate, Pentadecanoic acid, Fenretinide, N-(4-hydroxyphenyl) retinamide, Beta Doradecin, Murolan-3, a(II)-diene-10-peroxy, Betulin, Binapacryl having antioxidant, antitumor, antibacterial, antifungal and anticancer properties have been identified from cumin extract.

In this study, we evaluated biofilm formation of *Aeromonas veronii* using three different methods. Tube method is 80% accurate for biofilm production. In tube method, biofilm formation of *Aeromonas* was found to be positive. Congo red agar method also showed production of black colonies considered as positive result. Microtiter plate method was reported to have high specificity and accurate values for biofilm formation. Indian ink staining was used as a rapid screening test for biofilm formation. Strong transparent halo zone production around the cells was observed in *Aeromonas*.



a - control; b – biofilm attachment; B: Black colonies on Congo red agar; C: m-moderate biofilm formation, s-strong biofilm formation, n-no biofilm formation; D: c-capsular EPS production (X10 light microscopy)

Fig. 8: Detailed examination of biofilm formation Aeromonas veronii using A) Tube assay, B) Congo red agar, C) Micro titer plate method, D) EPS production using Indian ink

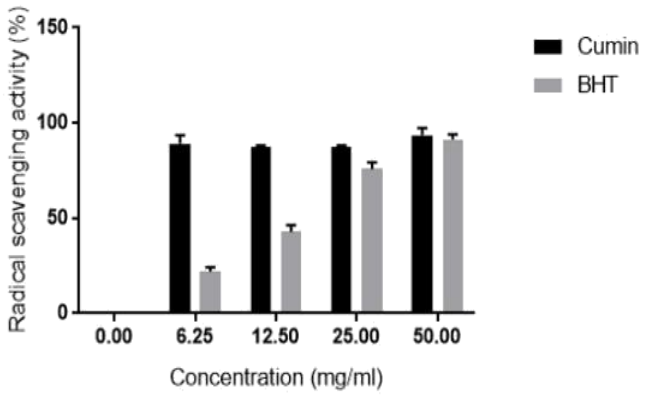


Fig. 9: Radical scavenging activity of cumin

In biofilm formation assay, a concentration dependent decrease in biofilm was observed for the bacterial strain when treated with cumin extract. Aeromonas showed less biofilm formation at concentration of 50mg/mL and 25mg/mL, 50mg/mL and 25mg/mL of plant extract treated with *Aeromonas* showed less production of biofilm. Lower concentration of extracts resulted strong biofilm formation. For DPPH assay, different concentrations of cumin extract were added and showed better antioxidant activity. Radical scavenging activity of cumin is higher than the BHT. As concentration of plant extract increases, the concentration of BHT also increases.

Current research suggests that, Gram negative bacteria are responsible for various infectious diseases, which trigger the mechanism called Quorum sensing. Communication of bacterial cells through some signaling molecules called autoinducers. Accumulation of autoinducers depends on the activation of specific gene. Screening of Quorum sensing inhibitors from plant sources is much safer than chemically synthesized compounds because of their antibacterial and antimicrobial properties. For anti-quorum sensing assays, they are also effectively involved in inhibition of biofilm

formation of pathogenic bacteria. *Chromobaterium violaceum* (MCC2290) a bioreporter, violacein pigmented strain, it produces C4 and C6 AHLs. *Chromobaterium violaceum* CV026 is a mini Tn5 mutant strain. By the addition of exogenous AHLs, it can produce its own signaling molecules. Production and inhibition of violacein pigment was carried out by using these two indicator strains [17]. *Aeromonas veronii* showed natural AHL production because of its quorum sensing activity. In our study, cumin extract interferes with AHL regulated function along with biofilm formation. Biofilm formation of *Aeromonas* treated with cumin observed less attachment towards at a concentration of 50mg/mL and 25mg/mL. Various phytochemical compounds containing different properties were identified from cumin seed extract by GC-MS analysis and also cumin extract showed better antioxidant scavenging activity (DPPH).

4. CONCLUSION

Quorum sensing can affect biofilm formation. High amount of AHL increases pathogenicity. The quorum sensing inhibitors isolated from natural sources can decrease the virulence of pathogenic organisms. Quorum sensing signalling, molecules can be used as targets for developing new naturally occurring inhibitory compounds which can replace the existing synthetic agents. Identification of such natural compounds can lead to the development of antibacterial drugs to be used for anti-virulence therapy against pathogenic organisms. Therefore, an attempt is being undertaken to study the inhibition of natural products isolated from plants on these autoinducer signaling molecules in quorum sensing regulating function and biofilm formation of selected Gram-negative bacteria.

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