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Research Article

Screening of Quorum Sensing (Qs) Modulatory Effect of Medicinal Plant Extracts Against Quorum Sensing Mediated Virulence Factors of Human Pathogenic Gram Negative Bacteria

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ABSTRACT

Anti qourum sensing agents would offer a way of controlling microbial infections with the advantage of reducing risks of resistance development.Searching of new anti quorum sensing agents derived from plants as an antimicrobial agents against various quorum sensing mediated virulence factors of pathogenic microorganism is an emerging area of medicine.In the present study, anti quorum sensing activity of ethanolic extract of *Curcuma longa Ocium tenuiflorum*, *Aegle marmelos*, *Eucalyptus globules*, *Azadirachta indica*, *Cynodon dactylon* against quorum sensing mediated virulence factors of human pathogenic bacteria *Proteus vulgaris* and *Salmonella paratyphi* has been carried out. Among the plants *Eucalyptus globules* revealed maximum inhibition of QS mediated virulence factors of *Proteus vulgaris*. In the case of *Salmonella paratyphi*, *Eucalyptus globules*, *Ocium tenuiflorum* and *Aegle marmelos* brought about maximum effect on QS mediated virulence factors. The present study revealed potential of these plant extracts in treating microbial infections through cell growth inhibition or quorum sensing inhibitors would suggests the possible utilization for the prevention of bacterial infections.

Key Words. Quorum sensing, plant extracts, virulence factors, *Proteus vulgaris*, *Salmonella paratyphi*

INTRODUCTION

The increasing occurrence of multi resistant pathogenic bacterial strains has gradually rendered traditional antimicrobial treatment ineffective. Today, a global concern has emerged that we are entering a post-antibiotic era with a reduced capability to combat microbes, and, hence, the development of novel therapeutic approaches to the treatment of bacterial infections constitutes a focal point of modern research. The alternative to antibiotic-mediated bacteria killing or growth inhibition is attenuation of bacterial virulence such that the organism fails to establish successful infection and, in consequence, is cleared by the host immune response. Compounds with such abilities are the result of rational drug design and are termed antipathogenic drugs as opposed to antibacterial drugs (i.e., most traditional antibiotics). Antipathogenic drugs target key regulatory bacterial systems that govern the expression of virulence factors[1]. Quorum sensing (QS) is a process of cell to cell communication that allows bacteria to share information about cell density and control the gene expression accordingly[2]. Gram negative bacterial cell-to-cell communication regulates gene expression in a population density-dependent manner by quorum sensing" (QS). Typically, gram negative bacteria produce *N*-acyl homoserine lactones (AHLs) by AHL synthase (luxI homologue), and once AHLs reach threshold level AHLs will bind to its cognate receptor (luxR homologue) to regulate gene expression[3,4]. Quorum sensing process that involves the production, detection, and response to extracellular signalling molecules called auto inducers. These auto inducers accumulate in the surrounding environment and in the presence of a large population of cells, the concentration accumulates to a level needed for virulence (. Quorum sensing is thought to afford pathogenic bacteria a mechanism to minimize host immune responses by delaying the production of tissue-damaging virulence factors until sufficient bacteria have amassed and are prepared to overwhelm host defence mechanisms and establish infection[5] It is well documented that QS regulates diverse bacterial physiological processes, including virulence determinants, bioluminescence,swarming, antibiotic biosynthesis,biofilm differentiation, and *Agrobacterium* plasmid conjugal transfer[6]. Recently, several inhibitors of QS have been discovered from natural sources like microorganisms and plants which interfere with QS. Natural products especially plants used in traditional medicines are a promising source for deriving molecules that can potentially inhibit quorum sensing[7]. These plants can offer a large and attractive repertoire for the discovery of quorum sensing inhibitors. They are of particular importance as these have been used for thousands of years for the treatment and management of diseases and may have few side-effects and toxicity issues as with many antibiotic regimens and currently known QS inhibitors. Herbs, Spices and Medicinal Plants (HSMP) used in Hispanic cultures have been used for several centuries to treat common ailments, are well known for their

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Table 1: Percent composition (%) of constituents in the ethanolic extract of *Cynodon dactylon*

S. No	Name of the compound	Rt	Molecular Formula	Molecular Weight	Peak area %
1.	2-Penta,6,10,14-trimethyl	16.38	$C_{18}H_{36}O$	262.43	66.2%
2.	1-Dodecanol,3,7,1 1-trimethyl	17.18	$C_{15}H_{32}O$	382.29	36.8%
3.	Hexadecanoic acid- ethyl ester	17.98	$C_{18}H_{36}O_2$	284.5	49.6%
4.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	19.17	$C_{20}H_{40}O$	296.43	100%
5.	Ethyl Oleate	19.65	$C_{20}H_{40}O$	310.51	100%
6.	Heptadecanoic acid 15-methyl-ethyl ester	19.85	$C_{20}H_{40}O_2$	326.55698	100%
7.	Eichosanoic acid- ethyl ester	21.65	$C_{21}H_{42}O_2$	326.5570	74.1%

Table 2: Percent composition (%) of constituents in the ethanolic extract of of *Eucalyptus globulus*

Name of the compound	Rt	Molecular Formula	Molecular Weight	Peak area %
Patchoulene	11.6	$C_{15}H_{24}$	204.35106	18.3%
Globulol	13.57	$C_{15}H_{26}O$	222.36	37.1%
a-phellandrene	15.9	$C_{10}H_{16}$	136.23404	100%
Pentadecanoic acid,14-methyl-methyl ester	17.23	$C_{17}H_{34}O_3$	286.45	100%
1,2-benzenedicarboxilic acid, butyl octy ester	17.72	$C_{20}H_{30}O_4$	334.4498	100%
8,11-Octadecadienoic acid, methyl ester	18.92	$C_{19}H_{34}O_2$	294.4721	100%
Ethanol,2-(9-octadecenyloxy)-,(z)-	19.15	$C_{20}H_{40}O_2$	312.534	88.1%
Oleic acid	19.92	$C_{18}H_{34}O_2$	282.461360	100%
2,3-Dihydroxypropyl elidate	22.03	$C_{21}H_{40}O_4$	356.5399	57.3
Hexadeconoic acid,1-(hydroxymethyl).1,2-ethannediyl ester	23.18	$C_{35}H_{68}O_5$	568.91	39.1%
9-Octadecenoic acid(z)-,2-hydroxy-1-(hydroxymethyl)ethyl ester	25.93	$C_{18}H_{34}O_2$	282.461360	76.8%

Table 3: Percent composition (%) of constituents in the ethanolic extract of of *Azadirachta indica*

Name of the compound	Rt	Molecular Formula	Molecular Weight	Peak area %
9-Octadecene,1,1-(1,2-ethanediylbis(oxy))bis-,(ZZ).	4.7	$C_{22}H_{46}O_4$	310.6027	5.6%
Ethyl 9,9-diformylnona-2,4,6,8-tetraenoate	8.97	$C_{13}H_{14}O_4$	234.2491	4.4%

antimicrobial effects on a variety of human pathogens[8]

However, few reports have been studied regarding anti quorum sensing activities of medicinal plant extracts against human pathogens mainly Gram negative bacilli.the In the present study, anti quorum sensing activities of ethanol extract of medicinal plant extracts against quorum sensing mediated virulence factors of human pathogenic gram negative bacteria has been investigated.

MATERIALS AND METHODS

Bacterial strains

Proteus vulgaris and *Salmonella paratyphi* –human pathogenic gram negative enteric bacteria have been studied in the present investigation. Both the bacterial strains were obtained from Microbial Type Culture Collection (MTCC), Chandigurh, India. Bacterial strains were maintained on nutrient agar slants.

Inoculum preparation

Inoculum of respective bacterial strain was prepared by inoculating a loopful of bacteria from nutrient agar slant to nutrient broth, kept under shaking condition (REMI, India) for 18 to 24 hours at 30°C and 150 rpm and the grown bacterial culture was adjusted to 0.5 McFarland standard (Ca. 108 CFU/mL).

Evaluation of anti quorum sensing activity of medicinal plant extracts against Quorum sensing mediated virulence factors.

Plant materials

Leaves and rhizome of *Ocium tenuiflorum*, *Aegle marmelos*, *Eucalyptus globules*, *Azadirachta indica*, *Cynodon dactylon* and *Curcuma longa* were collected from Agriculuture college and research institute Madurai.

Preparation of Plant Extract

The collected plant material were air-dried under shade at room temperature, finely ground into powder using

Table 4: Percent composition (%) of constituents in the ethanolic extract of *Ocium tenuiflorum*

S. No	Name of the compound	Rt	Molecular Formula	Molecular Weight	Peak area %
1.	Benzene,1-methyl-4-(1,2,2-trimethylcyclopentyl)-,(R)	12.17	$C_{15}H_{22}$	202.3352	100%
2.	6-(p.Toly)-2-methyl-2-heptenol	13.72	C11H14O	162.231	100%
3.	6-(p-Toly)-2-methyl-2-heptenol	14.7	C11H14O	162.231	100%
4.	7-Oxabicyclo(4.1.0)heptane,1-(1,3-dimethyl-1,3-butadienyl)-2,2,6-trimethyl-(E)	15.43	$C_{15}H_{24}O$	220.3519	100%
5.	Acetic acid,3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-yl ester	15.75	$C_{17}H_{26}O_3$	278.3882	100%
6.	7-(1,3-Dimethylbuta-1,3-dienyl)-1,6,6-trimethyl-3,8-dioxatricyclo[5.1.0.0(2,4)]loctane	16.08	C38H64O3	568.9165	100%
7.	10-Octadecenoic acid, methyl ester	18.97	$C_{19}H_{36}O_2$	296.4879	100%
8.	Heptadecanoic acid, 16-methyl-,methyl ester	19.2	$C_{19}H_{38}O_2$	298.5038	78.4%

Table 5: Percent composition (%) of constituents in the ethanolic extract of of C*urcuma long*

S. No	Name of the compound	Rt	Molecular Formula	Molecular Weight	Peak area %
1.	Pentadecanoic acid, 14-methyl-, methyl ester	15.25	$C_{17}H_{34}O_2$	270.450660	100%
2.	Hexadecanoic acid, ethyl ester	15.95	$C_{18}H_{36}O_2$	284.5	100%
3.	l-[+]Ascorbic acid 2,6-dihexadecanoate	16.35	$C_{38}H_{68}O_8$	652.9417	100%
4.	Oleic acid	17.68	$C_{18}H_{34}O_2$	282.46136	100%
5.	9-Octadecenoic,(E)-	18.18	$C_{18}H_{34}O_2$	282.46136	100%
6.	9-Octadecenoic,(E)-	20.22	$C_{18}H_{34}O_2$	282.46136	96.7%
7.	9-Octadecenoic,(E)-	20.77	$C_{18}H_{34}O_2$	282.46136	100%
8.	Hexadecanoic acid,2,3-dihydroxypropyl ester,(n)-	21.4	$C_{19}H_{38}O_4$	330.50262	100%
9.	9-Octadecenoic acid(Z)-,2-hydroxy-1.(hydroxymethyl)ethyl ester	24.42	$C_{18}H_{34}O_2$	282.461360	50%

domestic mixture and stored in an airtight plastic sampling bags for further studies. Extraction was carried out by the modified method of Hussaini et al[9]. The plant materials were separately extracted twice at room temperature with ethanol 95%(100 mL/10 g of plant material each run). The final ethanol extract of each plant part was filtered using (Whatman No.1) filter paper and evaporated under vacuum at 40°C using a rotary vacuum evaporator, the concentrated extract thus obtained was collected in screwcap vial and used for further studies.

GC-MS analysis

GC-MS analysis of ethanol extract of concentrated plant extracts was performed using a Perkin-Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a gas chromatograph interfaced to a mass spectrometer (GC-MS).

Anti quorum sensing assay

Effect of plant extracts on quorum sensing mediated virulence factors such as swarming motility, biofilm formation, cell adhesion, proteolytic activity and biofilm formation, cell adhesion, proteolytic activity of *Proteus vulgaris* and *Salmonella paratyphi* respectively was studied in the present work.

Swarming motility

Effect of plant extracts on swarming motility of *Proteus vulgaris* was carried out by the modified method of Ren et al[10,11]. To the top of 0.3% nutrient agar plates, 0.5 ml of respective plant extracts were added and allowed to dry for three hours at 30°C. The plates were point inoculated with *P. vulgaris* inoculums thus prepared in nutrient broth and incubated at 30°C for 24 hours. The extent of swarming was determined by measuring the diameter of the motility swarms.

Biofilm Inhibition

Biofilm inhibitory effect of plant extracts was studied against both the tested bacterial strains by crystal violet spectrophotometric microtitre plate assay[12]. Respective bacterial inoculum was prepared in tryptic soy broth as described earlier and 100 µL of respective bacterial inoculums was transferred to the 96 well microtitre plate under aseptic condition. 100 µL of nano suspension with different concentration was added to the bacterial inoculm and the microtitre plate was incubated at 37°C for 48

Table 6: Percent composition (%) of constituents in the ethanolic extract of of *Aegle marmelos*

S. No	Name of the compound	Rt	Molecular Formula	Molecular Weight	Peak area %
1	Benzene,1-methyl-4-(1,2,2-trimethylcyclopentyl)-,(R)	12.17	$C_{15}H_{22}$	202.3352	100%
2	6-(p.Toly)-2-methyl-2-heptenol	13.72	C11H14O	162.231	100%
3	6-(p-Toly)-2-methyl-2-heptenol	14.7	C11H14O	162.231	100%
4	7-Oxabicyclo(4.1.0)heptane,1-(1,3-dimethyl-1,3-butadienyl)-2,2,6-trimethyl-(E)	15.43	$C_{15}H_{24}O$	220.3519	100%
5	Acetic acid,3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-yl ester	15.75	$C_{17}H_{26}O_3$	278.3882	100%
6	7-(1,3-Dimethylbuta-1,3-dienyl)-1,6,6-trimethyl-3,8-dioxatricyclo[5.1.0.0(2,4)]loctane	16.08	C38H64O3	568.9165	100%
7	10-Octadecenoic acid, methyl ester	18.97	$C_{19}H_{36}O_2$	296.4879	100%
8	Heptadecanoic acid, 16-methyl-,methyl ester	19.2	$C_{19}H_{38}O_2$	298.5038	78.4%

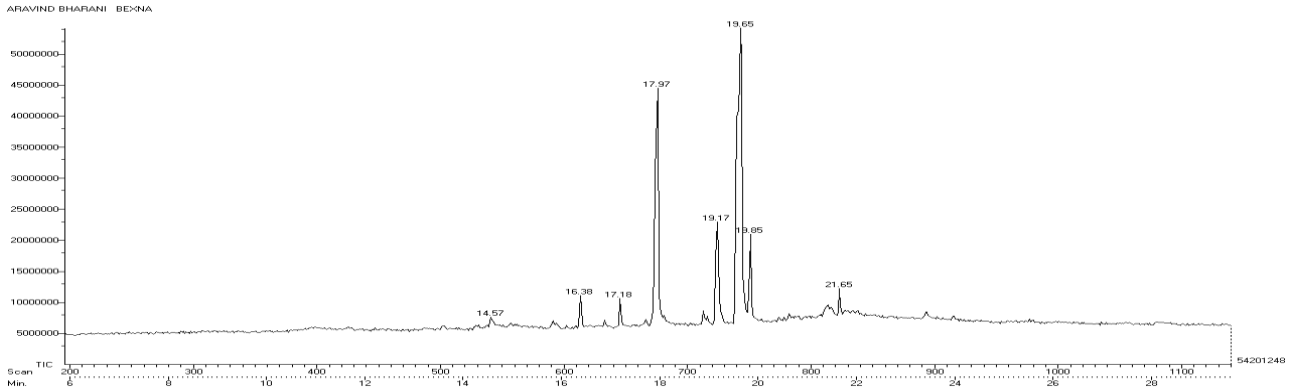

Figure 1: GC-MS chromatogram of *Cynodon dactylon*

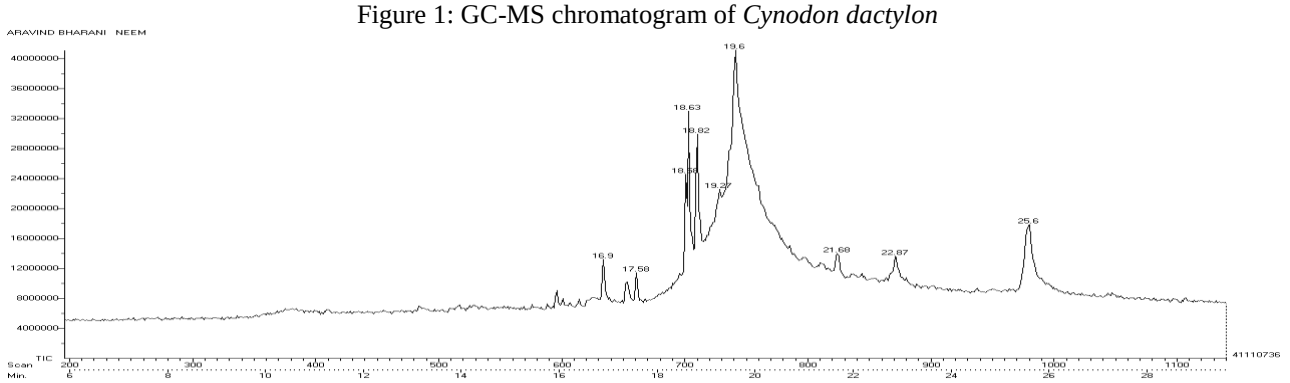

Figure 2: GC-MS Chromatogram of ethanolic extract of *Eucalyptus globulus*

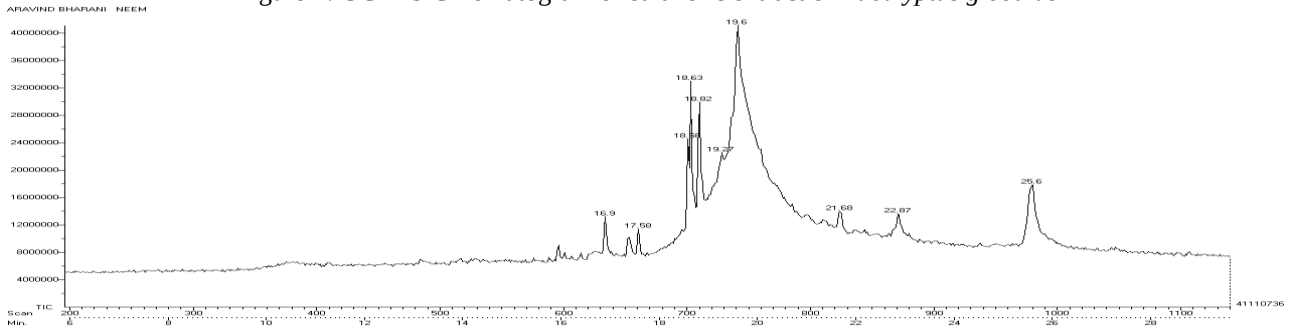

Figure 3: GC-MS Chromatogram of ethanolic extract of *Azadirachta indica*

hours. After the incubation period, the content in the well was completely removed and the wells were washed with phosphate buffered saline (PBS) followed by sterile distilled water.After washing, 100 µl of 0.1% aqueous solution of crystal violet was added, incubated at room temperature for 30 minutes .Followed by the incubation period, crystal violet was removed and washed using sterile distilled water. 200 µL of 95% ethanol. was added to the wells, incubated for 15 minutes at room temperature. Absorbance of the ethanol solubilised mixture in the well was read at 540 nm in an UV-Visible spectrophotometer. Control and triplicates were maintained.

Cell Adhesion

Plant extracts mediated inhibition of cell adhesion of both the tested bacterial strains was carried out by modified method of NCCLS[13] using 96 well flat bottom micro well plate previously coated with bovine serum albumin (BSA). Wells were coated with 150µl of freshly prepared 1.0% BSA, incubated at 30ºC for 30 minutes. After the incubation period, wells were washed thrice with sterile phosphate buffered saline (PBS).50 µl of bacterial inocula thus prepared was transferred to the well followed by the addition of 50µl of the respective plant extracts. Seeded microtitre plate was incubated at 37°C for 24 hours. Cells were allowed to adhere and the non-adhered cells were washed 5 times with PBS at room temperature. Adhered cells were detected by adding 50µl of 0.1% crystal violet per well, incubated at room temperature for 30 minutes. Wells were washed with sterile distilled water to remove excess stain. 10µl of ethanol was added to fix the adhered cells.50µl of 0.2% Triton X was added to the wells for the lyse of cells and the absorbance was read at 570nm.

Proteolytic activity

Evaluation of plant extracts on the proteolytic activity of *Salmonella paratyphi* was carried out by modified method of Lowry'set al[14].

Crude enzyme preparation

0.1 ml of tryptic soy broth bacterial culture was inoculated into 100 ml of protease production media (Yeast extract-5mg/l, Peptone-10mg/l, Glucose-10mg/l, Caesin-15mg/L)

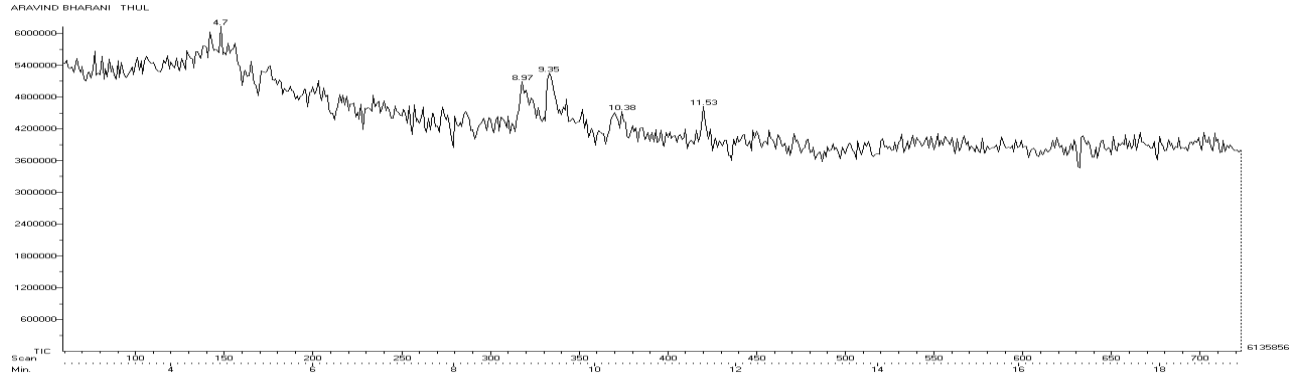

Figure 4: GC-MS Chromatogram of ethanolic extract of *Ocimum tenuiflorum*

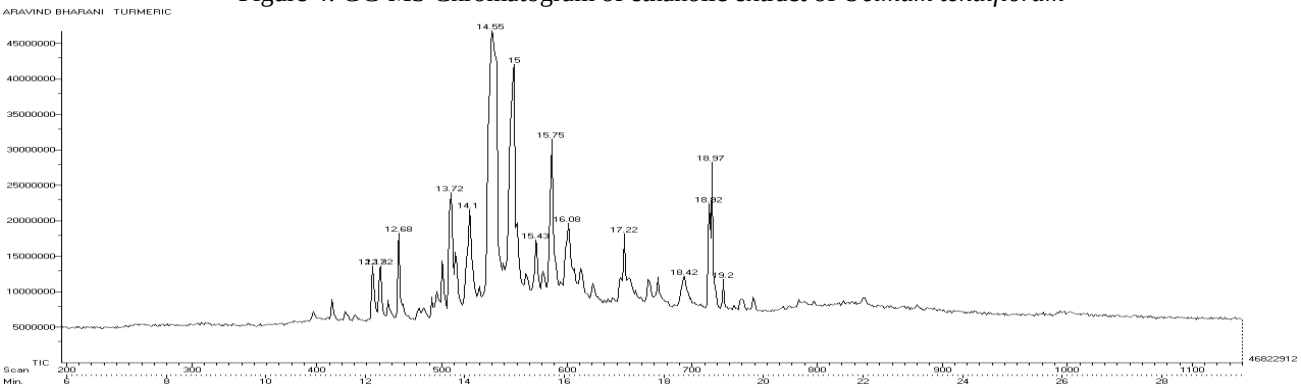

Figure 5: GC-MS Chromatogram of ethanolic extract of *Curcuma longa*

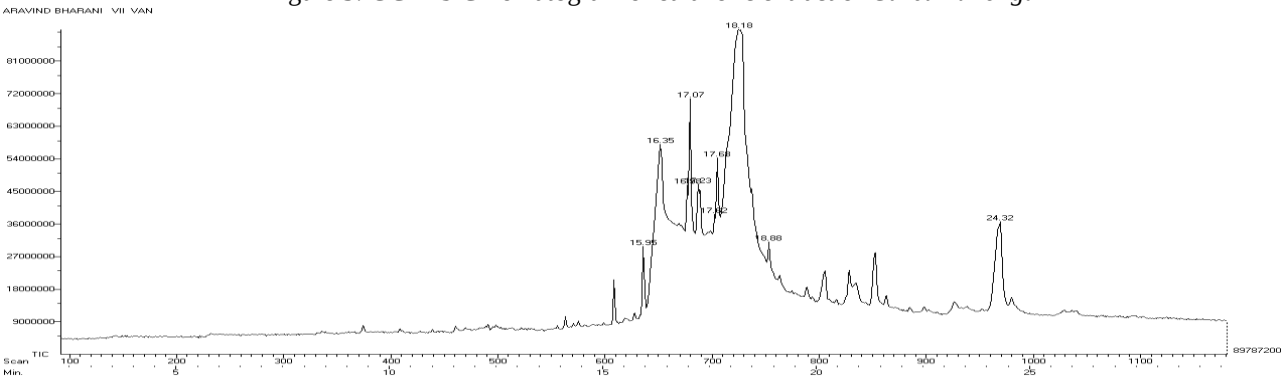

Figure 6: GC-MS Chromatogram of ethanolic extract of *Aegle marmelos*

supplemented with 200µl of respective plant extracts. Flasks were incubated at 37ºC for 48 hours. Broth was centrifuged after the incubation period at 10,000rpm for 10 minutes, the collected supernatant was used as the source of protease enzyme.

Enzyme Activity

Enzyme activity was assayed using casein as the substrate.The reaction mixture consisted of 0.25 ml of 50mM sodium phosphate buffer (pH 7.0) containing 2.0% (w/v) of azocasein and 0.15 ml of enzyme solution.After incubating at 25ºC for 15 min, the reaction was stopped by adding 1.2 ml of 10.0% (w/v) TCA, incubating at room temperature for an additional 15 min, and then the precipitate was removed by centrifugation at 8,000 ´ g for 5min. 1.4ml of 1.0M NaOH was added to 1.2 ml of the supernatant, and its absorbance was measured at 440nm.The protein concentrations were determined according to the Bradford method using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Quorum sensing modulatory activity of ethanolic extracts of medicinal plants was studied against QS mediated virulence factors of *P. vulgaris* and *S. paratyphi*. In the present investigation, solvent extraction and bioassays have led to the identification of potential compounds with anti quorum sensing activity. GC-MS was carried out to characterize the bioactive compounds. The name, molecular weight and structure of the components of the test materials were ascertained. The various phytochemicals which contribute to the medicinal activity of the plant are listed in Table 1 to 6. GC-MS analysis of the ethanol extract of *Cynodon dactylon* and *Eucalyptus globulus* revealed eight (Figure 1) and eleven major peaks (Figure 2) and the retention time for the major peaks was in the range of 16.8 to 21.65 and 11.6 to 25.93 respectively. The peaks constituted 36.8 to 100 %.and 18.3 to 100%. Nine (figure 3) and two (figure 4) major peaks was recorded in *Azadirachta indica* and *Ocium tenuiflorum* extracts with the retention time range of 16.9 to 25.6 (57.0 to 100.0 % constitution and 4.7 to 8.97 % (5.6 and 4.4 % of constitution respectively. Rhizome extract of C*urcuma long* (Figure 5) and leaf extract of *Aegle marmelos* (Figure 6) revealed nine major peaks of 12.17 to 19.2(78.4 to 100 % constitution) and 15.25 to 24.42 retention time (50 to 100 % constitution. Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 65,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library which revealed the presence of various constituents. Medicinal use of extracts obtained from plants in general have recently gained popularity, inducing scientific interest exemplified in screening

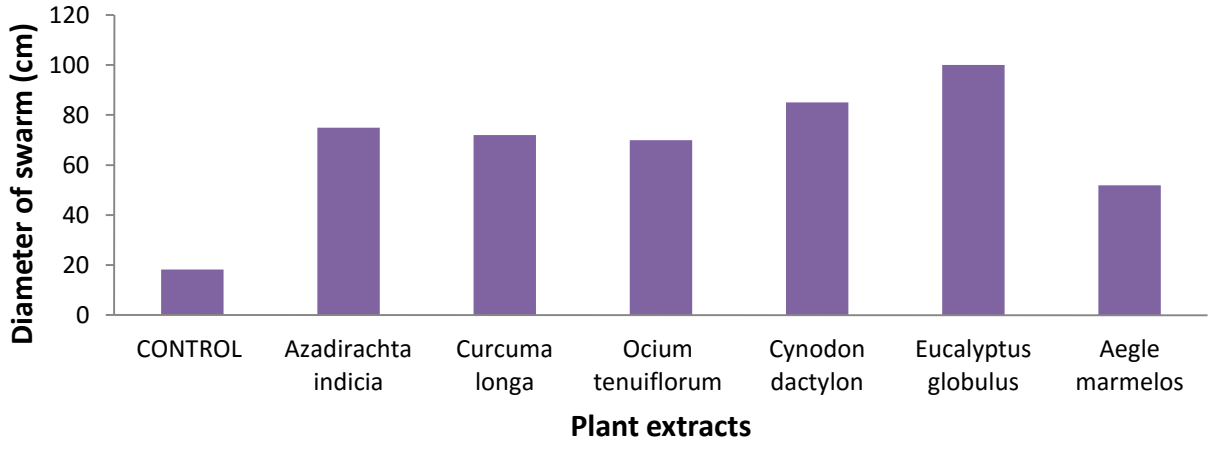

Figure 7: Effect of plant extracts on swarming motility of *Proteus vulgaris*

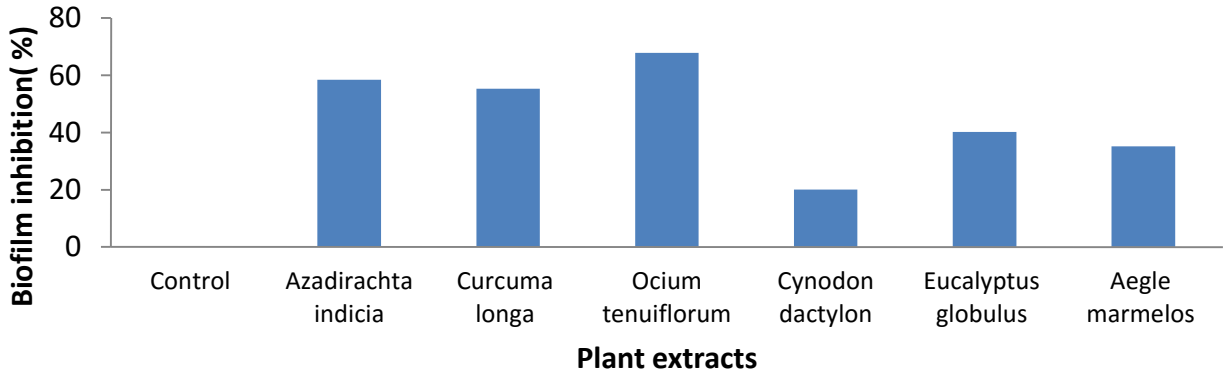

Figure 8: Effect of plant extracts on the biofilm inhibition (%) of *Proteus vulgaris*

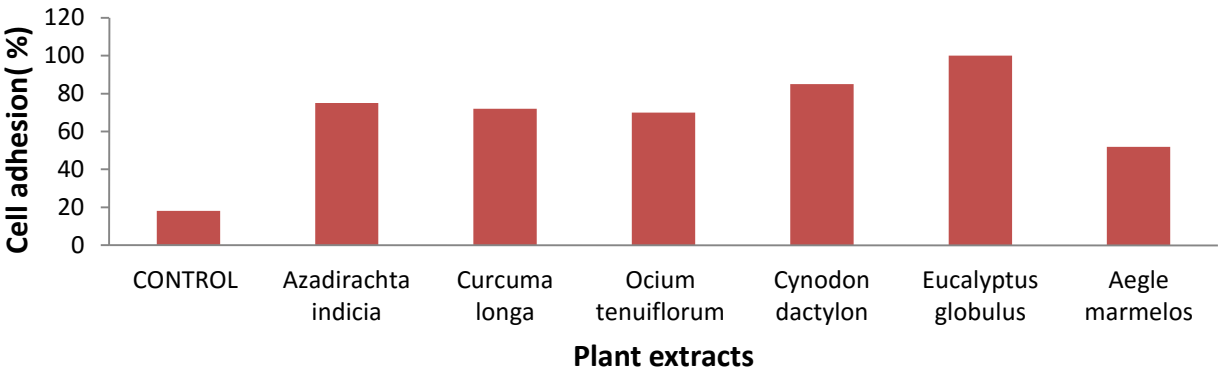

Figure 9: Effect of plant extracts on cell adhesion of *Proteus vulgaris*

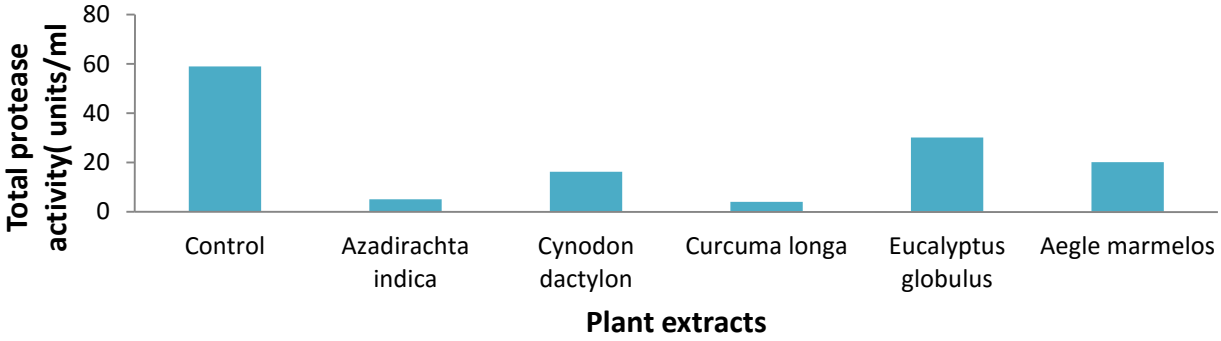

Figure 10: Effect of plant extracts on total proteolytic activity of *Proteus vulgaris*

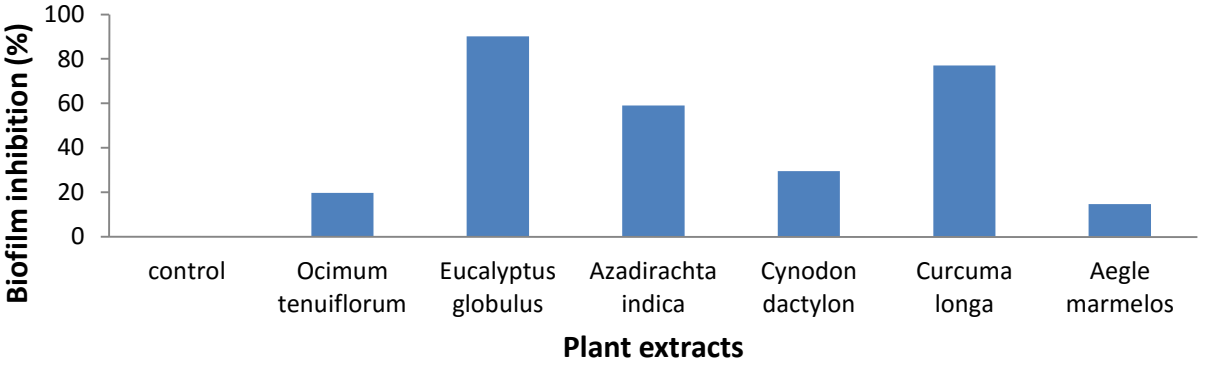

Figure 11: Effect of plant extracts on biofilm inhibition (%)of *Salmonella paratyphi*

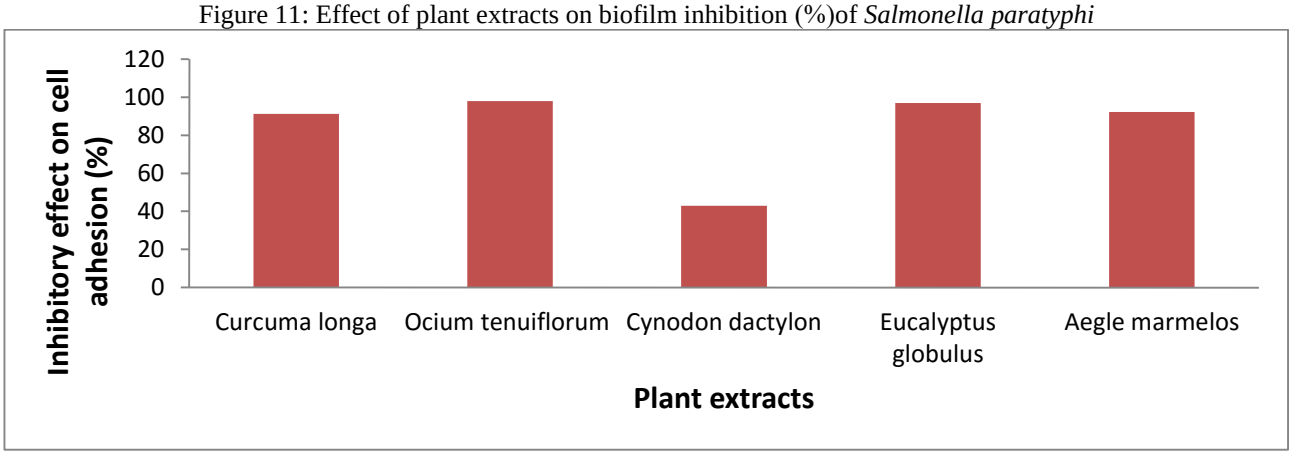

Figure 12: Effect of plant extracts on cell adhesion of Salmonella paratyphi

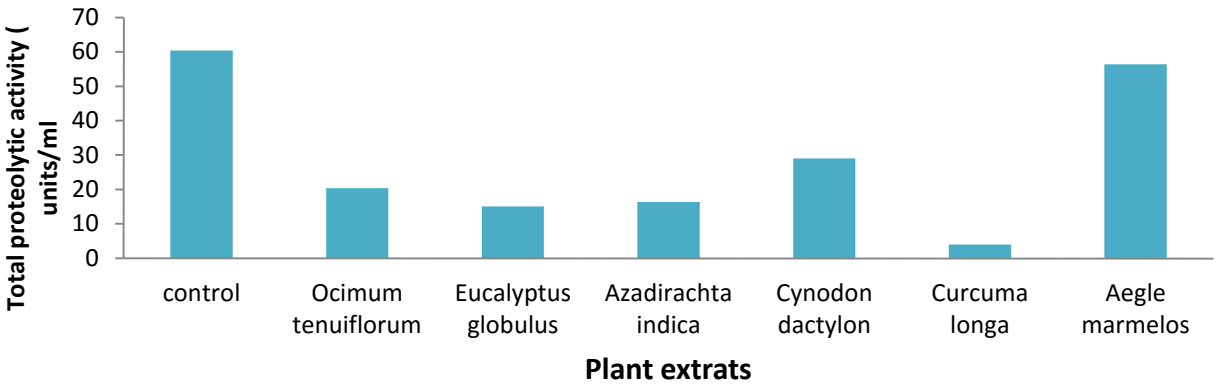

Figure 13: Effect of plant extracts on total proteolytic activity of *Salmonella paratyphi*

programs for novel and new components and uses pertaining to microbial growth or bacterial quorum sensing inhibition. The ability of the extracts to reduced the swarming motility of *P. vulgaris* was carried out by the modified method of Ren et al [10,11]using semi solid agar. The results indicate that *Eucalyptus globulus* extract significantly inhibited bacterial swarming followed by the *Aegle marmelos* **(**Figure**)** 7**).** Biofilm**-** an important QS mediated virulence factor of *P. vulgaris* and its susceptibility to plant extracts was presented in figure 8**.** The results indicates that *Ocium teniflorum* brought about maximum biofilm inhibition **(**67.9**%)** followed by *Azadirachta indicia* (58.49%). Cell adhesion inhibitory effect of plant extracts was studied by the method of NCCLS[13] using 96 well flat bottom micro well plate (Figure 9). Among the different plant extracts, maximum inhibition has been reported in *Ocium teniflorum* (97.37%) and *Eucalyptus globules* **(**94**.**64**%).** Proteolytic activity was found to be reduced in plant extracts treatment *Eucalyptus globulus* and *Ocium teniflorum* revealed maximum reduction of proeolytic activity (Figure 10). Anti quorum sensing activity of plant extracts against the quorum sensing mediated virulence factors of *S. paratyphi* revealed tested plant extracts showed varying degrees of anti quorum sensing activities. *Eucalyptus globulus* (90.16%) and *Ocium tenuiflorum* (98.04%) brought about maximum inhibition on biofilm formation and cell

adhesion (Figure 11,12). *Aegle marmelos* revealed maximum reduction of proteolytic activity (56.4%) (Figure 13). Anti quorum sensing activity of herbal plant extracts against human pathogenic bacteria revealed the presence of various phytochemicals in the extracts extracts to interfere with the activity of acyl homoserine lactone (AHL) a signalling molecule controls quorum sensing activities[15]. Phytochemicals are modulate the bacterial synthesis of AHL and in turn inhibit QS. Many natural extracts are believed to inhibit QS by either interfering with AHL activity by competing with them due to their structural similarity and/or to accelerate the degradation of the LuxR/LasR receptors for the AHL molecules. Inhibition of quorum sensing mediated virulence factors of *P. aeruginosa* by bud extract of Clove (*Syzygium Aromaticum***)** has been reported[16,17]**.** The present study clearly revealed the effective inhibition of QS mediated virulence factors by the ethanolic extracts of the medicinal plants would suggests the possible utilization of medicinal plants as an effective anti pathogenic agents**.**

CONCLUSION

Researchers are increasingly looking at herbal products in the quest for new therapeutic and antipathogenic agents which might be nontoxic inhibitors of quorum sensing, thus controlling infections without encouraging the appearance of resistant bacterial strains. The presence of active compounds exhibiting anti-QS activity in the plant extracts may be useful for the development of anti-infective drugs. Our laboratory is currently elucidating the chemical structure of these active compounds to understand the anti-QS mechanism in QS bacteria.

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