

**ANTI - QUORUM SENSING POTENTIAL OF SEAGRASS BIOACTIVE  
CONSTITUENTS AGAINST *PSEUDOMONAS AERUGINOSA***

Submitted in partial fulfilment of the requirements of award of

Bachelor of Science degree in

BIOTECHNOLOGY

By

**S.MAHESH (40770028)**



DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF SCIENCE AND HUMANITIES

**SATHYABAMA**

**INSTITUTE OF SCIENCE AND TECHNOLOGY**

(DEEMED TO BE UNIVERSITY) Accredited with Grade "A" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600 119

**MAY-2023**



# SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY  
(DEEMED TO BE UNIVERSITY)

Accredited "A" Grade by NAAC | 12B Status by UGC | Approved by AICTE

[www.sathyabama.ac.in](http://www.sathyabama.ac.in)

## DEPARTMENT OF BIOTECHNOLOGY

### BONAFIDE CERTIFICATE

This is to certify that the Project Report is the bonafide work of **S.MAHESH (40770028)** who carried out the project entitled "**ANTI - QUORUM SENSING POTENTIAL OF SEAGRASS BIOACTIVE CONSTITUENTS AGAINST *PSEUDOMONAS AERUGINOSA***" under my/ our supervision from September 2022 to March 2023.

Internal Guide

Dr. M. BAVANILATHA

  
EXTERNAL GUIDE 29/04/23

DR.D.JEYAPRAGASH

HEAD OF THE DEPARTMENT

Dr. V. RAMESH KUMAR

Submitted for Viva Examination held on 12/3/23\_\_\_\_\_

INTERNAL EXAMINER

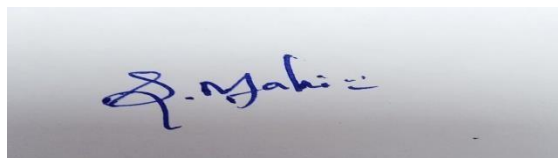
EXTERNAL EXAMINER

## DECLARATION

I **S.MAHESH** hereby declare that the Project Report entitled done by me **ANTI - QUORUM SENSING POTENTIAL OF SEAGRASS BIOACTIVE CONSTITUENTS AGAINST *PSEUDOMONAS AERUGINOSA*** under the guidance of **Dr.M.BAVANILATHA** (Internal) and **DR.D.JEYAPRAGASH** (External) at **CENTRE FOR OCEAN RESEARCH, SATHYABAMA UNIVERSITY** is submitted in partial fulfillment of the requirements for the award of Bachelor of Science degree in **BIOTECHNOLOGY**.

**DATE: 12/3/23**

**PLACE:Chennai**

A rectangular box containing a handwritten signature in blue ink. The signature appears to be 'S. Mahesh' written in a cursive style.

**SIGNATURE OF THE CANDIDATE**

## ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to the **Board of Management of SATHYABAMA** for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my Thanks to **Dr. V. RAMESH KUMAR. Head of the Department, Department of Biotechnology** for providing me necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project External guide **Dr. D. JEYAPRAGASH (CENTRE FOR OCEAN RESEARCH)** and project Internal guide **DR.M.BAVANILATHA** for their valuable guidance, suggestions and constant encouragement paved way for the successful completion of my project work.

I wish to express my thanks to all Teaching and Non-teaching staff members of the department of Biotechnology who were helpful in many ways for the completion of the project.

Lastly, I would like to thank my parents and family members for their continuous encouragement and support throughout my course of graduation. Without them, I would have not reached to this level and succeeded in all my achievements.

## ABSTRACT

Biofilm associated, multidrug resistant *Pseudomonas aeruginosa* infection remain a challenging problem in the clinical field since the conventional antibiotic therapy are largely inefficient and new approaches are needed. Seagrass *Halodule pinifolia* (Miki) Hartog has been shown to exhibit potential antimicrobial activities against harmful pathogens. Our study investigated the effects of seagrass *H. pinifolia* leaf extract and its bioactive constituents on QS-mediated biofilm formation in *P.aeruginosa* PAO1. Preliminary screening on antibiofilm activity showed that the methanolic extract of *H. pinifolia* exhibited potential inhibition of biofilm formation (91%) as compared to the control respectively. Further, the potential extract was column fractionated and the active fraction was separated using TLC. 4-hydroxy benzoic acid was profiled from fraction 11&12 and were purified by HPLC, structurally confirmed by NMR. 4-hydroxybenzoic acid (4-HBA) showed an effective inhibition of bacterial growth with minimum inhibitory concentration (MIC) of 31.25µg/ml. The present findings suggests that seagrasses may act as a newer source for the marine based drug discovery and the lead compound 4-HBA derived from *H. pinifolia* may act as anti-infective agent against *P. aeruginosa*.

**KEY WORDS:** Sea grass, *P.aeruginosa*, Biofilm, *H.pinifolia*, TLC.

## LIST OF CONTENTS

| CHAPTER NO | TITLE                         | PAGE NO |
|------------|-------------------------------|---------|
|            | LIST OF FIGURES               | I       |
|            | LIST OF ABBREVIATIONS         | li      |
|            | LIST OF SYMBOLS               | lii     |
| 1.         | INTRODUCTION                  | 1       |
| 1.1        | ANTIMICROBIAL RESISTANCE      | 1       |
| 1.2        | BIOFILM FORMATION             | 2       |
| 1.3        | <i>PSEUDOMONAS AERUGINOSA</i> | 3       |
| 1.4        | PLANT DERIVED COMPOUNDS       | 4       |
| 1.5        | SEAGRASS                      | 5       |
| 1.6        | SEAGRASS ACTIVE CONSTITUENTS  | 6       |
| 2.         | LITERATURE SURVEY             | 7       |
| 2.1        | ANTIBIOFILM ACTIVITY          | 7       |
| 2.2        | SEA GRASS BIOACTIVE COMPOUNDS | 7       |
| 2.3        | ANTIMICROBIAL RESISTANCE      | 8       |
| 3          | AIM AND OBJECTIVE             | 10      |

|           |                                                                                                                  |           |
|-----------|------------------------------------------------------------------------------------------------------------------|-----------|
| <b>4.</b> | <b>MATERIALS AND METHODS</b>                                                                                     | <b>11</b> |
| 4.1       | BACTERIAL STRAINS AND CULTURE                                                                                    | 11        |
| 4.2       | PLANT MATERIAL AND EXTRACTION                                                                                    | 13        |
| 4.3       | DETERMINATION OF ANTIMICROBIAL ACTIVITY OF<br><i>H.PINIFOLIA</i> CRUDE EXTRACT                                   | 11        |
|           | STATISTICAL ANALYSIS                                                                                             |           |
| 4.44      | ANTIBIOFILM ACTIVITY OF CRUDE EXTRACT                                                                            | 12        |
| .<br>4    | COLUMN FRACTIONATION OF ACTIVE <i>H.PINIFOLIA</i>                                                                | 12        |
| 4.54      | ANTI-MICROBIAL AND ANTI-BIOFILM ACTIVITY OF<br>COLUMN FRACTIONS                                                  | 13        |
| .<br>5    | THIN LAYER CHROMATOGRAPHY                                                                                        | 13        |
| 4.64      | HPLC AND NMR ANALYSIS                                                                                            | 13        |
| .<br>6    | MINIMUM INHIBITORY CONCENTRATION (MIC) OF 4-<br>HYDROXY BENZOIC ACID (4-HBA)                                     | 14        |
| 4.74      | ANTI-ADHERENCE POTNTIAL OF 4-HBA                                                                                 | 14        |
| .<br>7    |                                                                                                                  |           |
| 4.84      |                                                                                                                  |           |
| .<br>8    |                                                                                                                  |           |
| 4.94      |                                                                                                                  |           |
| .<br>9    |                                                                                                                  |           |
| 4.10      |                                                                                                                  |           |
| <b>5.</b> | <b>RESULTS AND DISCUSSION</b>                                                                                    | <b>15</b> |
| 5.1       | EFFECT OF <i>H.PINIFOLIA</i> CRUDE EXTRACTS ON<br><i>P.AERUGINOSA</i> ANTIBACTERIAL ACTIVITY OF<br>CRUDE EXTRACT | 15        |
| 5.2       | EFFECT OF <i>H.PINIFOLIA</i> CRUDE EXTRACTS ON<br><i>P.AERUGINOSA</i> BIOFILM INHIBITION                         | 15        |
| 5.3       | ANTIMICROBIAL ACTIVITY OF COLUMN                                                                                 | 17        |

|     |                                                 |    |
|-----|-------------------------------------------------|----|
|     | FRACTIONATION                                   |    |
| 5.4 | ANTIBIOFILM ACTIVITY OF COLUMN<br>FRACTIONATION | 17 |
| 5.5 | THIN LAYER CHROMATOGRAPHY (TLC)                 | 18 |
| 5.6 | HPLC &NMR ANALYSIS                              | 20 |

|     |                                                                                  |    |
|-----|----------------------------------------------------------------------------------|----|
| 5.7 | MINIMUM INHIBITORY CONCENTRATION OF 4-HBA<br>ON <i>P.AERUGINOSA</i>              | 21 |
| 5.8 | ANTIADHERENCE POTENTIAL OF QS INHIBITOR<br>AGAINST <i>PSEUDOMONAS AERUGINOSA</i> | 22 |
|     | DISCUSSION                                                                       | 23 |
| 6.  | <b>SUMMARY AND CONCLUSION</b>                                                    | 26 |
|     | <b>REFERENCES</b>                                                                | 27 |

## LIST OF FIGURES

| FIGURE NO | TITLE                                                                                                                | PAGE NO |
|-----------|----------------------------------------------------------------------------------------------------------------------|---------|
| 5.1       | Antimicrobial activity of <i>H.pinifolia</i> extracts on <i>P.aeruginosa</i>                                         | 15      |
| 5.2       | A) Antibiofilm activity of <i>H.pinifolia</i> extracts on <i>P.aeruginosa</i><br>B) Percentage of biofilm inhibition | 16      |
| 5.3       | Effect of <i>H.pinifolia</i> MeoH fractions on <i>P.aruginosa</i> antimicrobial activity                             | 17      |
| 5.4       | Effect of <i>H.pinifolia</i> MeoH fractions on <i>P.aruginosa</i> antibiofilm activity                               | 18      |
| 5.5       | Thin layer chromatography of active fractions                                                                        | 19      |
| 5.6       | HPLC & NMR Analysis                                                                                                  | 20      |
| 5.7       | Minimum inhibitory concentration of 4 -HBA on <i>P.aeruginosa</i> biofilm inhibition                                 | 21      |
| 5.8       | Anti-adherence potential of 4-HBA on <i>P.aeruginosa</i>                                                             | 22      |

## LIST OF TABLES

| TABLE NO | TITLE                        | PAGE NO |
|----------|------------------------------|---------|
| 5.1      | RF Value of active fractions | 19      |

## LIST OF ABBREVIATIONS

PA01 - *Pseudomonas aeruginosa*

MeOH - Methanol

H.pinifolia – *Halodule pinifolia*

4-HBA - 4 – Hydroxy Benzoic Acid

MIC - Minimum Inhibitory Concentration

QS - Quorum Sensing

DMSO - Dimethyl sulfoxide

PC- positive control

NC - Negative control

LB - Luria Bertani

TLC -Thin Layer Chromatography

HPLC – High Performance Liquid Chromatography

NMR – Nuclear Magnetic Resonance

CFU – Colony Forming Unit

RF – Retention Factor

UV – Ultra Violet

## LIST OF SYMBOLS

°C - Degree celcius.

% - Percentage

× - Multiple

= - Equal

- - Minus

## CHAPTER 1

### ANTI-QUORUM SENSING POTENTIAL OF SEAGRASS BIOACTIVE CONSTITUENTS AGAINST *PSEUDOMONAS AERUGINOSA*

#### 1. INTRODUCTION:

##### 1. ANTIMICROBIAL RESISTANCE:

Antibacterial resistance is described as bacteria's resistance to antibiotic medications that were first discovered to be successful in treating infections caused by that germ. In fact, the mortality rate for individuals with non-resistant bacteria infections is less than half that of people with a resistant version of the same infection. The rise and rapid global spread of antibiotic resistance in pathogenic bacteria is one of the most significant concerns in clinical treatment, necessitating coordinated efforts at the regional, national, and international levels (Kupferschmidt, K 2016; Fernández, J *et al.*, 2016;). Antibiotic resistance is widespread, and new resistance mechanisms are constantly emerging, increasing the likelihood of resistant strains spreading (Barbieri *et al.*, 2017). The emergence of multidrug-resistant pathogenic bacteria remains a difficult problem for antibiotic therapy in the twenty-first century, increasing the risk of infections (Jayapragash *et al.*, 2020). The most difficult hurdle for the healthcare industry is antibiotic resistance in pathogenic germs. Antibiotic efficacy against pathogenic microorganisms is currently diminishing due to the evolution of multidrug resistance (Adonizio *et al.*, 2008). Antibiotics become ineffective against resistant bacteria, allowing infections to remain in patients and increasing their risk of poor clinical outcomes and mortality. In fact, the mortality rate for individuals with non-resistant bacteria infections is less than half that of people with a resistant version of the same infection. One of the ways utilized by bacteria to build resistance is biofilm formation (Fuqua and Greenberg, 1998). It is generally known that treating diseases caused by biofilm forming bacteria necessitates extended treatment, which may result in antibiotic resistance due to significant evolutionary pressure. (Vijay K. Singh *et al.*, 2014 ;). Antimicrobial resistance is a major factor in infections caused by *Pseudomonas aeruginosa* biofilms, such as

urinary and pulmonary tract infections. Despite long-term antibiotic treatment, lung infections caused by *P.aeruginosa* biofilms frequently become chronic in patients with cystic fibrosis (Eliana Drenkard., 2003).

## **1.2 BIOFILM FORMATION:**

Cell-to-cell communication, also known as quorum sensing, regulates the production of biofilms. Inhibiting quorum sensing is one of the various ways used to reduce biofilm-forming microbes without developing medication resistance (Singh *et al.*, 2013, 2016b).

Biofilm formation makes the bacteria to become many fold resistance to antibiotics and resulted in the development of chronic infections in human beings (Jayapragash dhanaraj *et al.*,2020)Autoinducers (AIs), which are oligopeptides and acylated homoserine lactones (AHLs) in gram-positive and gram-negative bacteria, respectively, mediate the QS system(Francesca Guzzo *et al.*,2020;).

Biofilms are dense bacterial populations surrounded by an exopolysaccharide matrix that are bonded to a solid surface. Persistent bacterial biofilm infections have been linked to a variety of medical disorders, including periodontal disease, endocarditis, osteomyelitis, cystic fibrosis, and biomaterial-related bacterimias. Resistance to antimicrobial drugs and components of the human immune system is one of the most essential characteristics of bacterial biofilms. As a result, biofilm-related disorders are typically chronic and difficult to treat (Eliana Drenkard., 2003).

Bacterial biofilms grow on a variety of biotic (plants, animals, and people) and abiotic (plastic, glass, metal, and minerals) surfaces and have a structurally complex and dynamic architecture (Thenmozhi *et al.*, 2009).

### 1.3 PSEUDOMONAS AERUGINOSA:

*Pseudomonas aeruginosa* is a gram-negative bacterium that is an opportunistic human pathogen responsible for a variety of illnesses. *Pseudomonas aeruginosa* is a widespread bacteria that can cause illness in humans and other animals. It has citrate, catalase, and oxidase activity. Several environmental conditions have been shown to promote *Pseudomonas aeruginosa*'s polysaccharide intercellular adhesion (PIA) production and biofilm formation. It can be found all over the planet in soil, water, skin flora, and most man-made habitats. It thrives not just in normal but also in hypoxic settings, and has so colonised numerous natural and artificial environments. For food, it employs a diverse range of organic materials. In animals, its adaptability allows the pathogen to infect injured or immune-compromised organs (R.Nancy immaculate 1 *et al.*, 2013 ;). It accounts for around 57% of all nosocomial infections (Oncul *et al.*, 2009; Sarabhai *et al.*, 2013). *P. aeruginosa* was found to use a variety of numerous mechanisms, including biofilm formation, to successfully infect a wide range of hosts and defend itself against environmental stress and antibiotics (Driscoll *et al.*, 2007; Vandeputte *et al.*, 2010; Lee and Zhang, 2015). *P. aeruginosa*'s ability to cause disease is dependent on the synthesis of virulence factors, which actively induce harm to host tissues (Ysobel R. Baker *et al.*, 2013 ;). This bacteria is the major cause of potentially fatal infectious disorders such as urinary tract infections, respiratory tract infections, dermatitis, chronic wounds, and severe burn wound infection, and it also causes cystic fibrosis in immunocompromised persons (Hejazi A *et al.*, 1997, Kumar RJ *et al.*, 2004;). This bacterium thrives on damp surfaces and is also found on and in medical equipment, causing cross-infections in hospitals and clinics. It can also degrade hydrocarbons and has been used to break down tar balls and oil spills (R.Nancy immaculate *et al.*, 2013 ;). *Pseudomonas aeruginosa* is an opportunistic human pathogen that is the National Nosocomial Infection Surveillance System's second most prevalent gram-negative bacterial pathogen (Annapoorani A *et al.*, 2012 ;).

## 1.4 PLANT DERIVED COMPOUNDS:

Marine creatures are an amazing source of novel bioactive chemicals with a wider range of structural and chemical properties than terrestrial natural goods (Blunt, J.W *et al.*, 2018 ;).

Many plants have been known to have curative powers against human illnesses since ancient times due to their concentration of secondary metabolites, which have recently been discovered to serve as antimicrobial agents against human pathogens (Ramona Barbieri *et al.*,2017).

Secondary metabolites produced by seagrasses are recognised to operate as defence mechanisms under stress conditions. Several seagrass species produce active metabolites such as polyphenols, terpenoids, and halogenated compounds that have been shown to have anticancer (antitumor), antifungal, anti-inflammatory, antimicrobial, and antiviral, anti diabetic, antimalarial, antioxidant, anti-aging, and cytotoxic properties. These species are also useful for illness prevention in humans (Kim, D.H *et al.*, 2016 ;).

Alkaloids, terpenoids, flavonoids and coumarins, peptides, glycosides, nucleosides, and polyphenols are only a few of the physiologically active, low-molecular-mass (5 kDa) compounds ("parvome", parv = small, -ome = group) found in aquatic and terrestrial plants. Antibiotics, allosteric regulators, catalysis, catalytic cofactors, regulatory activities at the DNA, RNA, and protein levels, pigments, mutagens, antimutagens, receptor agonists, antagonists, signal molecules, siderophores, detergents, metal complexing/transporting agents, pheromones, toxins, and other interesting activities are all possible (Davies *et al.*, 2012).

## 1.5 SEAGRASS:

Seagrasses are real marine flowering plants that belong to the angiosperm family, which includes 72 species classified into four groups (Zosteraceae, Hydrocharitaceae, Posidoniaceae, and Cymodoceaceae) (Short, F *et al.*, 2016; Dhanaraj J *et al.*, 2021;). Except in the polar regions, seagrasses are eukaryotic organisms found in shallow water sections of temperate, subtropical, and tropical seas (Mehari Ghebretinsae, M *et al.*, 2019;). In Asia and Africa, seagrasses, a paraphyletic type of marine angiosperm, have long been utilized in traditional medicine (Torrecatro *et al.*, 2004;). Seagrasses were utilized as food in the form of nutrient-rich seeds, fertilizer in the form of nutrient-rich biomass, and livestock feed for goats and sheep in India. They were also used to treat heart disease and sea sickness (A.F. Newmaster *et al.*, 2011). According to reports, *H. pinifolia* seagrass may be more effective in combating human illnesses than other seagrass species like *C. serrulata* and *H. ovalis* (Kannan *et al.*, 2010, Kannan *et al.*, 2013;). Seagrass meadows have the potential to store carbon, enhance water quality, offer food and habitat, and serve as biological indicators (Unsworth, R *et al.*, 2014;).

Secondary metabolites produced by seagrasses are recognised to operate as defence mechanisms under stress conditions. Seagrasses have been used in folk medicine for a range of therapeutic purposes, including the treatment of fevers, stomach problems, muscle pain, wounds, and skin disorders; they are also used as a remedy for ray stings and as sedatives for babies (Grignon-Dubois, M *et al.*, 2015;). In addition to its medicinal activity, seagrasses have been used for basket making, salt extraction, mattress filling material, thatched roofs, fertilizer, paper materials for transporting sensitive products, and nitrocellulose, among other things (Jafriati, J *et al.*, 2019;).

There are two families of seagrasses. Hydrocharitaceae and Cymodoceaceae, with 14 species identified in India, are disappearing due to environmental and geopolitical instabilities (Dhanaraj *et al.*, 2018;).

## 1.6 SEAGRASS ACTIVE CONSTITUENTS:

Seagrass, a distinctive asset of the beachfront biological community, provides numerous opportunities for innovative, economically significant constituents and has received little attention in terms of phytochemical constituents. Antimicrobial, antioxidant, metabolite synthesis, and nutritive evaluations have brought sea grass to the forefront as a source of new bioactive chemicals (Athiperumalsami *et al.*, 2010; Kannan *et al.*, 2010; Pradheeba, M., *et al.*, 2011 ;)

Bioactive chemicals can be either primary or secondary. Secondary metabolites are byproducts of the metabolic process in plants. Primary data profiling Secondary metabolites are not needed for plant growth and development, but they are critical for plants to resist stress by maintaining the subtle balance in the surroundings (dhanaraj *et al.*, 2018).

Several seagrass species produce active metabolites such as polyphenols, terpenoids, and halogenated compounds that have been shown to have anticancer (antitumor), antifungal, anti-inflammatory, antimicrobial, antiviral, anti diabetic, antimalarial, antioxidant, anti-aging, and cytotoxic properties(Christina *et al.*,2022;).

## CHAPTER -2

### 2. LITERATURE SURVEY

#### 1. ANTIBIOFILM ACTIVITY:

The preliminary studies on Antibiofilm activity evidenced that methanolic extract of *H.pinifolia* exhibited 96% of potential biofilm formation when compared to that of the control. Eight active compounds were profiled from fraction 2 and were further purified by HPLC and NMR results confirmed the structure of those compounds. Out of which 4-HBA showed an effective inhibition against *Pseudomonas aeruginosa* in which MIC was showed at concentration of 62.5 µg/mL. This study reveals the way that seagrass can act as a good source for the purpose of drug discovery and in which the 4-MBA derived from *H. pinifolia* may act as active fractionate against the growth of *Pseudomonas aeruginosa* (Danaraj *et al.*,2020;).

Biofilms have great significance in healthcare-associated infections owing to their inherent tolerance and resistance to antimicrobial therapies. New approaches to prevent and treat unwanted biofilms are urgently required. In this study they used three species were investigated for their effects in mediating non-lethal interactions on sessile bacterial (*Escherichia coli*) and fungal (*Candida albicans*) cultures. This study focused on anti-biofilm activities of seagrass extracts, without killing cells (Vincent *et al.*, 2018 ;).

#### 2. SEAGRASS BIOACTIVE COMPOUNDS:

Marine angiosperms produce a diverse range of secondary metabolites with unique structural characteristics that have the potential to be developed into effective and potent drugs for a number of diseases. Hence it is important to study the sea grass bioactive metabolites, medical properties, and underlying mechanisms when considering their pharmacological activity. The results of the studies on this reported pharmacological activities of numerous seagrass species, their secondary metabolites, pharmacological properties, and mechanism of action. They concluded the highlights

the potency of seagrasses as a promising source of natural therapeutically products for preventing or inhibiting human diseases (Christina Mutiara *et al.*, 2022 ;).

At present, anti-virulence drugs are being considered as potential therapeutic alternatives and adjuvants to currently failing antibiotics. These drugs do not kill bacteria but inhibit virulence factors essential for establishing infection and pathogenesis through targeting non-essential metabolic pathways reducing the selective pressure to develop resistance .in this study they reported the effect of naturally isolated plant compounds on the repression of the quorum sensing (QS) system which is linked to virulence/pathogenicity in *Pseudomonas aeruginosa*. The results of the studies on this reported the trans-cinnamaldehyde (CA) and salicylic acid (SA) significantly inhibit expression of QS regulatory and virulence genes in *P. aeruginosa* PAO1 at sub-inhibitory levels without any bactericidal effect they concluded the study shows the significant inhibitory effect of natural plant-derived compounds on the repression of QS systems in *P. aeruginosa* (Syed *et al.*, 2019 ;).

## **2.3 ANTIMICROBIAL RESISTANCE:**

The development of antibiotics is considered among the most important advances of modern science. Antibiotics have saved millions of lives. However, antimicrobial resistance (AMR) threatens this progress and presents significant risks to human health. The results of the studies on this reported the increase in AMR has been driven by a diverse set of factors, including inappropriate antibiotic prescribing and sales, use of antibiotics outside of the health care sector, and genetic factors intrinsic to bacteria. Alternative approaches to address the AMR threat include new methods of antibacterial drug identification and strategies that neutralize virulence factors. They concluded the Antimicrobial resistance poses significant challenges for current clinical care. Modified use of antimicrobial agents and public health interventions, coupled with novel antimicrobial strategies, may help mitigate the effect of multidrug-resistant organisms in the future (Marston *et al.*, 2016;).

*Pseudomonas aeruginosa* is a human opportunistic pathogen known to build biofilm and is able to proliferate within the biofilm. Antibacterial substances are of natural origin, and

it is thought that their influences on the environment are important and can be used as biological control agents. In the present study, multi-drug resistant and the high yielding biofilm *Pseudomonas aeruginosa* strains have been isolated from the hospital waste. Antibiofilm and Antimicrobial activity of medicinal plants *Centella asiatica* (Thankuni), *Mentha spicata* (Mentha), *Azadirachta indica* (Neem), *Psidium guajava* (Guava) and spices *Syzygium aromaticum* (Cloves), *Cinnamomum zeylanicum* (Cinnamon) extracts were studied with modified crystal violet biofilm assay and Well diffusion assay respectively. the results of the studies recommends the potency of Methanol and ethyl acetate extract of Cinnamon and could be a target for further research to search antibiofilm bioactive compound for therapeutic uses (Jahan *et al.*,2018;).

The preliminary studies on the Evaluation of anti-quorum sensing (anti-QS) and anti-biofilm activity of ethanol, methanol, acetone and ultra-sonicated extracts of *Hypericum perforatum* L. (HP) which is a well-known wound healer, against *P. aeruginosa*. This study reveals the HP's ethanol, methanol and acetone extracts (250 µg/ml doses) inhibited LasIR signalling pathway up to 65.43%, 59.60%, 55.95% and same solvent extracts obtained with ultrasonication inhibited 71.33%, 64.47%, 57.35% respectively. Moreover, inhibition rates of RhlIR pathway were ethanol, methanol, acetone extracts (250 µg/ml doses) and ultrasonication extracts (250 µg/ml doses), compared to untreated controls. In the experiments, ethanol, methanol, acetone and ultra-sonicated extracts of HP did not inhibit biofilm formation. They concluded the HP plant is capable for blocking of las and rhl QS systems of *P. aeruginosa*. However, it was observed that ethanol, methanol and acetone extract of the plant samples did not show anti-biofilm activity against *P. aeruginosa*. This led us to thinking that biofilm formation was caused via another pathway such as IQS or PQS. Further studies with isolated active compounds of HP might give a better understanding of the effects on biofilm formation of *P. aeruginosa*.

## CHAPTER 3

### AIM AND OBJECTIVE

#### AIM:

To determine the Anti-quorum sensing potential of seagrass bioactive constituents against *Pseudomonas aeruginosa*.

#### OBJECTIVE:

- To perform Antibiofilm activity of seagrass against *Pseudomonas aeruginosa*
- To fractionate the active constituents from Seagrass *Halodule pinifolia* by column chromatography
- To identify the active fractions and isolate the quorum sensing inhibitors of *Pseudomonas aeruginosa*
- To determine the MIC Value of active compounds

## CHAPTER - 4

### 4. MATERIALS AND METHODS

#### 1. Bacterial strains and culture:

The ATCC reference number ATCC31480 was used to obtain *Pseudomonas aeruginosa* PAO1 strain culture material. It was grown in Luria Bertani broth and incubated at 37° C for 24hrs.

#### 2. Plant Material and Extraction:

*Halodule pinifolia* collected from Olaikuda region, Rameswaram region was washed with native seawater to remove epiphytes, sand particles, followed by the distilled water wash. The samples were transported to the laboratory and shade dried. Dried sample (2 gm) of *H. pinifolia* was weighed and transferred to each conical flask containing methanol and ethyl acetate. It was then incubated at 45° C for 24 hours and after filtration the samples were filtered using Whatmann No.1 filter paper and concentrated using rotary evaporator. The final yield of each solvent extracts were obtained and the concentration of 1 mg/ ml crude extracts was used for further assays.

#### 3. Determination of antimicrobial activity of *H.pinifolia* crude extract:

*Pseudomonas aeruginosa* was inoculated on the LB agar plates and incubated at 37° C for 24 hours in the inverted position and the obtained growth were stored at 4°C for future use. Antibacterial activity of *H. pinifolia* crude extracts (MeOH and EtAc) was performed by well diffusion method following the methods of CLSI guidelines (2009). The bacterial suspension was diluted to 10<sup>8</sup> CFU/mL using a sterile physiological solution and 50 µl of bacterial suspension were swabbed uniformly on the surface of LB agar plates. The agar plates were allowed to dry for five minutes and the well was created. Following. 20 µl of *H. pinifolia* crude extracts were added to the wells and oxofloxacin, levofloxacin was used as positive controls respectively. The inoculated plates were incubated at 37° C for 24 hours and the zone of inhibition (mm) was

measured. Triplicates were done and the data obtained for antimicrobial activity were expressed as the mean zone of inhibition of the extracts was interpreted.

#### **4.4. Anti-biofilm activity of crude extract**

To determine the effects of crude *H. pinifolia* extracts on *P. aeruginosa* biofilm, LB broth (3ml) along with the bacterial suspension (20 µl) were added to the six well plates in the presence and absence of crude extracts (100 µg/ ml). Oxicillin and levofloxacin (50 µg/ ml) was used as positive control. Wells containing LB broth and bacterial suspension were used as negative control, while the wells with only LB broth acted as blank respectively. The plates were incubated at 37° C for 24 hours and after incubation the planktonic cells were gently removed. All the wells were washed with sterile distilled water and the plates were dried. The surface bound cells were stained with crystal violet (0.2%) solution for 5 min followed by washing with distilled water and destaining with 20% glacial acetic acid and the biomass of biofilm was quantified by measuring the OD at 570 nm.

#### **4.5 Column fractionation of active *H. pinifolia*:**

The crude methanolic extract of *H. pinifolia* which exhibited the potential inhibition against *P. aeruginosa* biofilm was fractionated using column chromatography. For the fractionation, a vertical glass column (40 mm width x 60 mm length) made of borosilicate material was used. The column was thoroughly washed with methanol and thoroughly dried before packing. A glass rod was used to place a piece of glass wool at the bottom of the column. Slurry of silica was prepared with methanol and was poured from the top of the column around 2/3rd of the column with simultaneous draining of the solvent to help proper packing of the column. Further, methanol was added to the top of the silica slurry to 1cm height. Dried extracts of methanol was mixed with minimum quantity of silica and was poured down in to column. Different fractions were collected, air-dried and stored at -20° c for further bioassays against *P. aeruginosa*.

## 6. Anti-microbial and Anti-biofilm activity of column fractions:

The anti-microbial and anti-biofilm activity of column fractions was performed following the methodology mention in section 4.3. & 4.4. The fractions which showed the potential inhibition of *P. aeruginosa* biofilm was taken up for further analysis.

## 7. Thin layer chromatography (TLC):

The active constituents exhibited activity against *P. aeruginosa* was separated by preparative TLC using silica gel 60 F<sub>254</sub> glass plates, 20 x 20 cm as stationary phase and a mobile phase of methanol:chloroform:glacial acetic acid (1.5:3:0.5). A small spot of solution containing a sample extracts of the *H. pinifolia* is applied on a silica plate using a capillary tube, about 1.5 centimeters from the bottom edge. The individual bands from the active fractions were observed under UV light and the purity of the compound was confirmed by HPLC following the structural elucidation by <sup>1</sup>H and <sup>13</sup>C NMR.

## 8. HPLC AND NMR ANALYSIS:

HPLC purification and structural elucidation of active compounds from *H. pinifolia* were done using the compounds isolated from the active fractions of *H. pinifolia*. The purification of active compound was performed using Agilent 1260 series preparative HPLC (Agilent Technologies Inc., Chemetrix) equipped with binary pump including photodiode array detector. The separation was achieved by a reverse phase Acclaim<sup>TM</sup> 120C18 column (Phenomenex; 4.6 × 250 mm, 5 mm particle size). The mobile phase contains 1% aqueous acetic acid solution (Solvent A) and acetonitrile (Solvent B). Flow rate was adjusted to 0.8 mL/min and the column was thermostatically controlled at 280 °C, while the injection volume was kept at 10 µl. A gradient elution was performed by varying the proportion of solvent B to solvent A, which was changed from 10 to 40% B in a linear gradient for the duration of 25 min, from 40 to 60% B of 38 min and from 60 to 90% B in 50 min. The mobile phase composition back to initial condition (solvent B: Solvent A: 10: 90) in 55 min and allowed to run for another 10min, prior to the injection of another samples. The analysis per sample was carried out for 65 min.

The structural elucidation was carried out by  $^{13}\text{C}$  and  $^1\text{H}$  NMR according to the method of Mitra *et al.*, (2006).

### **9. Minimum Inhibitory Concentration (MIC) of 4-Hydroxy benzoic acid (4-HBA):**

The active compound isolated from *H. pinifolia* was identified as 4-hydroxybenzoic acid (4-HBA) and the minimum inhibitory concentration of 4-HBA was determined according to the procedure described by EUCAST Definitive documents EDef 7.1. The cells at a density of  $0.5\text{--}2.5 \times 10^5$  cfu/ mL was cultured in 96 well plates and treated with a gradient concentration of 4-HBA (1000 - 3.91  $\mu\text{g}/\text{mL}$ ) respectively. The plates were incubated at room temperature for 24 h and the absorbance was measured at 570 nm. The results were expressed in percentage of inhibition.

### **10. Anti-adherence potential of 4-HBA:**

Anti-adherence activity of 4-HBA on *P. aeruginosa* PAO1 biofilm formation was observed using Fluorescence microscopy. In brief, the glass slides coated with *P. aeruginosa* biofilm was immersed in a solution containing 4-HBA at MIC concentration (31.25  $\mu\text{g}/\text{mL}$ ) and was incubated for 6 hours at room temperature. The glass slides were then washed thrice with distilled water and stained with acridine orange (0.1%). The excess stain was removed by washing with distilled water and the bound film was observed under Fluorescence microscopy.

## CHAPTER 5

### 5. RESULTS AND DISCUSSION

#### 5.1 Effect of *H.pinifolia* crude extracts on *P.aeruginosa* Antibacterial activity of crude extract:

The antibacterial assay was carried out by the above mentioned method (4.3) to check the efficiency of compound that are prepared with 2 solvents .i.e., Methanol and Ethyl acetate .From which methanol seagrass extract showed higher zone of inhibition. This indicates the high antibacterial activity.



Figure 5.1. Antimicrobial activity of *H.pinifolia* extracts on *P.aeruginosa*

#### 5.2 Effect of *H.pinifolia* crude extracts on *P.aeruginosa* biofilm inhibition:

The leaves of *H.pinifolia* was extracted with two different solvents such as and methanol (MeOH) and ethyl acetate (EtAc) the percentage yield of the extract was achieved to be 0.79% and 1.68% respectively. The effect of crude extracts on *P. aeruginosa* biofilm inhibition was observed in 6 well microtiter plates and the results observed that the maximum inhibition of biofilm was achieved by the methanolic extract (100µg/ml) as compared to that of positive control (oxycillin50µg/ml). Furthermore, the crude MeOH

extract of *H.pinifolia* was taken up for column chromatographic fractionation and 12 different fractions were collected and assayed for *P. aeruginosa* biofilm inhibition.

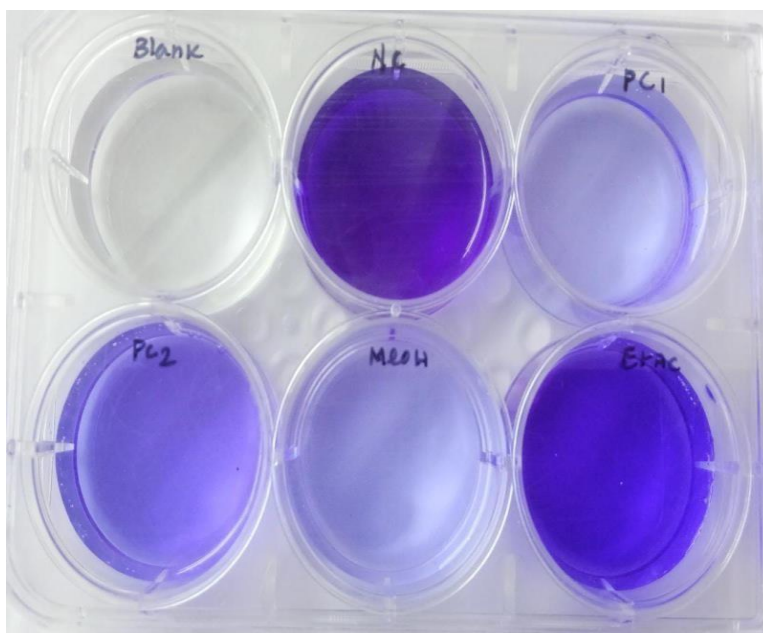


Figure 5.2 A) Antibiofilm activity of *H.pinifolia* extracts on *P.aeruginosa*

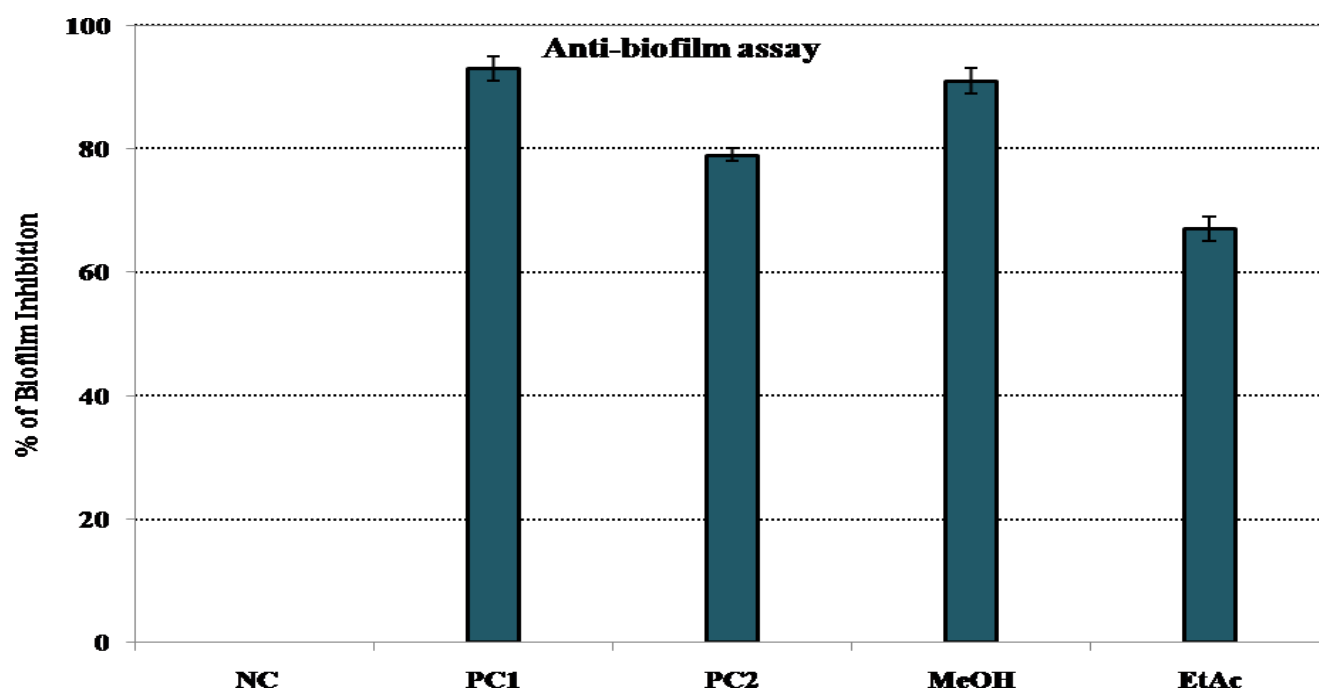
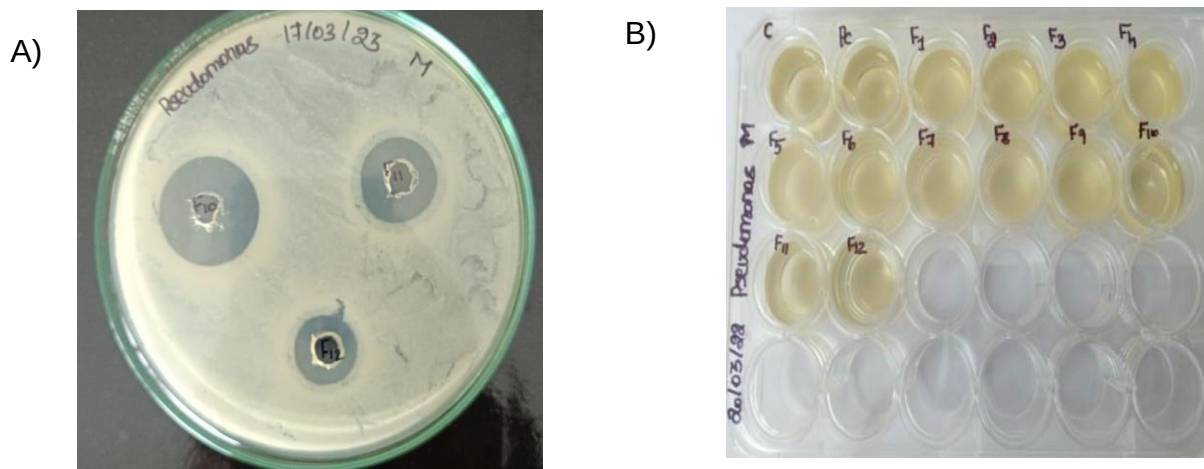


Figure 5.2 B) Percentage of Biofilm Inhibition

### 5.3 Antimicrobial activity of column fractionation:

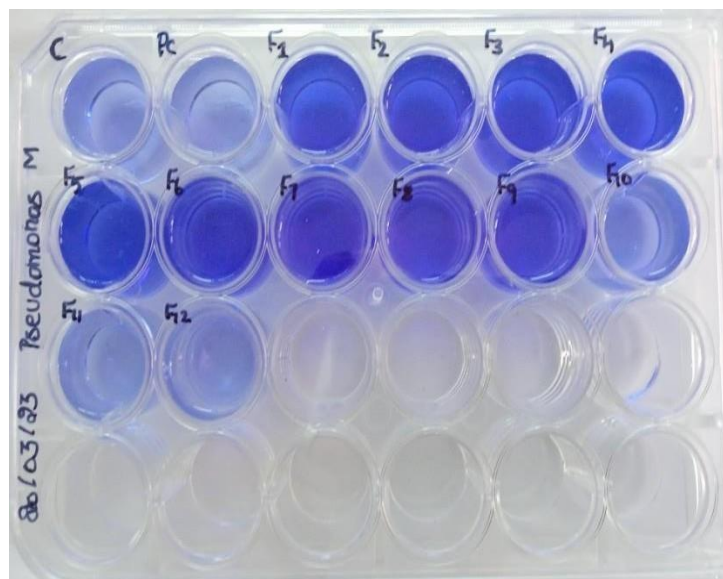
The 12 different fractions obtained from *H. pinifolia* were tested against *Pseudomonas aeruginosa* for antimicrobial activity. The results interpreted was the zone of inhibition occurred in F10, F11 & F12. Which contained the methanolic fractions of 200µg/ml, 100µg/ml & 50µg/ml respectively.



**Figure 5.3 Effect of *H. pinifolia* MeOH fractions on *P. aeruginosa* antimicrobial activity**

### 5.4 Antibiofilm activity of column fractionation:

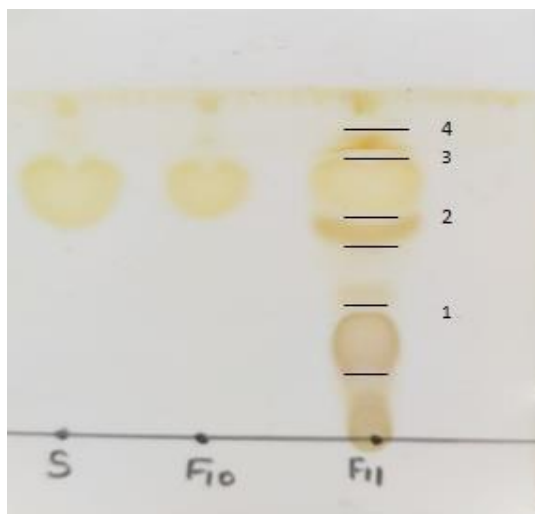
12 fractions obtained from *H. pinifolia* were tested against *Pseudomonas aeruginosa* biofilm inhibition and the result found that the fraction 10, 11 & 12 showed an effective inhibition on biofilm formation. And hence the active fraction was further taken up for the TLC.



**Figure 5.4 Effect of *H.pinifolia* MeOH fractions on *P.aeruginosa* for biofilm inhibition.**

## **5.5 THIN LAYER CHROMATOGRAPHY (TLC):**

To find the active constituents of seagrass extract *H.pinifolia* above mentioned method was carried out (4.6).By column chromatography and TLC we got different active constituents namely, 4HBA, rosamarnic acid, Vanillic acid and ethanol extract directly from the methanol extract of *H.pinifolia*. When seagrass extract Was separated on TLC plate each band produced molecular ion peak corresponding to 4HBA, rosamarnic acid, Vanillic acid and ethanolic extract was shown in table 5.1.Each plate was developed to a height of about 8cm using mobile phase methanol: chloroform: glacial acetic acid in the ratio 1.5:3:0.5. Column chromatography of methanol extract using chloroform, methanol and glacial acetic acid mobile phase show the separation of 4 different active constituents.



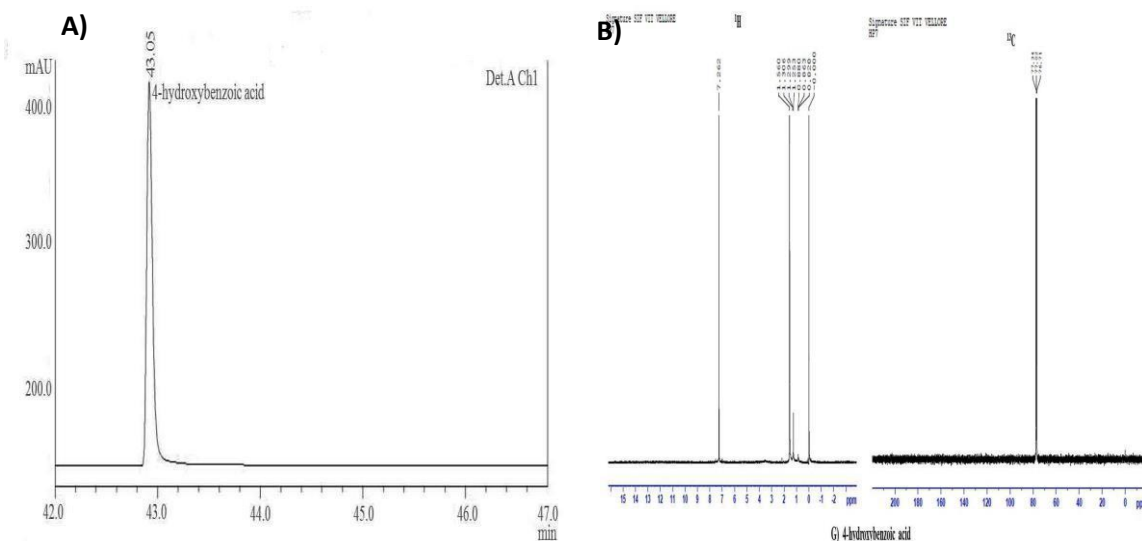
**Figure 5 .5 Thin layer chromatography of active fractions.**

**Table 5.1: R<sub>F</sub> value of active fractions:**

| Mixture         | R <sub>F</sub> value |
|-----------------|----------------------|
| Standard        | $3/3.9 = 0.76$       |
| Fraction 10     | $3.2/3.9 = 0.82$     |
| Fraction 11 (1) | $1.5/3.9 = 0.38$     |
| Fraction 11 (2) | $2.5/3.9 = 0.64$     |
| Fraction 11 (3) | $3.1/3.9 = 0.79$     |
| Fraction 11 (4) | $3.7/3.9 = 0.94$     |

## 5.6 HPLC & NMR Analysis:

4-hydroxybenzoic acid (41.73%) was found to be a lead molecule in the active fractions which was further purified by HPLC for the screening of these compounds as inhibitors of *P. aeruginosa* yeast to hypha conversion. The purity of compound obtained in the range of 97.29%–99.17%. The chromatogram for pure compound 4-HBA derived from *H.pinifolia* fractions is shown in Figure 5.6A. A typical chromatogram demonstrating the separation of 4-HBA compound was recorded at 260 nm is shown. The retention time repeatability for all the standards and that for the peak areas for two concentration levels (10 µg/mL and 20 µg/mL) was found below 1%. The structure of the each compound was further confirmed by <sup>13</sup>C and <sup>1</sup>H NMR (Figure 5.6B).



**Figure 5.6 HPLC and NMR Analysis of 4-HBA**

### 5.7 Minimum inhibitory concentration of 4-HBA on *P.aeruginosa*:

The minimum inhibitory concentration of 4-HBA was obtained in LB broth micro dilution method. The maximum biofilm inhibitory concentration of 4-HBA was found to be 31.25µg/ml.

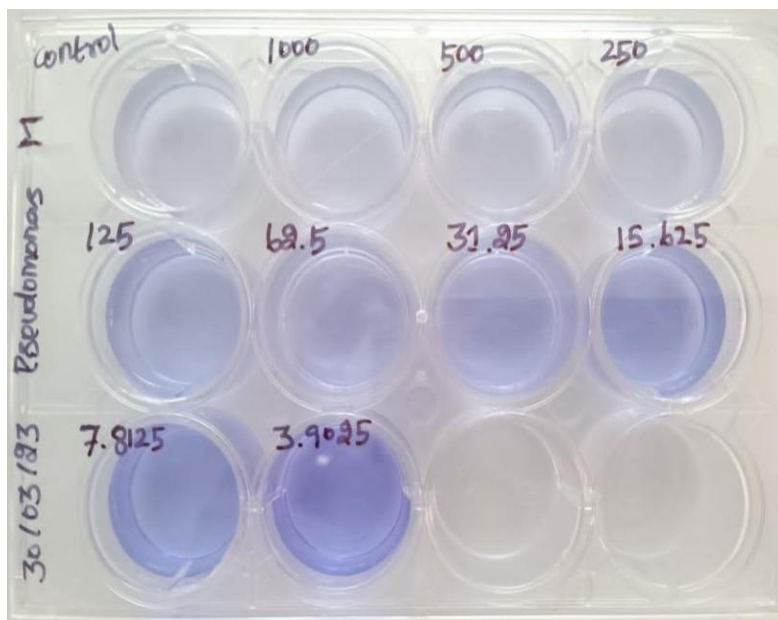
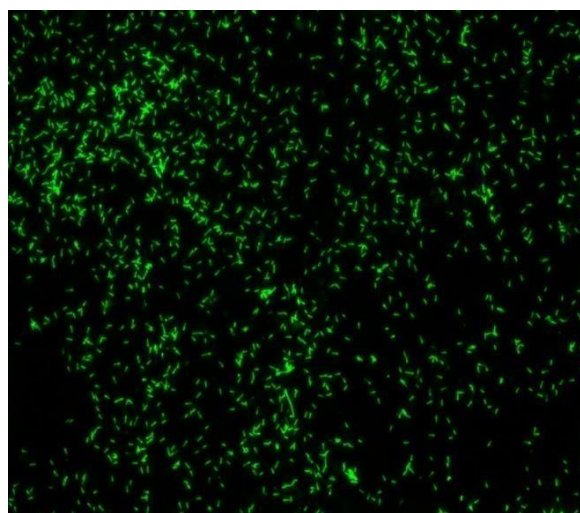


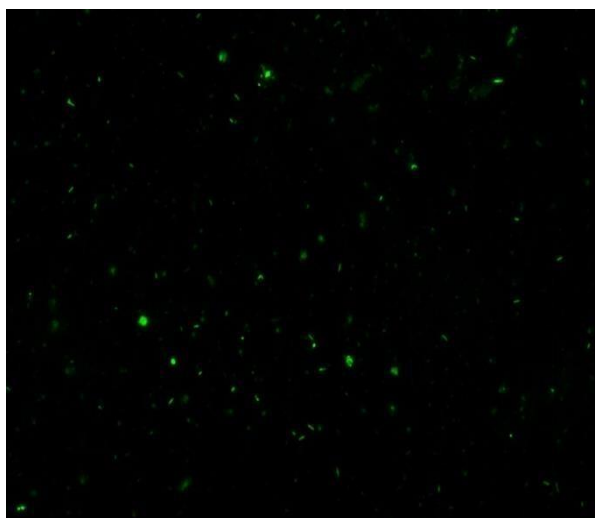
Figure 5.7 Minimum Inhibitory Concentration of 4-HBA on *P.aeruginosa* biofilm inhibition

### 5.8 Antiadherence potential of QS inhibitor against *Pseudomonas aeruginosa*:

The adherence of *P. aeruginosa* biofilm on a glass slide coated with 4-hydroxybenzoic acid at a concentration of 31.25 g/L was tested, and the results showed that 4-HBA inhibited biofilm adherence on the coated glass slide, while the slide coated with DMSO (control) promoted *P. aeruginosa* adherence. The slides observed under fluorescence microscopy.



Control



4-HBA (31.25 µg/ml)

**Figure 5.8. Anti-adherence potential of 4-HBA on *P. aeruginosa*.**

## DISCUSSION

Seagrasses have long been used as a folk medicine for a numerous remedial purposes, e.g., for the treatment of skin diseases, wounds, muscle pains, stomach problems and tranquilizers for babies (Torre-Castro *et al.*, 2004 ;). In addition, seagrass *H. pinifolia* reported to exhibit a potential antibacterial and antioxidant activities against different harmful pathogens (Kannan *et al.*, 2010;]. Though, reports available on the antimicrobial activity of seagrass *H. pinifolia*, no information has been documented yet on the effect of seagrass *H. pinifolia* extract and their derived compounds that represses QS- mediated biofilm inhibition of *P. aeruginosa*.

### **Anti-biofilm potential and characterization of active compounds from *H. pinifolia*:**

Different solvent extracts (MeOH and EtAc) of *H. pinifolia* were used for determining the *P.aeruginosa* biofilm inhibition and the preliminary study revealed that the methanolic extract of *H. pinifolia* (100µg/mL) exhibited a promising anti-biofilm activity of *P. aeruginosa* when compared to the control respectively (Fig 5.1). In most cases, *P. aeruginosa* show resistance towards commercial antibiotics (J.W. Costerton *et al.*, 1999 ;) and so new approaches have to be executed in order to prevent the biofilm-mediated infections. Que *et al.*, (2016) reported that the biofilm formation in *P. aeruginosa* occurred in two steps such as cell-surface attachment which initiates the biofilm formation by attaching on the surface and cell to cell communication in which the adhered cells interacts with the nearby cells with chemical signals. In the present study, methanolic extract of *H. pinifolia* (100 µg/mL) found to inhibit the *P. aeruginosa* biofilm adhesion on the surface of the micro titre well plates of about 91% respectively. It was reported that the methanolic extract of Halodule, Halophila and Zostera were found to be more effective against *S. aureus*, *K. pneumonia* and *P. aeruginosa* ( C. Sreenath Kumar *et al.*,2008;), while Balasubramanian *et al.*,( Y.R. Baker *et al.*, 2013;] reported that the water soluble phenolic extract *H. pinifolia* exhibited a strong antibacterial activity against *P. aeruginosa* which was in agreement with the present study.Further, the active compounds from the methanolic extract were determined by column fractionation.

Among the 12 methanolic fractions studied, fraction 11 showed potential biofilm inhibition of about 96% as compared to other fractions and hence the active compound present in the fraction 11. maximum to minimum concentrations were profiled from the fraction 11 of *H. pinifolia* was further purified by HPLC and structurally confirmed by <sup>1</sup>H and <sup>13</sup>C NMR.

### **Effect of lead compound 4-HBA on virulence factors in *P. aeruginosa***

The active compounds purified from *H. pinifolia* were tested for the growth inhibition of *P. aeruginosa* PAO1 and the result found that 4-Hydroxybenzoic acid (4-HBA) at a concentration of 100 µg/mL apparently reduced the growth of *P. aeruginosa* PAO1 respectively. Therefore, the minimum inhibitory concentration of 4-HBA was determined as 31.25 µg/mL and is considered direct evidence as a lead molecule for controlling *P. aeruginosa* infections.

### **Ecological significance**

In recent years, seagrass ecosystem is considered to be highly productive with massive ecological and socio-economic importance. It provides habitats for numerous marine organisms and is a major energy source in the coastal marine food web for herbivores. In addition, seagrass play a significant role in nutrient regeneration and shore stabilization process. It was also reported that seagrass as marine angiosperm involved in protecting the marine organisms from ocean warming (S.O Ylva *et al.*, 2018 ;). Earlier reports documented that seagrass phenolics plays a significant role in the folk medicine in Asia and Africa. The present study corroborates with the earlier reports that seagrass *H. pinifolia* produce a numerous bioactive metabolites which showed potential against *P. aeruginosa* by attenuating their biofilm matrix involved during pathogenesis. The present study is the first report in documenting the influence of seagrass *H. pinifolia* constituents 4- HBA on inhibiting the QS-mediated the biofilm inhibition. Furthermore, the metabolites from other seagrass species and their bioactive potential need to be studied in future which paves a new facet in the marine based drug discovery. Though the seagrass *H. pinifolia* plays a major ecological role in the marine environment, this

study is a proven record that it also has ability to control the harmful pathogenic microorganisms.

## CHAPTER 6

### SUMMARY AND CONCLUSION

This study concludes that seagrasses and its active constituents act a potential candidate for controlling microbial infections as antipathogenic agents in the modern phytomedicine. The bioactive compound 4-HBA from the *H. pinifolia* may consider as anti-infective agent for *P. aeruginosa* .Further, molecular docking and animal experimental studies on 4-HBA may promote it as an effective drug against *P. aeruginosa* infections. Moreover, identifying and exploring the active constituents or anti-QS agent from other seagrass species against harmful pathogens paves a way for marine based drug discovery which may efficiently inhibit virulence and demonstrate the utility against drug resistant bacteria.

## REFERENCES

1. Adonizio, Allison, Kok-Fai Kong, and Kalai Mathee. "Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* by South Florida plant extracts." *Antimicrobial agents and chemotherapy* 52, no. 1 (2008): 198-203.
2. Annapoorani, B. Kalpana, K.S. Musthafa, S.K. Pandian, A.V. Ravi, Antipathogenic potential of *Rhizophora* spp. against the quorum sensing mediated virulence factors production in drug resistant *Pseudomonas aeruginosa*, *Phytomedicine* 20 (2013) 956–963, <https://doi.org/10.1016/j.phymed.2013.04.0>
3. Athiperumalsami, Thirumalai, V. Devi Rajeswari, S. Hastha Poorna, Venkataraman Kumar, and L. Louis Jesudass. "Antioxidant activity of seagrasses and seaweeds." (2010): 251-257.
4. Baker, Y.R., Galloway, W.R., Hodgkinson, J.T. and Spring, D.R., 2013. Design and synthesis of a biotinylated chemical probe for detecting the molecular targets of an inhibitor of the production of the *Pseudomonas aeruginosa* virulence factor pyocyanin. *Molecules*, 18(10), pp.11783-11796.
5. Barbieri, R., Coppo, E., Marchese, A., Daglia, M., Sobarzo-Sánchez, E., Nabavi, S.F. and Nabavi, S.M., 2017. Phytochemicals for human disease: An update on plant-derived compounds antibacterial activity. *Microbiological research*, 196, pp.44-68.
6. Barbieri, Ramona, Erika Coppo, Anna Marchese, Maria Daglia, Eduardo Sobarzo-Sánchez, Seyed Fazel Nabavi, and Seyed Mohammad Nabavi. "Phytochemicals for human disease: An update on plant-derived compounds antibacterial activity." *Microbiological research* 196 (2017): 44-68
7. Blunt, John W., Anthony R. Carroll, Brent R. Copp, Rohan A. Davis, Robert A. Keyzers, and Michèle R. Prinsep. "Marine natural products." *Natural product reports* 35, no. 1 (2018): 8-53.
8. Chandrika, B.R., Kulkarni-Almeida, A., Katkar, K.V., Khanna, S., Ghosh, U., Keche, A., Shah, P., Srivastava, A., Korde, V., Nemmani, K.V. and Deshmukh,

- N.J., 2012. Identification of novel isocytosine derivatives as xanthine oxidase inhibitors from a set of virtual screening hits. *Bioorganic & medicinal chemistry*, 20(9), pp.2930-2939.
9. Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin-Scott, H. M. (1995). Microbial biofilms. *Annual Reviews in Microbiology*, 49, 711–745. Clinical and Laboratory Standards Institute (2006). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard. (7th ed.). Clinical and Laboratory Standards Institute document M7-A7.
  10. Cullen-Unsworth, Leanne C., Lina Mtwana Nordlund, Jessica Paddock, Susan Baker, Len J. McKenzie, and Richard KF Unsworth. "Seagrass meadows globally as a coupled social–ecological system: Implications for human wellbeing." *Marine pollution bulletin* 83, no. 2 (2014): 387-397.
  11. D. Jeyapragash, P. Subhashini, S. Raja, G. Abirami, T. Thangaradjou, Seagrass as a Potential Source of Natural Antioxidants. *Free Radicals and Antioxidants*, 2016
  12. D. Jeyapragash, P. Subhashini, S. Raja, G. Abirami, T. Thangaradjou, Seagrass as a Potential Source of Natural Antioxidants. *Free Radicals and Antioxidants*, 2016.
  13. Danaraj, J.; Saravanakumar, A.; Mariasingarayan, Y. Seagrass Metabolomics: A New Insight towards Marine Based Drug Discovery. In *Metabolomics-Methodology and Applications in Medical Sciences and Life Sciences*; IntechOpen: London, UK, 2021; ISBN 978-1-83969-083-9.
  14. Danaraj, Jeyapragash, Yosuva Mariasingarayan, Saravanakumar Ayyappan, and Vijayakumar Karuppiyah. "Seagrass *Halodule pinifolia* active constituent 4-methoxybenzoic acid (4-MBA) inhibits quorum sensing mediated virulence production of *Pseudomonas aeruginosa*." *Microbial Pathogenesis* 147 (2020): 104392.
  15. Drenkard, Eliana. "Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms." *Microbes and infection* 5, no. 13 (2003): 1213-1219.

16. Driscoll, James A., Steven L. Brody, and Marin H. Kollef. "The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections." *Drugs* 67 (2007): 351-368.
17. Duffy, J.E., Benedetti-Cecchi, L., Trinanes, J., Muller-Karger, F.E., Ambo-Rappe, R., Boström, C., Buschmann, A.H., Byrnes, J., Coles, R.G., Creed, J. and Cullen-Unsworth, L.C., 2019. Toward a coordinated global observing system for seagrasses and marine macroalgae. *Frontiers in Marine Science*, 6, p.317.
18. E. Drenkard, F.M. Ausubel, *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation, *Nature* 416 (2002) 740–743.
19. Fernández, J., Tandon, P., Mensa, J., & Garcia-Tsao, G. (2016). Antibiotic prophylaxis in cirrhosis: Good and bad. *Hepatology*, 63(6), 2019–2031.  
doi:10.1002/hep.28330
20. Fuqua C, Greenberg EP. Self perception in bacteria: quorum sensing with acylated homoserine lactones. *Current opinion in microbiology*. 1998 Apr 1;1(2):183-9.
21. Gono, Christina Mutiara Putri, Peni Ahmadi, Triana Hertiani, Eris Septiana, Masteria Yunovilsa Putra, and Giuseppina Chianese. "A comprehensive update on the bioactive compounds from seagrasses." *Marine Drugs* 20, no. 7 (2022): 406.
22. Grignon-Dubois, Micheline, Bernadette Rezzonico, and Hugues Blanchet. "Phenolic fingerprints of the Pacific seagrass *Phyllospadix torreyi*-Structural characterization and quantification of undescribed flavonoid sulfates." *Phytochemistry* 201 (2022): 113256.
23. Guzzo, Francesca, Monica Scognamiglio, Antonio Fiorentino, Elisabetta Buommino, and Brigida D'Abrosca. "Plant derived natural products against *Pseudomonas aeruginosa* and *Staphylococcus aureus*: Antibiofilm activity and molecular mechanisms." *Molecules* 25, no. 21 (2020): 5024.
24. Harwansh, R.K., Mukherjee, K., Bhadra, S., Kar, A., Bahadur, S., Mitra, A. and Mukherjee, P.K., 2014. Cytochrome P450 inhibitory potential and RP-HPLC standardization of trikatu—A Rasayana from Indian Ayurveda. *Journal of ethnopharmacology*, 153(3), pp.674-681.

25. Hejazi, A. and Falkiner, F.R., 1997. *Serratia marcescens*. *Journal of medical microbiology*, 46(11), pp.903-912.
26. J.W. Costerton, P.S. Stewart, E.P. Greenberg, Bacterial biofilms: A common cause of persistent infections, *Science* 284 (1999) 1318–1322.
27. Jafriati, J., Hatta, M., Yuniar, N., Ade, R.J., Dwiyantri, R., Sabir, M. and Muhammad, R.P., 2019. *Thalassia hemprichii* seagrass extract as antimicrobial and antioxidant potential on human: a mini review of the benefits of seagrass. *Journal of Biological Sciences*, 19(5), pp.363-371.
28. Jeyapragash, D.; Subhashini, P.; Raja, S.; Abirami, K.; Thangaradjou, T. Evaluation of in-vitro antioxidant activity of seagrasses: Signals for potential alternate source. *Free Radic. Antioxid.* 2016, 6, 77–89. [CrossRef].
29. K. Paramasivam, K. Venkataraman, C. Venkataraman, R. Rajkumar, S. Srinivasaasu, Diversity and distribution of sea grass associated macrofauna in gulf of mannar biosphere reserve, southern India, in: *Marine Faunal Diversity in India*, 2015, pp. 137–160
30. K. Vijayakumar, T. Ramanathan, Antiquorum sensing and biofilm potential of 5-Hydroxymethylfurfural against Gram positive pathogens, *Microb. Pathog.* 125 (2018) 48–50.
31. kannan Rengasamy, R.R.; Rajasekaran, A.; Micheline, G.-D.; Perumal, A. Antioxidant Activity of Seagrasses of the Mandapam Coast, India. *Pharm. Biol.* 2012, 50, 182–187. [CrossRef] [PubMed].
32. Kannan RR, Arumugam R, Anantharaman P. Antibacterial potential of three seagrasses against human pathogens. *Asian Pacific Journal of Tropical Medicine*. 2010 Nov 1;3(11):890-3.
33. Kannan, Rengasamy Ragupathi Raja, Radjasagarin Arumugam, Thirunavukarasu Thangaradjou, and Perumal Anantharaman. "Phytochemical constituents, antioxidant properties and p-coumaric acid analysis in some seagrasses." *Food research international* 54, no. 1 (2013): 1229-1236.
34. Kannan, Rengasamy Ragupathi Raja, Rajasekaran Arumugam, and Perumal Anantharaman. "Antibacterial potential of three seagrasses against human pathogens." *Asian Pacific Journal of Tropical Medicine* 3, no. 11 (2010): 890-893.

35. Kavita, Kumari, Vijay Kumar Singh, Avinash Mishra, and Bhavanath Jha. "Characterisation and anti-biofilm activity of extracellular polymeric substances from *Oceanobacillus iheyensis*." *Carbohydrate polymers* 101 (2014): 29-35.
36. Kumar, R.J., Kimble, R.M., Boots, R. and Pegg, S.P., 2004. Treatment of partial-thickness burns: a prospective, randomized trial using Transcyte™. *ANZ journal of surgery*, 74(8), pp.622-626.
37. Kupferschmidt, K. (2016). *Resistance fighters*. *Science*, 352(6287), 758– 761. doi:10.1126/science.352.6287.758
38. Kupferschmidt, Kai. "Resistance fighters." (2016): 758-761.
39. Lee, Jasmine, and Lianhui Zhang. "The hierarchy quorum sensing network in *Pseudomonas aeruginosa*." *Protein & cell* 6, no. 1 (2015): 26-41.
40. Limmer, Stefanie, Samantha Haller, Eliana Drenkard, Janice Lee, Shen Yu, Christine Kocks, Frederick M. Ausubel, and Dominique Ferrandon. "Pseudomonas aeruginosa RhIR is required to neutralize the cellular immune response in a *Drosophila melanogaster* oral infection model." *Proceedings of the National Academy of Sciences* 108, no. 42 (2011): 17378-17383.
41. M. de la Torre-Castro, P. Ronnb € € ack, Links between humans and seagrasses-an example from tropical East Africa, *Ocean Coast Manag.* 47 (2004) 361–387
42. M. Vandeputte, M. Kiendrebeogo, T. Rasamiravaka, C. Stevigny, P. Duez, S. Rajaonson, M. El Jaziri, The flavanone naringenin reduces the production of quorum sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1, *Microbiology* 157 (2011) 2120–2132
43. Marston, H. D., Dixon, D. M., Knisely, J. M., Palmore, T. N., & Fauci, A. S. (2016). *Antimicrobial Resistance*. *JAMA*, 316(11), 1193. doi:10.1001/jama.2016.11764
44. Mary, R. NANCY IMMACULATE, and N. Banu. "Screening of antibiofilm and anti-quorum sensing potential of *Vitex trifolia* in *Pseudomonas aeruginosa*." *Int J Pharm Pharm Sci* 7, no. 8 (2015): 242-5.

45. Mehari Ghebretinsae, M.; Awet Adhanom, B.; Henok Ghebremedhin, G.; Biniam Tsegay, B. A Review on Bioactive Secondary Metabolites of Seagrass of the Southern Red Sea, Eritrea. *Int. J. Adv. Res.* 2019, 7, 306–314. [CrossRef]
46. Nagabhatla, Nidhi, Ngo Tho Hung, Luong Thi Tuyen, Vu Tran Ngoc Cam, Juliet Dhanraj, Nguyen Thi Thien, and Fredric William Swierczek. "Ecosystem-based approach for planning research and capacity development for integrated coastal zone management in Southeast Asia." *APN Science Bulletin* 9, no. 1 (2019): 3-9.
47. Newmaster, A. F., Berg, K. J., Ragupathy, S., Palanisamy, M., Sambandan, K., & Newmaster, S. G. (2011). Local knowledge and conservation of seagrasses in the Tamil Nadu State of India. *Journal of Ethnobiology and Ethnomedicine*, 7, 1- 17.
48. Nile, Shivraj Hariram, et al. "Screening of ferulic acid related compounds as inhibitors of xanthine oxidase and cyclooxygenase-2 with anti-inflammatory activity." *Revista Brasileira de Farmacognosia* 26 (2016): 50-55.
49. Oncul, O., E. Ulkur, A. Acar, V. Turhan, E. Yeniz, Z. Karacaer, and F. Yildiz. "Prospective analysis of nosocomial infections in a burn care unit, Turkey." *Indian Journal of Medical Research* 130, no. 6 (2009): 758-764.
50. Pradheeba, M., Dilipan, E., Nobi, E.P., Thangaradjou, T. and Sivakumar, K., 2011. Evaluation of seagrasses for their nutritional value.
51. Pursiainen, Annie, Lari Veneranta, Sanna Kuningas, Anniina Saarinen, and Meri Kallasvu. "The more sheltered, the better—Coastal bays and lagoons are important reproduction habitats for pike in the northern Baltic Sea." *Estuarine, Coastal and Shelf Science* 259 (2021): 107477.
52. Queenan, Anne Marie, Chris M. Pillar, Jennifer Deane, Daniel F. Sahm, A. Simon Lynch, Robert K. Flamm, Janet Peterson, and Todd A. Davies. "Multidrug resistance among *Acinetobacter* spp. in the USA and activity profile of key agents: results from CAPITAL Surveillance 2010." *Diagnostic microbiology and infectious disease* 73, no. 3 (2012): 267-270.
53. R.R.R. Kannan, R. Arumugam, P. Anantharaman, Antibacterial potential of three Seagrasses against Human pathogens, *Asian Pac. J. Tropical Med.* 11 (2010) 890–893

54. R.R.R. Kannan, R. Arumugam, T. Thangaradjou, P. Anantharaman, Phytochemical constituent, antioxidant properties and p-coumaric acid analysis in some seagrasses, *Food Res. Int.* 54 (1) (2013) 1229–1236.
55. Ragupathi Raja Kannan, R.; Arumugam, R.; Anantharaman, P. Chemical Composition and Antibacterial Activity of Indian Seagrasses against Urinary Tract Pathogens. *Food Chem.* 2012, 135, 2470–2473. [CrossRef].
56. Sarabhai, Sajal, Prince Sharma, and Neena Capalash. "Ellagic acid derivatives from *Terminalia chebula* Retz. downregulate the expression of quorum sensing genes to attenuate *Pseudomonas aeruginosa* PAO1 virulence." *PLoS one* 8, no. 1 (2013): e53441.
57. Short, F.; Short, C.; Novak, A. Seagrasses. In *The Wetland Book: II: Distribution, Description and Conservation*; Springer Science: New York, NY, USA, 2016; ISBN 978-94-007-6173-5.
58. Singh VK, Mishra A, Jha B. Anti-quorum sensing and anti-biofilm activity of *Delftia tsuruhatensis* extract by attenuating the quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa*. *Frontiers in cellular and infection microbiology*. 2017 Jul 26; 7:337.
59. Thenmozhi, Ramalingam, Paramasivam Nithyanand, Janarthanam Rathna, and Shunmugiah Karutha Pandian. "Antibiofilm activity of coral-associated bacteria against different clinical M serotypes of *Streptococcus pyogenes*." *FEMS Immunology & Medical Microbiology* 57, no. 3 (2009): 284-294.
60. Vandeputte, Olivier M., Martin Kiendrebeogo, Sanda Rajaonson, Billo Diallo, Adeline Mol, Mondher El Jaziri, and Marie Baucher. "Identification of catechin as one of the flavonoids from *Combretum albiflorum* bark extract that reduces the production of quorum-sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1." *Applied and environmental microbiology* 76, no. 1 (2010): 243-253.