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Inhibition of quorum sensing mediated pigment production in *Serratia marcescens* by secondary metabolite of *Aspergillus* sp. identified by metagenomic studies

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Abstract
Bacteria communicate with each other with the help of signaling molecules called as autoinducers (AI) by a process known as Quorum sensing (QS). The production of virulence factors of the pathogenic bacteria is the result of QS regulated gene expression of the bacterial consortium at a higher density. *Serratia marcescens* is a prominent opportunistic pathogen which are found to cause nosocomial infections. The Virulent factors secreted by *Serratia marcescens* under the influence of QS are lecitinase, lipase, chitinase, chloroperoxidase, hemolysin and pigment prodigiosin.
The continuous and inappropriate use of antibiotics made *S. marcescens* a multi-drug resistant bacteria. An alternative for the antibiotic treatment was inhibition of QS, which indirectly inhibits the virulent nature of the pathogenic bacteria. In this study, inhibition of prodigiosin, a pigment produced by *S. marcescens* was tested with a fungal extract from fungi isolated from dump site (extreme environment). The fungal extract was found to inhibit QS by causing a reduction in the production of pigment. The fungal extract was subjected to GCMS analysis to find the active compounds present and five major compounds were detected. The extract was also examined for its anti-tumor effect on lung Adenocarcinoma cell line A549 and was found to inhibit proliferation of the cells.

Keywords: Quorum sensing, serratia marcescens, prodigiosin, autoinducers

Introduction
Fungi are known to produce small molecules, after active growth referred to as secondary metabolites which are not essential for their growth. These secondary metabolites are used to inhibit other organisms competing for ecological niche, metabolism and reproduction. Secondary metabolites produced by fungi are developed into drugs, including antibiotics, vasodilators, immune suppressants, anticancer, and anti-multiple sclerosis agents [1]. Soil from waste dump site have enormous amount of nutrients from various sources of wastes including agricultural and farm waste, domestic waste, industrial waste, hospital waste and they may contain heavy metals. Fungi growing in such site are subjected to extreme environmental conditions and that many of the compounds they synthesize are specific to them. Hence fungi from such environments seem to be good potential candidates for isolation of novel bioactive compounds.
Emergence of multi-drug resistant pathogens has generated an interest in inhibiting the virulence factor rather than destroying the pathogen. *Serratia marcescens*, a gram-negative bacteria belonging to *Enterobacteriaceae* family, is a prominent opportunistic pathogen that cause a wide array of infections in immunocompromised patients. *S. marcescens* has been noted to produce virulence factors controlled through cell to cell mediated quorum sensing which includes a major pigment prodigiosin (2-methyl-3-75 amyl/pentyl-6-methoxyprodigiosin). The biofilm formation which is a major virulence factor controlled by QS, helps *Serratia* to adhere and grow on the surface of medical devices. And bacteria growing inside biofilm are tough to remove and are highly resistant to antibiotics compared to their planktonic forms. Inability of *S. marcescens* to produce prodigiosin and other exoenzymes in QS mutants confirms the role of QS system in its pathogenesis [2]. Novel way to control infections apart from new antibiotics is to target communication between cells through quorum sensing. Anti-quorum sensing activity does not develop any resistance in *S. marcescens* as it only mitigates the virulence factor but not the growth of the organism [3].
Fungal metabolites have great potential as antimicrobial agents. The fungal metabolite

produced by fungi isolated from municipal dump site in Chennai was examined for its anti-quorum sensing activity against prodigiosin production of *S. marcescens*.

Materials and Methodology

Sample collection

Soil sample was collected from three different areas in Municipal dump site located in Chennai. The soil was mined to remove the top layer and soil from subterranean layer was collected by sterile means. The soil sample from three different regions were mixed together for further process.

Isolation of fungi

Soil sample weighing 1g was serially diluted in dilutions ranging from 10^{-1} - 10^{-7} to make a soil suspension. 0.1ml of the soil suspension from tubes were pipetted and plated on potato dextrose agar. The plates were incubated at room temperature for 4 days. Four different colonies were obtained from the dilution 10^{-5} and the isolated colonies were reaped and inoculated on sterile PDA plates to obtain pure culture.

Molecular identification of unknown fungi

The genomic DNA was extracted from 5 to 7 days old unidentified fungal cultures grown in Potato Dextrose broth. 18S rRNA gene region was amplified with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [4].

PCR amplification was performed in Agilent Sure Cycler 8800® gradient PCR machine. The following cyclic conditions were performed: initial denaturation for 5 minutes at 95 ºC followed by 35 cycles of denaturation for 45 seconds at 94 ºC; annealing for 1 minute at 59 ºC; extension for 1 minute at 72ºC followed by a final extension at 72 ºC for 5 min.

The amplified PCR products were electrophoresed along with 1 kb Ready- to -Use DNA marker on 1.2% gel at 100 volts for 45 minutes at room temperature using 1X TAE buffer (Tris–acetate 40mM pH 8.0, EDTA 1mM, pH 8.0). The PCR products were stained with ethidium bromide (ETBR) and visualized and photographed using Gelstan gel documentation system.

Production of fungal biomass

The fungal isolate was inoculated in 1000ml broth medium (Potato Dextrose) and incubated 30º C for 10 days in a shaker incubator. The flask was taken out after 10 days, the mycelium was separated by filtration and the filtrate was collected in a separate conical flask.

Extraction of secondary metabolite

The filtrate was filled in a separatory funnel with equal volume of ethyl acetate and shaken vigorously, and then the mixture was allowed to stand for one hour. The metabolites dispersed in ethyl acetate was pipetted out and collected in a separate sterile container. This crude extract was allowed to dry by evaporation to obtain extract of fungal metabolite. The extract was stored at 4 ºC for further use [5].

Antimicrobial activity

Antimicrobial activity was tested by standard agar well diffusion technique with the ethyl acetate fungal extract. The test organisms (*Enterococcus faecalis, staphylococcus aureus, Klebsiella pneumonia, Escherichia coli*) to be inhibited was seeded in nutrient agar plates. 20µl of the ethyl acetate fungal extract was poured in wells at different concentration (25mg/ml, 50mg/ml, 75mg/ml,100mg/ml). The plates were incubated at 37 ºC for 24 hours and observed for zone of inhibition.

Preparation of stock solution of extract

1mg of the fungal extract was weighed separately in a sterile tube. The extract was dissolved in 100µl of DMSO and 900µl of LB medium to make 1ml of stock solution and mixed thoroughly. The stock solution was syringe sterilized and stored for further use [3].

Anti-quorum sensing activity

The ethyl acetate extract was serially diluted in two-fold dilution with sterile LB nutrient to obtain different concentrations 2000, 1000, 500, 250, 125, 62.5, 31.25 and 15.625 µg/ml to determine the MIC of the extract. These diluted extracts were taken in separate tubes and were made up to 2ml in a tube. 100µl of test organism was inoculated in each tube. The tubes were incubated at 37º C for 18 hours. Positive control was a tube with only LB broth and negative control was a tube with LB broth and organism. The tubes were tested for presence or absence of pigment Prodigiosin [3].

Confirmation of anti-quorum sensing activity

The inhibitory effect of the ethyl acetate extract observed in tubes were tested by inoculating a loop full of culture from the tubes on a sterile LB media plate without extract. The plates were incubated at 37º C for 18 hours. The plates were checked for pigmentation after incubation [3].

Qualitative analysis of fungal extract

Gas Chromatography-Mass Spectrometry (GC-MS) is an analytical method that is employed for profiling different components present in the test sample. GC-MS analysis was performed to identify the bioactive constituent present in the most effective fraction of the fungal extract with anti-quorum sensing property.

Anticancer activity

Lung adenocarcinoma cell line (A549) was used in the experiment to estimate the anticancer activity of the fungal extract. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), incubated at 37 ºC in 5% CO_2. Cells were treated with different concentration of ethyl acetate extract (2000µg/ml and 250µg/ml) and incubated for 24 hours. Cells were treated with 10µl of the reaction mixture (1:1 Acridine orange - Ethidium bromide) and incubated for 5mins. Cells were then observed under inverted microscope [6].

Results

Isolation of fungi

Four different fungal colonies were obtained from 10^{-5} dilution PDA plates. The most prevalent species was isolated from the plates and sub-cultured in separate PDA plates.

Molecular identification of fungi

Genomic DNA was extracted from the fungal isolate by the standard method. PCR amplification of 18S rRNA gene region was standardized with 55 °C as annealing temperature. The purity and integrity was confirmed by Agarose gel electrophoresis. The PCR product was visualized as thick bands in the Ethidium Bromide -stained gel under UV

illumination and photographed using Gelstan gel documentation system (Fig. 1). Sequencing of PCR product resulted in 520 base pairs in size.

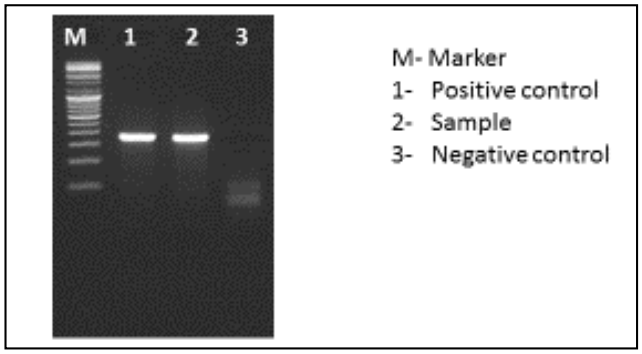


Fig 1: PCR analysis of the amplified product of 18S rRNA gene region. Lanes: M= 1Kb DNA marker; 1 and 2 – amplified PCR product of 18S rRNA gene.

The sequences were edited using Bioedit software v 7.0.9[7]. The edited sequences were blast with GenBank Nucleotide Database (http://www.ncbi.nlm.nih.gov/BLAST/) using the algorithm Blast N[8]. The output of BLAST search was sorted based on maximum identity with other genus or species names in GenBank records. The sequence based identity showed cutoff of 99% or greater was considered as significant, and best hit was defined as the sequence with highest maximum identity to the query sequence.

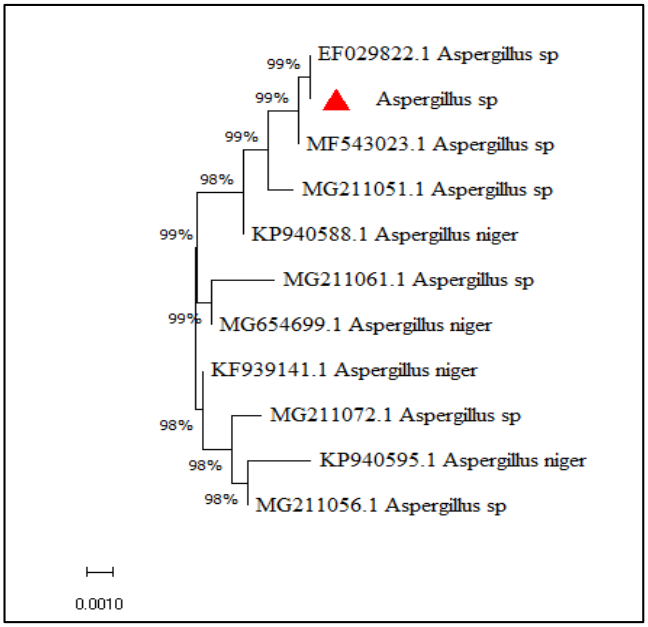


Fig 2: Phylogenetic tree

Antimicrobial activity

The ethyl acetate fungal extracts were tested for their antibacterial activity against clinical isolates *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis and Klebsiella pneumoniae*. There was no significant zone of inhibition observed in the plates.

Antiquorum sensing activity

The anti-quorum sensing effect of ethyl acetate fungal extract was analyzed by broth dilution method. The extract inhibited the production of prodigiosin pigment without any effect on growth of the organism (Fig:3).

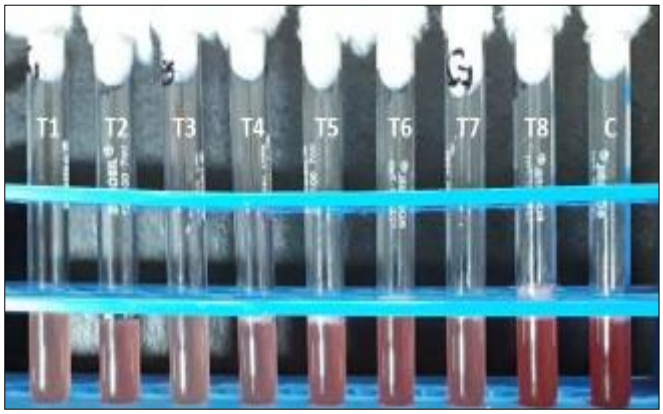
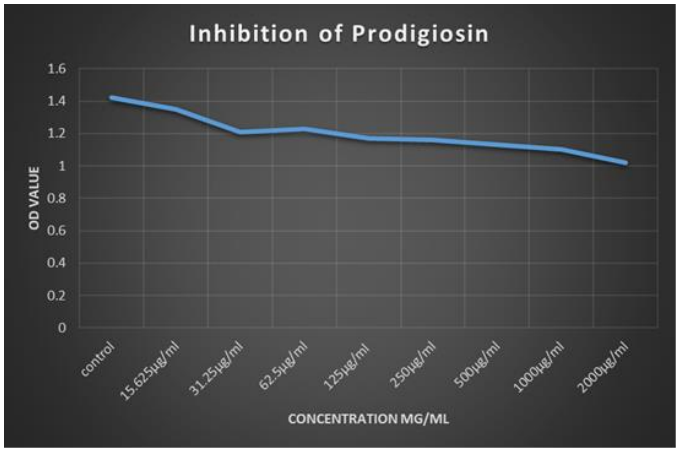


Fig 3: Inhibition of prodigiosin production.

The inhibition of pigment of *S. marcescens* was carried out in concentration dependent manner and the OD value for each concentration was recorded at 600nm (Graph 1).



Graph 1: Graphical representation of inhibition of prodigiosin by fungal extract.

Confirmation of anti-quorum sensing activity

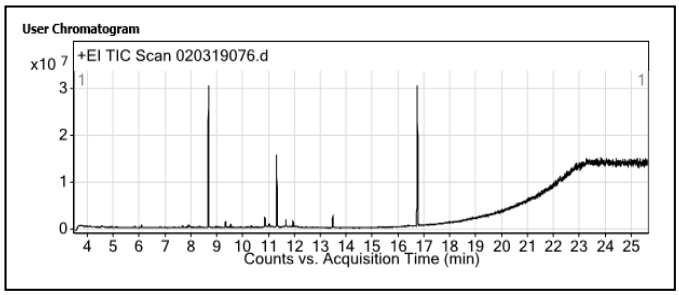
The extract was found to inhibit the prodigiosin pigment of *S. marcescens* only in its presence. The test organism produced pigment when inoculated in extract free nutrient medium plate (Fig. 4)

Fig 4: Anti-quorum sensing confirmation plate.

Qualitative analysis of fungal extract

GCMS analyses revealed the presence of 5 compounds in the

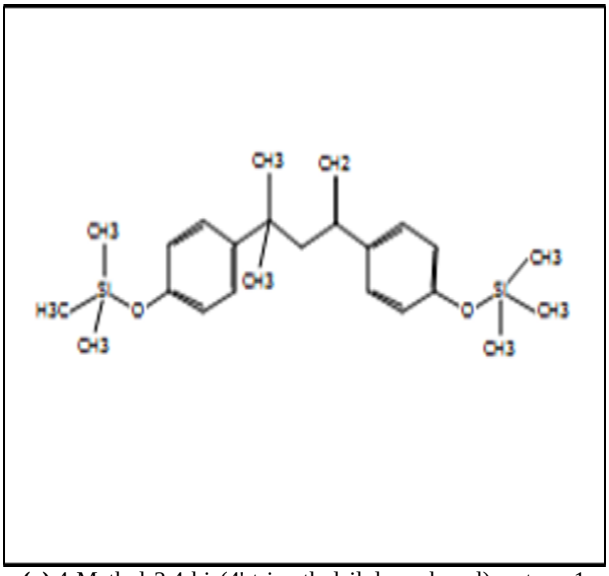
partially purified fungal extract. The major compounds present in the extract were identified as 4-Methyl-2,4-bis(4'-trimethylsilyloxylphenyl) pentene-1 with RT 25.06 (85.65%); Phthalic acid, di (2-propylpentyl) ester with RT 16.71 (6.85%); Phthalic acid, cyclobutyl tridecyl ester with RT 11.92 (0.15%); 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester with RT 11.29 (2.5%) and 2,4-bis(1,1-dimethylethyl)-phenol with RT 8.66 (4.7%). The details of compounds identified (Table 1), structure (Fig. 5.), retention time, area percentage are indicated in Graph 2.



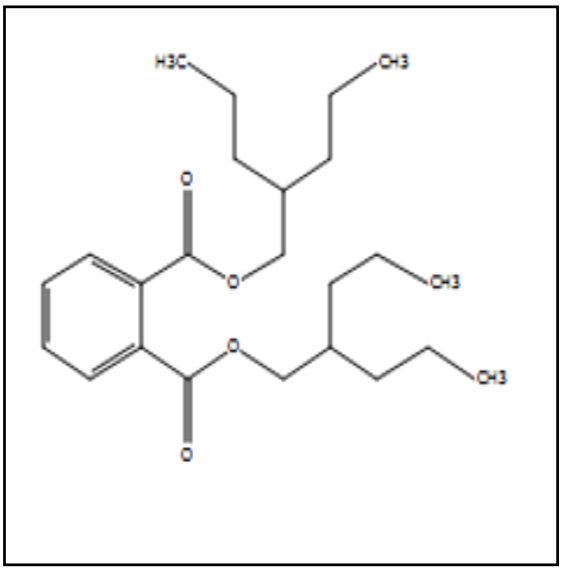
Graph 2: GCMS chromatogram of Fungal Extract

Table 1: GCMS analysis of fungal extract.

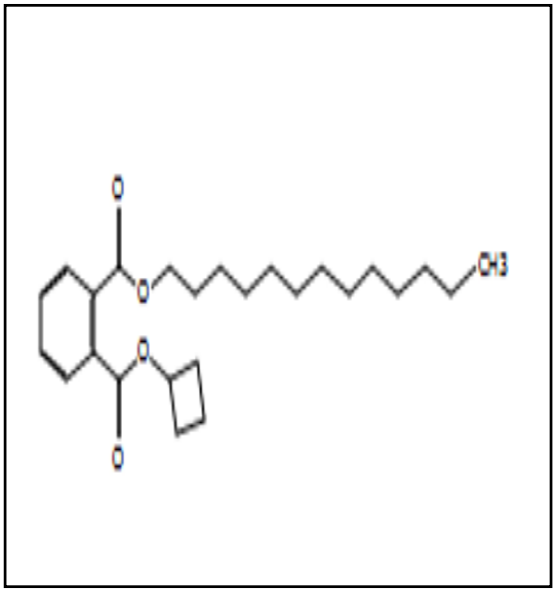
RT	Name	Formula	Area	Height	Mass
8.66	Phenol,2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	4.7%	10743118	206.2
11.29	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	2.5%	9914088	278.2
11.92	Phthalic acid, cyclobutyl tridecyl ester	$C_{25}H_{38}O_4$	0.15%	842542	402.3
16.71	Phthalic acid, di(2-prophypentyl) ester	$C_{24}H_{38}O_4$	6.85%	10325791	390.3
25.06	4-Methyl-2,4-bis(4'-trimethylsilyloxylphenyl)pentene-1	$C_{24}H_{36}O_2Si_2$	85.6%	2588120	412.2



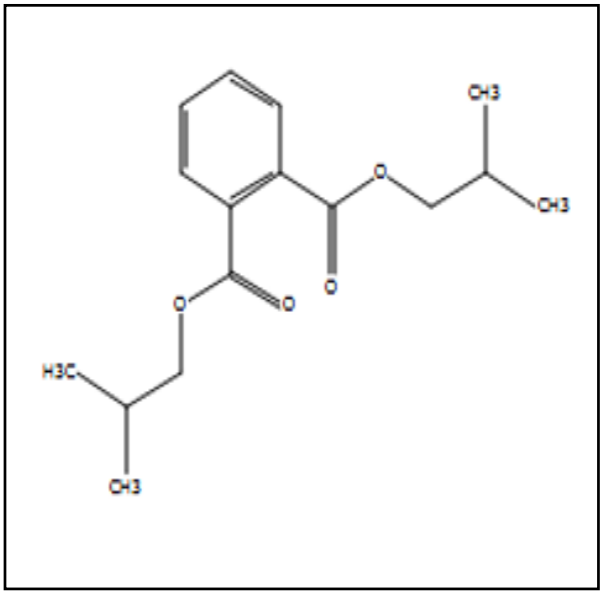
(a) 4-Methyl-2,4-bis(4'-trimethylsilyloxyphenyl)pentene-1



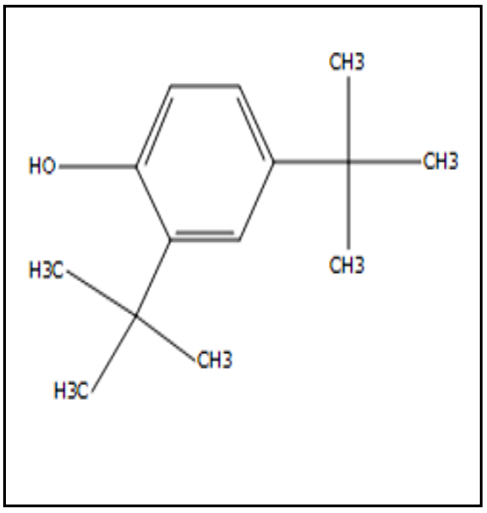
(b) Phthalic acid, di(2-propylpentyl) ester



(c) Phthalic acid, cyclobutyl tridecyl ester



(d) 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester



(e) Phenol, 2,4-bis(1,1-dimethylethyl)

Fig 5: Structure of major compounds

Anticancer activity of fungal extract

The estimation of *in vitro* anticancer activity of the ethyl acetate extract was done on Lung adenocarcinoma cell line (A549) in varying concentrations from 2000µg/ml to 250µg/ml. The ethyl acetate extract inhibited the proliferation of A549 cells significantly in a dose dependent manner from 2000 µg/ml (70 %), to 250µg/ml (34%) compared to positive control (Fig. 6); (Graph 3).

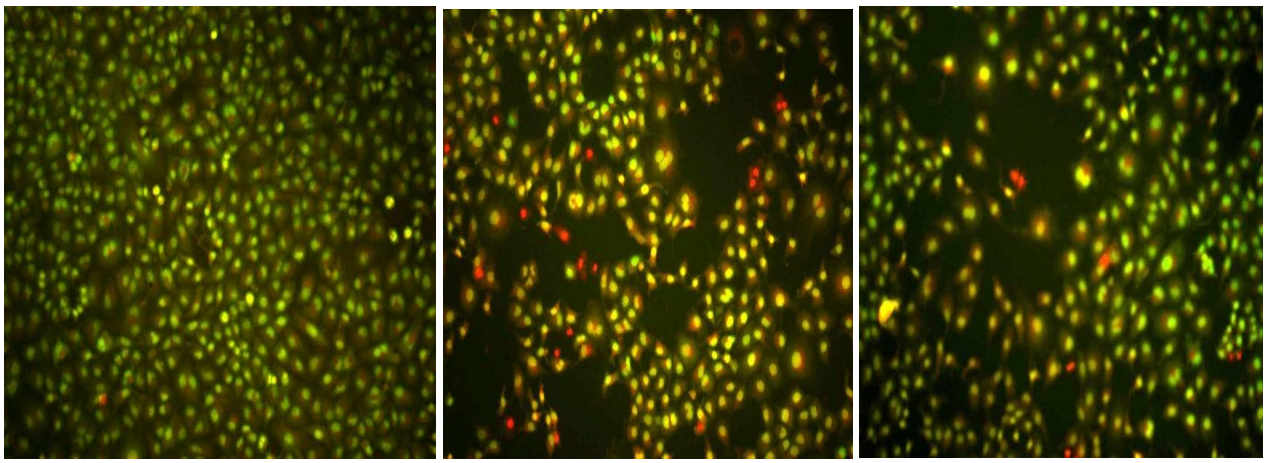
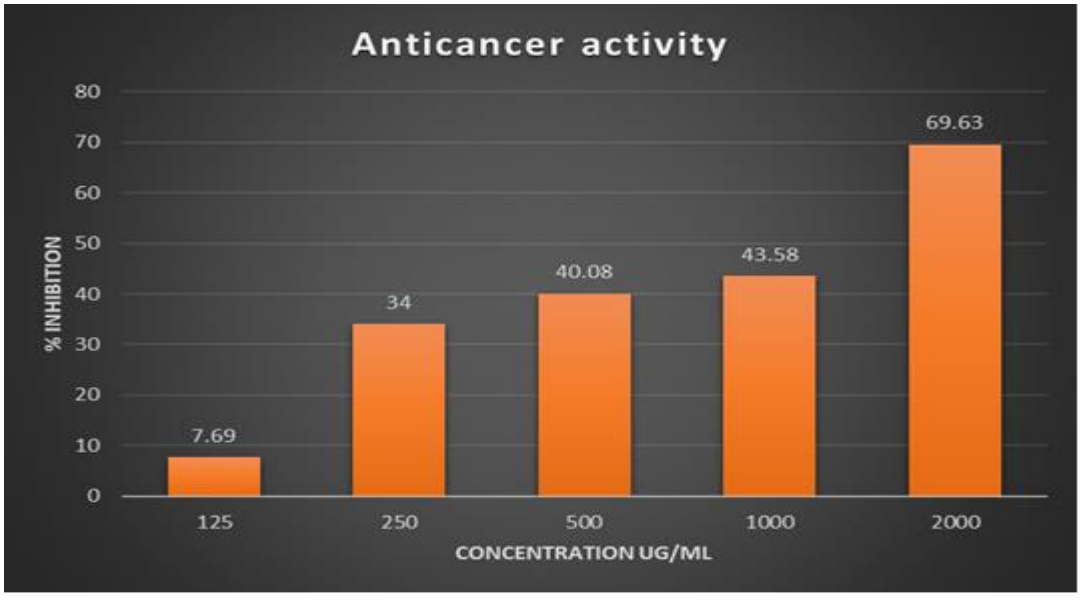


Fig 6 (a): Control **(b):** 2000µg/ml – 69.63% **(c):** 250µg/ml – 34%



Graph 3: Graphical representation of Anticancer activity of fungal extract.

Discussion

Fungal metabolites are diverse and exhibit various biological activities. A total of four fungi were obtained from dump site soil. The *Aspergillus* strain which was dominant among the four was chosen for extraction of bioactive compounds.

Amplification and sequencing of short, standard DNA regions (metabarcoding) is becoming an increasingly popular tool for the characterization of fungal communities. Various molecular approaches have been used for the detection of *Aspergillus* from environmental samples. Targets for the genus level detection of *Aspergillus* include the internal transcribed spacer (ITS) regions. The ITS regions are located between the 18S and 28S rRNA genes and offer distinct advantages over other molecular targets due to the existence of approximately 100 copies per genome. In the current study the internal transcribed spacer (ITS) region, ITS 1-5.8S- ITS 4, from isolates of aspergilli were amplified, sequenced, and compared with strain sequences in GenBank. ITS amplicons from Aspergillus species ranged in size from 565 to 613 bp. Comparison of reference strains and GenBank sequences demonstrated that both ITS 1 and ITS 4 regions were found to be similar to those of *Aspergillus* sp. The ITS regions have been used as targets for phylogenetic analysis [9]. Patrick Woo *et al*., 2010 [10] and Amutha and Godavari 2014 [4]., have researched on 18S rRNA genes, 26S rRNA genes, 5.8S rRNA genes, 28S rRNA genes and ITS1, ITS 4 sequences of different fungi for the study of genetic relationship.

In the present study the anti-quorum sensing potential of fungal metabolite was assessed for its ability to hinder QS mediated pigment production in *S marcescens*. The QS activity was determined by monitoring the prodigiosin pigment production in *S. marcescens*. The *in vitro* trial showed that the selected fungal strain produced metabolite that exhibited antiquorum sensing activity at varying degrees. The prodigiosin production was significantly reduced compared to that of control. Our results are in accordance with findings of Musthafa *et al*, 2011 [11] who have reported the reduction in prodigiosin production with the extract from a marine *Bacillus* sp. The anti-virulence effect observed in the tubes were confirmed further by subculturing loop full of inoculum on a fresh extract free Nutrient agar medium and incubated at 37 °C for 18 hours developed the prodigiosin pigment, confirming the inhibition in the tubes was due to the effect of the extracts. Similar results have been reported by [3] Pavithra *et al.,* 2018.

The GC MS analysis of the ethyl acetate extract yielded five major peaks inclusive of phenol,2,4-bis(1,1-dimethylethyl); 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester; Phthalic acid, cyclobutyl tridecyl ester; Phthalic acid, di(2-propylpentyl) ester; and 4-Methyl-2,4-bis(4'-trimethylsilyloxyphenyl) pentene-1 ester. Out of which Phenol,2,4-bis(1,1-dimethylethyl) has been reported to have QS mediated reduction of prodigiosin pigment production in uropathogen *S. marcescens* by 84% by Padmavathi *et al*., 2014 [12] from a marine bacterium hence our results are in conformity with the earlier report. The *in vitro* anticancer activity was carried out using A549 cell lines revealed that bioactive secondary metabolite in the ethyl acetate extract is capable of inhibiting proliferation of A549 cell with IC_{50} = 1000 µg/ml.

Conclusion

To the best of our knowledge, fungal extracts have not been explored for its anti QS activity against uropathogen *S. marcescens*. Therefore, this report shows QSI activity of ethyl acetate extract of *Aspergillus* sp. by possibly targeting QS pathway. The results confirm the potential use of fungal metabolite as an efficient anti virulence agent to treat persistent infections of *S. marcescens.*

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