

**SEAGRASS DERIVED ACTIVE CONSTITUENTS AS QUORUM
SENSING INHIBITORS OF METHICILLIN RESISTANT
STAPHYLOCOCCUS AUREUS**

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Science degree in Biotechnology

by

MAHALAKSHMI R (40770027)



DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO AND CHEMICAL

SATHYABAMA

TITUTE OF SCIE CE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade "A" by NAAC

JEPPIAR NAGAR, RAJIV GANDHI SALAI, CHE NNAI – 600 119

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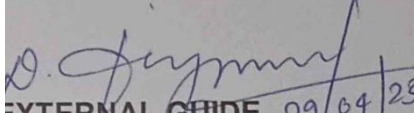
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BONAFIDE CERTIFICATE

This is to certify that this project report is the bonafide work of
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**"SEAGRASS DERIVED ACTIVE CONSTITUENTS AS QUORUM SENSING
INHIBITORS OF *METHIC/LLIN RESISTANT STAPHYLOCOCCUS
AUREUS*"** under our supervision from November 2022 to April 2023

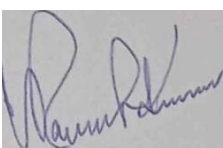


Internal guide
Dr. C .VALLI NACHIYAR



EXTERNAL GUIDE 29/04/23

Dr .D. JEYAPRAGASH



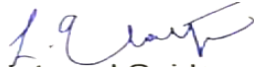
HEAD OF THE DEPARTMENT

Dr. RAMESH KUMAR

Submitted for viva voice examination held on



INTERNAL EXAMINER



EXTERNAL EXAMINER

DECLARATION

I **MAHALAKSHMI R (40770027)** hereby declare that the Project Report titled **SEAGRASS DERIVED ACTIVE CONSTITUENTS AS QUORUM SENSING INHIBITORS OF *METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS*** done by me under the guidance of **Dr.C. VALLI ACYAR** (Internal) and **Dr.D.JEYAPRAGASH** (External) at Col. Dr. JEPPI R RESEARCH PARK, CENTRE FOR OCEAN RESEARCH, in Mahatma Institute of Science and Technology is submitted in partial fulfillment of the requirements for the award of Bachelor of Science Degree in Biotechnology.

DATE:

PLACE: CHENNAI

jv)ahnJo., - 1G

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., M.Phil., PhD. 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 Internal Guide Dr.C.Vallinaciyar and my External Guide Dr.D.Jeyapragas' o their valuable guidance, suggestions and constant encouragement throughout the 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.

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* infection continues to be a difficult problem in the clinical setting. Because of their embedded tolerance and resistance to antimicrobial treatments, biofilms play a significant role in infections related with healthcare. New approaches to prevent and treat unwanted biofilms are urgently required. An appealing strategy to stop bacterial infections without resistance development is to inactivate the QS virulence mechanism with an anti-infective drug. *Halodule pinifolia*, a kind of seagrass, the potential antibacterial effects against dangerous microorganisms have been demonstrated. In this work, we looked at how QS-mediated virulence factors and biofilm formation in *S. aureus* were affected by seagrass *H. pinifolia* leaf extract and its bioactive components. The methanolic extract of *H. pinifolia* shown potential prevention of biofilm development (96%) as compared to the control, according to preliminary screening on antibiofilm activity. The possible extract was also separated in a column. From fractions active compounds were profiled, purified by HPLC, and structurally verified by NMR. The Caffeic acid showed an effective inhibition of bacterial growth and was considered as a lead molecule with minimum inhibitory concentration (MIC) of 250 µg/mL. The present findings suggest that seagrasses may act as a newer source for the marine based drug discovery and the lead compound Caffeic acid derived from *H. pinifolia* may act as anti-infective agent against *S. aureus* as it controls the QS-mediated virulence production.

Keywords : Antimicrobial resistance, Antibacterial activity, Antibiofilm activity, Seagrass *Halodule pinifolia*, MRSA, Quorum sensing

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LIST OF ABBREVIATIONS

S.NO	ABBREVIATIONS	EXPANSION
1	LB	Luria Bertani
2	MeOH	Methanol
3	<i>S.aureus</i>	<i>Staphylococcus aureus</i>
4	MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
5	CV	Crystal Violet
6	<i>H.pinifolia</i>	<i>Ha/odule Pinifolia</i>
7	HA-MRSA	Healthcare Acquired Methicillin Resistant <i>Staphylococcus aureus</i>
8	CA-MRSA	Community Associated Methicillin Resistant <i>Staphylococcus Aureus</i>
9	QS	Quorum Sensing
10	<i>H.Ovalis</i>	<i>Halophila Ova/is</i>
11	<i>C.Serrulata</i>	<i>Cymodocea Serrulata</i>
12	CAPE	Caffeic Acid Phenethyl Ester
13	EtAC	Ethyl Acetate
14	DMSO	Dimethyl Sulfoxide
15	TLC	Thin Layer Chromatography
16	HPLC	High Performance Liquid Chromatography
17	H2O2	Hydrogen Peroxide
18	MIC	Minimum Inhibitory Concentration

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CHAPTER -1

INTRODUCTION

Antimicrobial resistance (AMR) has emerged as one of the most pressing public health threats, posing serious challenges to successful prevention and treatments, of intractable diseases. The transfer of resistance genes through natural evolution, bacterial mutation and horizontal gene transfer are major causes of antibiotic resistance (**Dadgostar et al.,2019**). However, the fact that bacteria can develop resistance to antibiotics has created a situation in which antibacterial agents have lost their efficacy due to the spread and persistence of drug-resistant bacteria (**Vranakis et al.,2014**). Although the emergence of antimicrobial resistance in microorganisms is a natural phenomenon, selection for antimicrobial resistance has been exposure to

antimicrobial in medicine, agriculture and the environment strategies to reduce antimicrobial resistance by eliminating antimicrobial selective pressure alone rely on resistance imposing fitness costs, and their efficacy is not

always clear (**Holmes et al.,2016**). Impact of environment diffusion of antimicrobial resistance genes on human health and ecosystems (**Keen et al.,2011**). Antimicrobial resistance is a burden on the global economy with economic losses due to lost productivity due to disease one of the reasons antimicrobial resistance is of concern is its economic impact on physicians, patients, health care administrators, pharmaceutical, manufacture and the general public (**Jee et al.,2018**). Prevention of antimicrobial resistance in individual hosts is a primary method for preventing the spread of resistant organisms in the community, In contrast, in many other important resistant pathogens, such as penicillin - resistant *streptococcus pneumoniae*, methicillin - resistant *staphylococcus aureus* and vancomycin - resistant *enterococcus faecium* resistance is mediated by the acquisition of genes or gene fragments. In these organisms, indirect select mechanisms at the population level are responsible for the spread of resistance (**Lipsitch et al.,2002**).

Antimicrobial-resistant strains pose a growing threat to human and animal health. Resistance mechanisms to avoid the toxic effects of antimicrobials have been identified and described for all known antimicrobials currently available for human clinical use (Harbolhe et al., 2006). Increasing antimicrobial resistance in respiratory pathogens may reduce the efficacy of standard dosing regimens for many oral agents. The goal of antibacterial therapy is to maximize bactericidal activity (Craig et al., 2017). The rate at which antimicrobial resistance and virulence factors develop in the microbial world can be slowed by lowering the selective pressure applied. This can be achieved by radically reducing the global use of current and future antimicrobials (Michael et al., 2014). Methicillin-resistant *Staphylococcus aureus* (MRSA) is emerging as a risk factor for patients in the general population, especially in immune-compromised patients. Vancomycin is a commonly used selective antibacterial agent for MRSA infections, but strains of *Staphylococcus aureus* with reduced susceptibility to vancomycin occur worldwide (Docci et al., 2007). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a well-known nosocomial bacterium. More than 10% of nosocomial bloodstream infections are due to MRSA. *Staphylococci* are grape-like structures of Gram-positive cocci. People are frequently occupied by *S. aureus* on the outer surface of the skin and upper respiratory tract, especially the nasal passages (Stapleton et al., 2002). Antimicrobial resistance occurs through several different mechanisms, including altered drug targets, enzymatic drug inactivation, altered drug accessibility (Vestergaard et al., 2019). To make matters worse, many *S. aureus* strains are resistant to multiple antibiotics making them more difficult to treat. Furthermore, the global spread of multidrug-resistant *Staphylococcus aureus* strains has led the World Health Organization to classify MRSA as a "High" threat to the global population (WHO 2017) (Hardy et al., 2012). *Staphylococci* are approximately 0.8 µm in diameter arranged in "Rows of grapes" under an aerobic or anaerobic microscope. Grows best at 37 °C and pH 7.4 (Edwards et al., 2012).

The problem of increasing antimicrobial resistance is even more ominous given the very limited number of new antimicrobial in development. As soon as new antibiotics were introduced, *Staphylococci* evolved efficient mechanisms to neutralize them. This inevitably reduces the efficacy of bactericidal antibiotics to treat these. Special rules were applied when defining antimicrobial resistance in *S. aureus*. As soon as a *S. aureus* isolate is classified as *MRSA*, it is classified as Multi Drug Resistance (MOR). This is because resistance to oxacillin or cefoxilin indicates insensitivity to all categories of antibacterials - lactams *MRSA* strains have been dubbed "Super bugs" in the medical field. There is an urgent need to discover new inhibitors against this clinically important pathogen. This study evaluates the antibacterial and antibiofilm activity of caffeic acid compounds against drug-resistant *staphylococcus aureus* isolates (Wei et al.,2022). Bacteria living in a biofilm growth mode have been shown to significantly contribute to the development of drug resistance, with up to 1000 - fold reduced susceptibility to most antibiotics. Biofilms can prevent the interaction of antibiotics with bacterial cells and protect bacteria from the formation of various environment stresses, Biofilm - associated infection of the host immune systems is very difficult to treat with conventional antibiotics. Causes with Chronic infections and non - healing wounds such as *MRSA* endocarditis {Wei et al.,2022}. The use of antimicrobial agents has undoubtedly contributed to the success of the industry, from many small farmers to a few large producers. Many plants have been used primarily for their antibacterials properties derived from compounds synthesized during the plant's secondary metabolism (Olasunkanni et al.,2017). The use of plants in the fight against diseases. Regarding botanical Extracts, the antibacterial activity of crude extracts, which may replace conventional antibiotics may consider medicinal plants with anti - *MRSA* activity for the treatment of *MRSA* infection {Augustine et al.,2019}.

Today, higher plants are still used as important sources of novel compounds that are directly useful as pharmaceuticals, as model compounds for synthetic or semi-synthetic structural modifications and optimization as biochemical or/and pharmacological probes. **(Ayuningrum 2019.,)**. Specific aspects of plant based drug discovery and development are also addressed, including supply issues in commercial development and the convention on Biological Diversity **(Olivier potterat et al., 2006)**. Plant - derived extracts may reveal elegant mechanism to disrupt sessile lifestyles by manipulating the expression of stage .. specific biofilm phenotypes **(Cheung et al.,2011)**. Seagrass - derived bioactive compounds may be relevant to conservation movements related to the ecological functions of this plant. To overcome this, studies on bioactive compounds from marine microbes have been conducted from antimicrobial agents **(Sibero et al.,2019)**. A large number of marine organisms have been reported to have antibacterial activity.. It contains several potential antibacteria compounds **(Kristiana et al.,2019)**. Seagrass that produce antimicrobial compounds enable their endophytic and epiphytic bacteria to produce antimicrobial compounds that can be used against *MRSA*. Seagrass are fundamental species and provide essential ecosystem services, E.g. Oxygen production, habitat provision and nutrient recycling **(Lamb et al.,2010)**. A plant - derived antibacterial agents that inhibits the formation of microbial biofilms. Seagrass populations are suffering from global declines, largely due to increasing pressures directly related to human activity [E.g. Ocean warming, Coastline alternation, Deteriorating water quality] **(Orthe et al.,2006)**. Their ribbon- like grassy leaves **(Pushpa et al.2005.,)**. In india, Seagrass was famous as a fertilizer and for was used for heart disease treatment and for nutritional purposes. Seagrass is associated with antibacterial activity. Example are *Cymodocea serruleta*, *Halodule pinifolia* and *Halophila stipulacea* **(Bien et al.,2011)**. Seagrass *H. pinifolia* is reported to have more potential antibacterial activity against human pathogens than other seagrass species such as *C. Serrulata* and *H. Ovalis* also have antiviral, antioxidant, antiinflammatory, insecticidal, larval extermination **(Jeyapragash Danaraj et al.,2020)**.

The severity of Bacterial infection depends on the virulence of the particular strain (**Cheung et al.,2011**). The virulence of *S. aureus* relies in part on the quorum sensing (QS) systems, which allows the pathogens to rapidly adapt to its environment and gene expression in bacterial populations (**Yarwood et al.,2003**). Quorum sensing (QS) is an important mechanism of bacterial protection, enabling bacteria to transmit specialized signals in response to changes in cell density in a given environment, leading to the formation of biofilms and other virulence factors (**Zhao et al.,2013**). In this study, I studied the anti-biofilm performance of seagrass extracts at inhibitory concentrations and how they affect functional characteristics of biofilms (Such as adhesion, biofilm, quorum sensing).

CHAPTER-2

LITERATURE SURVEY

1. Dadgostar P. Antimicrobial Resistance: Implications and Costs. *Infect Drug Resist.* 2019 Dec 20, Many studies have demonstrated the disastrous financial consequences of AMR including extremely high healthcare costs due to an increase in hospital admissions and drug usage. The literature review, which included articles published after the year 2012, was performed using Scopus, PubMed and Google Scholar
2. Vranakis, I., Goniou, I., Psaroulaki, A., Sandalakis, V., Tselentis Y., Gevaert, K., & Tsiotis, G. (2014). Proteome studies of bacterial antibiotic resistance mechanisms. *Journal of Proteomics*. The methodologies used, as well as lists key proteins identified, thus providing the reader not only a summary of research already done, but also directions for future research. This article is part of a Special Issue entitled: Trends in Microbial Proteomics.
3. Holmes AH, Moore LS, Sundsfjord A, Steinbakk M, Regmi S, Karkey A, Guerin PJ, Piddock LJ. Understanding the mechanisms and drivers of antimicrobial resistance. *Lancet*. 2016 Jan 9. Strategies to reduce antimicrobial resistance by removing antimicrobial selective pressure alone rely upon resistance imparting a fitness cost) an effect not always apparent. Minimising resistance should therefore be considered comprehensively, by resistance mechanism, microorganism, antimicrobial drug, host, and context; parallel to new drug discovery, broad ranging, multidisciplinary research is needed across these five levels, interlinked across the health-care, agriculture, and environment

sectors. Intelligent, integrated approaches, mindful of potential unintended results, are needed to ensure sustained, worldwide access to effective antimicrobials.

4. Montforts, M. H., & Keen, P. L. (2011). Antimicrobial resistance *in* the environment. John Wiley & Sons. This contributed work shows how the environment plays a pivotal role *in* the development of antimicrobial resistance traits in bacteria and the distribution of resistant microbial species, resistant genetic material, and antibiotic compounds. The impact of the distribution in the environment of antimicrobial resistance genes and antibiotics on both the ecosystem and human and animal health.
5. Jee, Y., Carlson, J., Rafai, E., Musonda, K., Huong, T. T. G., Daza, P.: ... Yoon, T. (2018). Antimicrobial resistance: a threat to global health. *The Lancet Infectious Diseases*, September 18. The aim of this study was to investigate antimicrobial resistance of bacteria isolated from MA. A total of 138 extracts from almonds ($n = 63$), cashew nuts ($n = 36$). and soybeans ($n = 39$) were analyzed for *Enterobacteriaceae*.
6. Lipsitch M, Samore MH. Antimicrobial use and antimicrobial resistance: a population perspective. *Emerg Infect Dis*. 2002 Apr;8. Erratum in: *Emerg Infect Dis* 2002 May;8 Indirect, population-level mechanisms of selection account for the increase in the prevalence of resistance. These mechanisms can operate even when treatment has a modest, or even negative, effect on an individual host's colonization with resistant organisms.
7. Drug-resistant infections a threat to our economic future; 2017. Available from: www.worldbank.org. Accessed September 16,

2019. Since the use of these "miracle drugs" started over 70 years ago, loss of drug-effectiveness because of antimicrobial resistance (AMR) is increasing in both developing and developed countries. If this trend continues unchecked, the world will confront a reality where many infectious diseases have "no cure and no vaccine."

8. Galbraith H., Takur S., Zhao, S., & Hite, O. G. (2006). Genetics of antimicrobial resistance. *Animal biotechnology*. The versatility with which bacteria adapt to their environment and exchange DNA between different genera highlights the need to implement effective antimicrobial strategies and infection control programs in both human and veterinary medicine.
9. Craig, William A. "Antimicrobial resistance issues of the future." *Diagnostic microbiology and infectious disease*. With the oral vaccine against penicillin-resistant *Streptococcus pneumoniae*, this vaccine is associated only with amoxicillin and amoxicillin/clavulanate. For *Haemophilus influenzae*, several -lactams including cefixime, ceftriaxone and cefuroxime provide serum levels with the target concentrations above the MIC.
10. Michael CA Dominey-Howes D, Labbate M. The antimicrobial resistance crisis: causes, consequences, and management. *Front Public Health*. 2014 Sep. This may be accomplished by radically reducing the global use of current and prospective antimicrobials. Current management measures to legislate the use of antimicrobials and to educate the healthcare world in the issues, while useful, have not comprehensively addressed the problem of achieving an overall reduction in the human use of antimicrobials.

- 11.** Pesavento, G., Ducci, B., Comodo, N., & Nostro, A. L. (200?). Antimicrobial resistance profile of *Staphylococcus aureus* isolated from raw meat: A research for methicillin resistant *Staphylococcus aureus* (MRSA). *Food control*, 18. Transmission of MRSA from food to people can represent a serious problem only for immunocompromised people. Vancomycin is the elective antimicrobial commonly used in case of MRSA infection but *S. aureus* strains with reduced sensibility to vancomycin are emerging worldwide.
- 12.** Stapleton PD, Taylor PW. Methicillin resistance in *Staphylococcus aureus*: mechanisms and modulation. *Sci Prog.* 2002. An understanding of the mechanism of methicillin resistance has led to the discovery of accessory factors that influence the level and nature of methicillin resistance. Accessory factors, such as Fem factors, provide possible new targets, while compounds that modulate methicillin resistance such as epicatechingallate, derived from green tea, and corilagin, provide possible lead compounds for development of inhibitors.
- 13.** Vestergaard M, Frees D, Ingmer H. Antibiotic Resistance and the MRSA Problem. *Microbial Spectr.* 2019 Mar;?. the current knowledge of the wide variety of resistance mechanisms that *S. aureus* employ to withstand antibiotics. Since resistance development has been inevitable for all currently available antibiotics, new therapies are continuously under development. Besides development of new small molecules affecting cell viability, alternative approaches including anti-virulence and bacteriophage therapeutics are being investigated and may become important tools to combat staphylococcal infections in the future.
- 14.** Hardy BL, Bansal G, Hewlett KH, Arora A, Schaffer SD, Kamau E, Bennett JW, Merrell OS. Antimicrobial Activity of Clinically Isolated

Bacterial Species Against *Staphylococcus aureus*. Front Microbial. 2022 Jan 15. Defined mechanistic studies on specific isolates revealed that the inhibitory activity was retained in conditioned cell free medium (CCFM) derived from the isolates. Furthermore, CCFM obtained from *S. saprophyticus* significantly decreased mortality of *S. aureus* infected *Galleria mellonella* caterpillars. While future studies will seek to define the molecular mechanisms of the inhibitory activities, our current findings support the study of polymicrobial interactions as a strategy to understand bacterial competition and to identify novel therapeutics against *S. aureus* and other pathogens.

15. Edwards AM, Massey RC, Clarke SR. Molecular mechanisms of *Staphylococcus aureus* nasopharyngeal colonization. Mol Oral Microbial. 2012 Feb;27. The evolutionary implications of this may explain why *S. aureus* is such a capable pathogen because many of the proteins involved in colonization have also been identified as virulence factors. This review describes the diverse molecular mechanisms used by *S. aureus* to colonize the host and discusses how the pressures that have selected for these may have led to its virulence.
16. Wei G, He Y. Antibacterial and Antibiofilm Activities of Novel Cyclic Peptides against Methicillin-Resistant *Staphylococcus aureus*. Int J Mol Sci. 2022 Jul 21. Lugdunin (cyclic peptide-1), a nosocomial cyclic peptide produced by *Staphylococcus lugdunensis*, exhibits potent antimicrobial activity against MRSA. Amazingly, cyclic peptide-1 and its analogues cyclic peptide-11 and cyclic peptide-14 have the ability to disperse mature MRSA biofilms and show anti-clinical MRSA activity, including MRSA persister cells. In addition, these three cyclic peptide compounds have non-toxicity, lower hemolytic activity and lack of resistance development

- 17.** Mastoor, S.; Nazim, F.; Rizwan-ul-Hasan, S.; Ahmed, K.; Khan, S.; Ali. S.N.; Abidi, S.H. Analysis of the Antimicrobial and Anti-Biofilm Activity of Natural Compounds and Their Analogues against *Staphylococcus aureus* Isolates. *Molecules* 2022, 27, to discover novel inhibitory compounds against this clinically important pathogen. In this study, we evaluated the antimicrobial and anti-biofilm activity of 11 compounds, including phenyl propenes and phenolic aldehydes eugenol, ferulic acid, sinapic acid, salicylaldehyde, vanillin, cinnamoyl acid, and aldehydes, against drug-resistant *S. aureus* isolates.

CHAPTER-3

AIM AND OBJECTIVES

AIM:

To Analyse Seagrass derived active constituents as quorum sensing inhibitors of *Methicillin resistant staphylococcus aureus*

OBJECTIVES :

1. To perform antibiofilm activity of seagrass extracts against *MRSA*
2. To fractionate the active constituents from seagrass *Halodule pinifolia* by Column Chromatography
3. To identify the active fractions and isolate quorum sensing inhibitors of *MRSA*
4. To determine MIC and quantify the virulence transcript levels of *MRSA* upon exposure to the QS inhibitor

CHAPTER-4

MATERIALS AND METHODS

1. SAMPLE COLLECTION

1. Bacterial strain

Methicillin resistant staphylococcus aureus (ATCC strain used)was grown in Luria bertani broth and incubated at 37° C. Overnight culture was used in overall experiments.

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 EXTRACTS

MRSA 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 10⁸ 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. ANTI-BIOFILM ACTIVITY OF CRUDE EXTRACT

To determine the effects of crude *H. pinifolia* extracts on *MRSA* 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.

5. COLUMN FRACTIONATION OF ACTIVE *H. PINIFOLIA*

The crude methanolic extract of *H. pinifolia* which exhibited the potential inhibition against *MRSA* 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 *MRSA*.

4.G ANTI-MICROBIAL AND ANTI-BIOFILM ACTIVITY OF COLUMN FRACTIONS

The anti-microbial and anti-biofilm activity of column fractions was performed following the methodology. The fractions which showed the potential inhibition of *MRSA* biofilm was taken up for further analysis.

7. THIN LAYER CHROMATOGRAPHY (TLC)

The active constituents exhibited activity against *MRSA* was separated by preparative TLC using silica gel 60 F₂₅₄ 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 centimetres from the bottom edge. The individual bands from the active fractions were observed under UV light and the purity of the compound were confirmed by HPLC following the structural elucidation by ¹H and ¹³C 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 TM 120C18 column (Phenomenex; 4.6 x 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 other samples. The analysis per sample was carried out for 65 min. The structural elucidation was carried out by ^{13}C and ^1H NMR according to the method of **Mitra et al. (2006)**.

9. MINIMUM INHIBITORY CONCENTRATION (MIC) OF CAFFEIC ACID

The active compound isolated from *H. pinifolia* was identified as caffeic acid and the minimum inhibitory concentration of Caffeic acid 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 Caffeic acid (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.

1. RING BIOFILM ASSAY

To assess the effect of caffeic acid on *MRSA* biofilm growth, ring biofilm assay was performed. *MRSA* was separately grown in the glass tubes in the LB broth (3ml) and 20 μL of bacterial suspension was added and incubated at 37°C for 24 hours. After incubation, caffeic acid at IC concentration was added to the culture tubes following incubation at 37°C for 8 hours. After incubation, the culture was discarded and the tubes were washed with distilled water and dried. Following, 3ml of a 0.4% CV solution, was added to each tube to stain the adhered biomass and the tubes incubated for 15min at room temperature. The Crystal Violet dye was discarded and the tubes were again rinsed three times with distilled water and air dried. Tubes were photographed to document the amount of biofilm that was adhered to the glass surface.

4.9.2 CATALASE ASSAY

An *MRSA* overnight culture was grown in test tube using LB broth. Cultures were inoculated by adding 20µL of an overnight culture of bacteria into 3ml LB broth. 250µg concentration of Caffeic acid were added to the each tubes and the tubes were incubated at 37° temperature. 100µL of Hydrogen peroxide (H₂O₂) were added to the each tubes. Visually observed for from formation. Tubes were photographed and documented.



4.10 ANTI-ADHERENCE POTENTIAL OF CAFFEIC ACID

Anti-adherence activity of caffeic acid on *MRSA* biofilm formation was observed using Fluorescence microscopy. In brief, the glass slides coated with *MRSA* biofilm was immersed in a solution containing caffeic acid at MIC concentration (250 µg/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

RESULTS AND DISCUSSION

5.1. ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF THE EXTRACTS AGAINST MULTIDRUG-RESISTANT (MDR) *MRSA*

Antibacterial activity is an action that prevents or kills or inhibits pathogens from growing. Antibiotics are a category of compounds that can be obtained naturally from fungus, transfer by diffusion of the antimicrobial agent from the TLC. Inter-species interactions between prokaryotic and eukaryotic cells, as well as between various prokaryote taxa, are what define anti-biofilm activities. Therefore, it is expected that bacterial anti-biofilm activities will serve to naturally control bacterial numbers in an ecological niche. MOR bacteria is a category of bacteria that have developed a resistance to several classes of antibiotics and compounds. In this study, we performed the susceptibility test to confirm the resistance status of *MRSA*. The antimicrobial and anibiofilm for susceptibility test. Tested against drug resistant *MRSA* bacteria showed resistance to Caffeic acid. The seagrass associated bacteria were tested to inhibit the growth of these pathogenic MOR bacteria. Methanolic crude extract exhibited the maximum zone of inhibition than EtAc respectively (**Figure 1**).

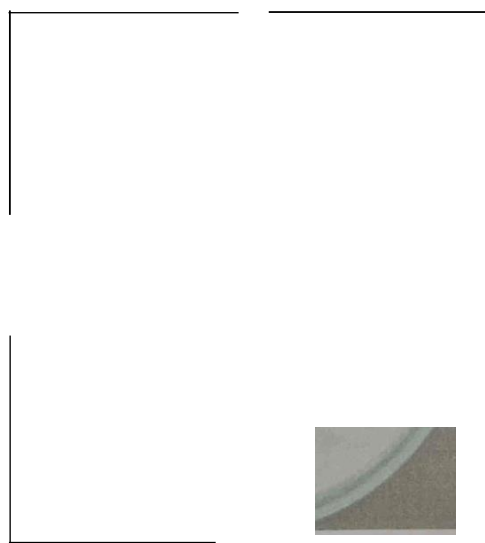


Figure 1: Anti-bacterial activity of *H. pinifolia* crude extracts

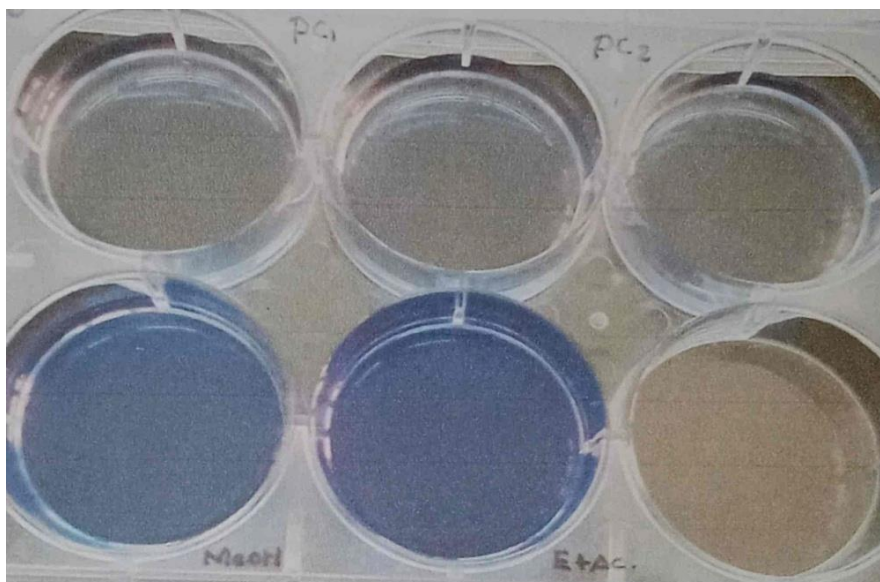
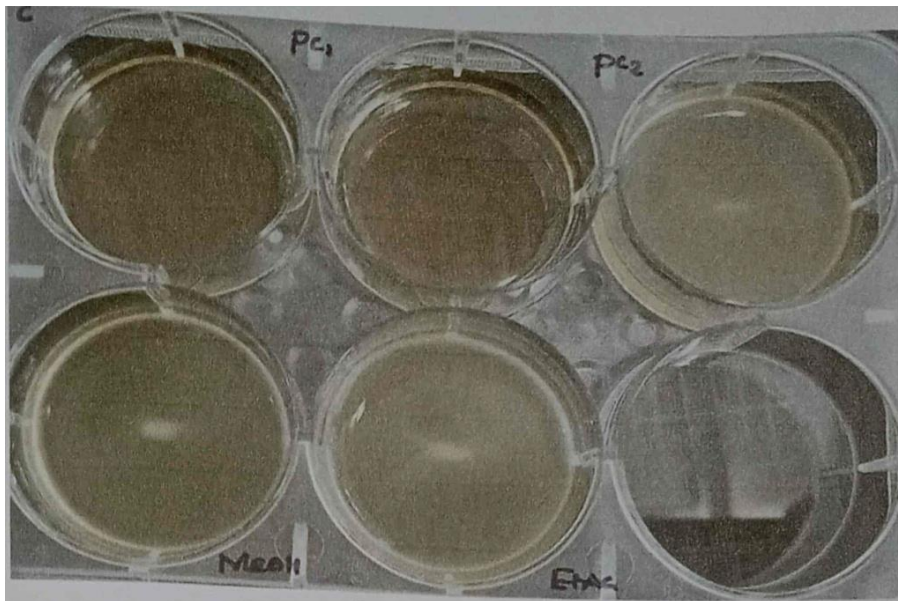


Figure 2: Anti-biofilm activities of the extracts against multi-drug resistant (MOR) *MRSA*

5.2 COLUMN FRACTIONATION OF POTENTIAL EXTRACTS

These are unique methods for separating compounds based on size, shape, and charge from mixtures. The idea of chromatography includes the use of a stationary phase, such as silica gel combined with calcium sulphate as a binder, and a mobile phase, which is the solvent of extraction. I have collected 15 fractions from the extract using methanol. I have performed antimicrobial and antibiofilm of fractionated extract.



Figure 3: Antimicrobial activity of fractionated extract from *H. pinifolia*

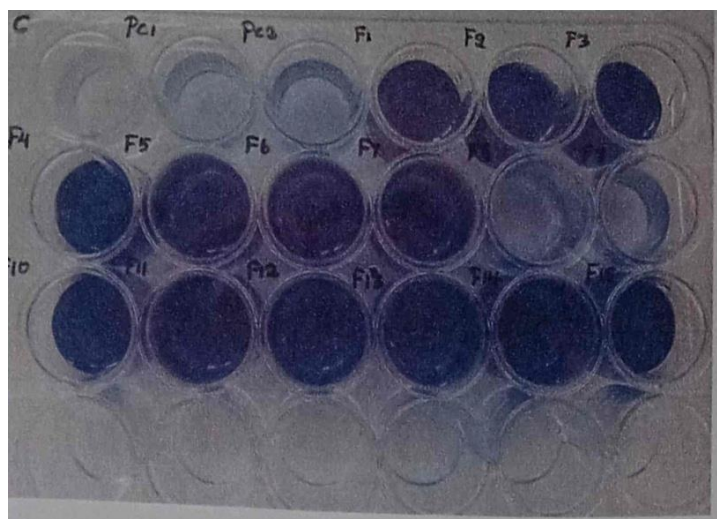


Figure 4: Antibiofilm activity of fractionated extract from *H.pinifolia*

Table 1 : Zone of inhibition in fractions

Zone of inhibition in fractions	Zone of inhibition
PC	0.6mm
Fa	0.3 mm
Fs	0.4 mm

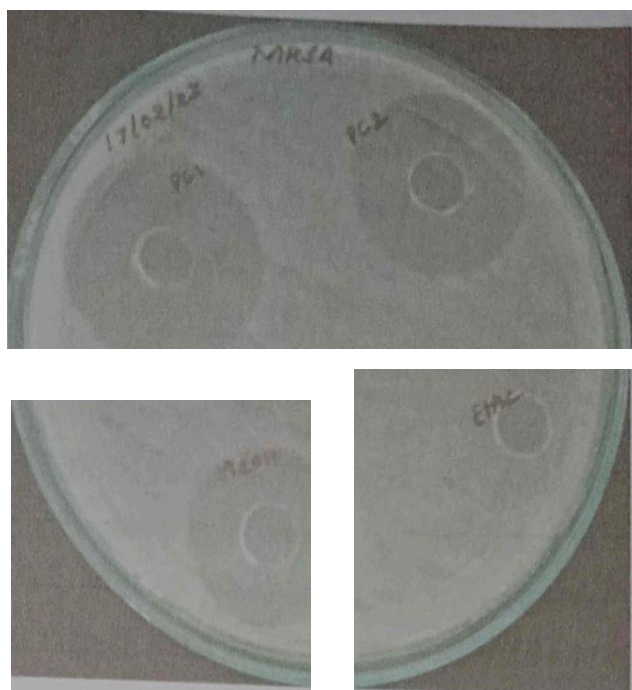


Figure 5: Inhibition zone *H. pinifolia*-associated bacteria against *Staphylococcus aureus*

Table : 2 Zone Of Inhibition

Species MRSA	Zone of inhibition
PC1	0.9 mm
PC2	0.7 mm
MeOH	0.6 mm
EtAC	Nil

5.3 THIN LAYER CHROMATOGRAPHY{TLC}

An important property of any compound, is its R_f - value (retention factor). This value is an indication of how far up a TLC-plate a compound has wandered. A high R_f - value indicates that the compound has travelled far up the plate and is less polar, while a lower R_f - value indicates that the compound has not travelled far, and is more polar. This value is easily calculated by measuring the distance the spot has wandered, and dividing this by the distance the solvent has travelled. The R_f _value is dependent on the both the compound and the solvent used for development. If we analyze two compounds and they give the same R_f - value with the same solvent system, the two compounds are most likely identical.

5.4 HPLC AND NMR ANALYSIS

The bioactive compounds present in the fraction 8 and 9 of MeOH extract was purified. The analysis per sample was carried out for 65 min. HPLC chromatograms were detected using photodiode array UV detector at three different wavelengths (260, 280 and 310) as per the absorption maxima of analyzed compounds and the structural elucidation was carried out by according to the method of **Mitra et al.,(2006)**.

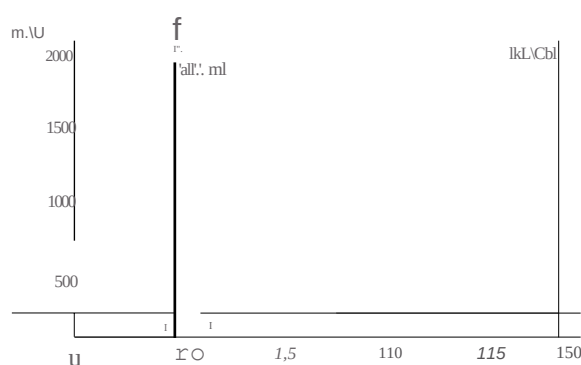


Figure 6(a): HPLC Analysis

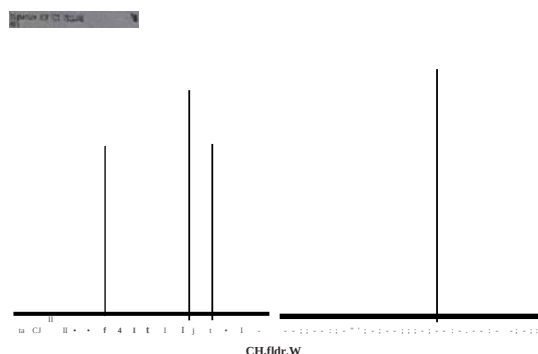


Figure 6(b): NMR Analysis

5.5 MINIMUM INHIBITORY CONCENTRATION (MIC)

A region of media where bacteria are unable to grow, due to presence of a drug that impedes their growth. The lowest antimicrobial dose necessary to prevent a microorganism's observable development following an overnight incubation. Determine the MIC Comparing the Gradient Method to other methods reveals how much simpler it is to use. The approach is quick, easy to use, and suitable for routine microbiological diagnostics since it uses a gradient of antibiotic concentrations that has been specified. We use Caffeic acid at a gradient concentration. This method usually produces lower MIC values than the reference broth microdilution method. Consequently, it typically does not enable the identification of resistant strains. With a minimum inhibitory concentration (MIC) of 250 g/ml, the caffeic acid studied in the current study had a variety of effects on *S. aureus* strains.

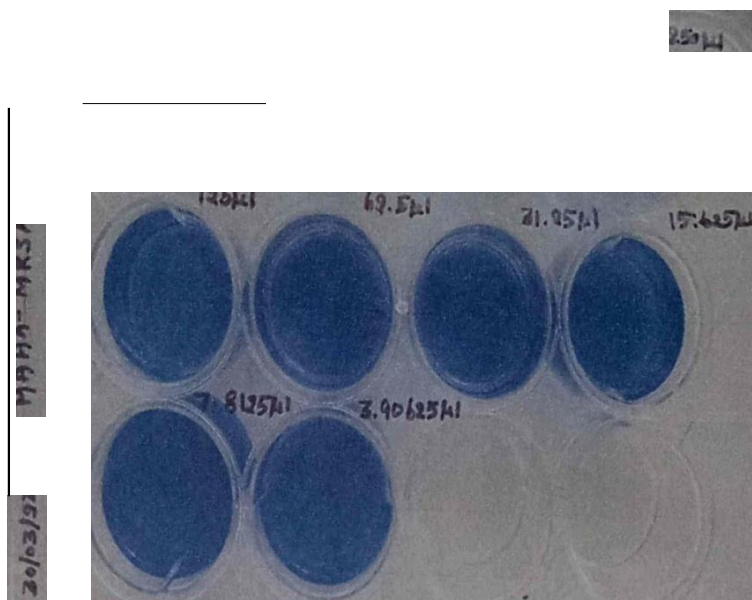
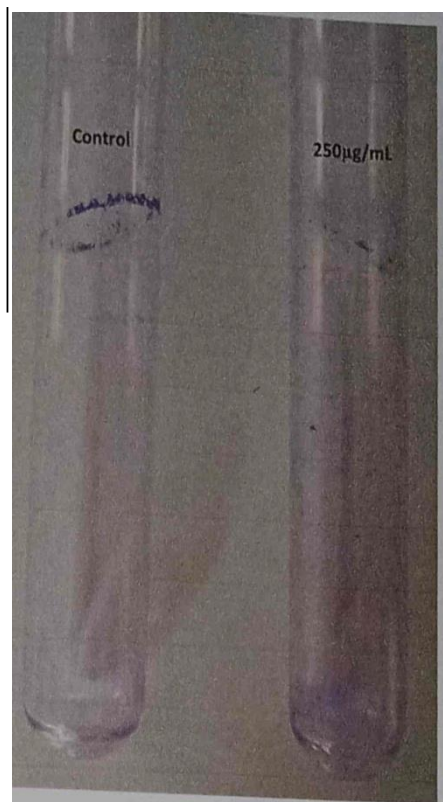


Figure 7: From the results obtained, 250µg/ml were taken as MIC for further assays

5.5.1 BIOFILM GROWTH IN GLASS TUBES

It is based on the ability of microorganisms to adhere and form a biofilm by indirect measurement of the mobility of magnetisable microbeads. IN THE PRESENCE OF BIOFILM. Testing was done on controls made of sterile medium with toner to demonstrate that beads could move about freely in the absence of sessile cells. The magnetic forces were insufficient to attract the beads to the bottom of the well or to free them from being trapped during biofilm development due to the bacterial cells' increasing adherence. Thus, the spot intensity was reduced during the time experiment until its disappearance. Caffeic acid showed reduction in ring biofilm formation on glass test tubes. The caffeic acid concentrations have shown an effective inhibition of MRSA ring biofilm as compared to the controls respectively.



(MIC 30µL-250µg/ml)

Figure 8: Ring biofilm assay of Caffeic acid

5.5.2 CATALASE ASSAY

Hydrogen peroxide's bactericidal actions are counteracted by Catalase, which presence in bacteria has been linked to pathogenicity. The catalase test makes it easier to find this enzyme in bacterial samples, that converts the toxic gas hydrogen peroxide to water and oxygen. Caffeic acid showed froth formation on glass tubes. The caffeic acid concentration have shown an froth formation of *MRSA* as compared to the control respectively.

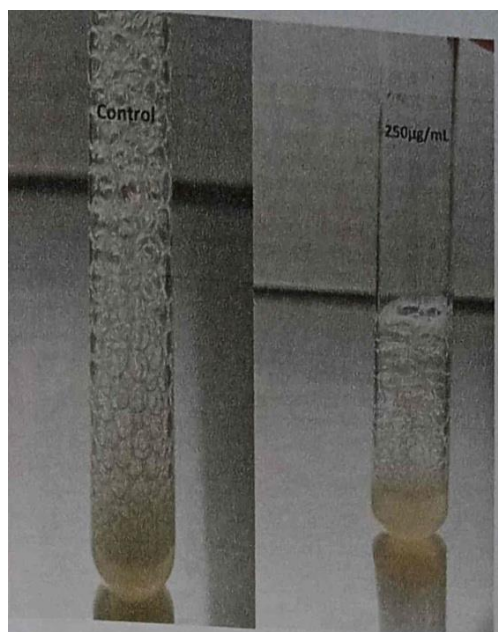


Figure 9: Catalase assay of caffeic acid against *MRSA*

5.6 ANTI ADHERENCE POTENTIAL OF QS INHIBITOR AGAINST *MRSA*

Caffeic acid at a concentration of 250µg/3ml coated on the glass slide was tested against the *MRSA* biofilm adherence and the result found that Caffeic acid inhibited the adherence of biofilm on the coated glass slide, while the slide coated with control promoted the adherence potential of *s.aureus* respectively. The slide observed under microscope where shown in **Figure 11 and 12.**

OBSERVATIONS

Control



Figure :10(a)

250 μ g/ml

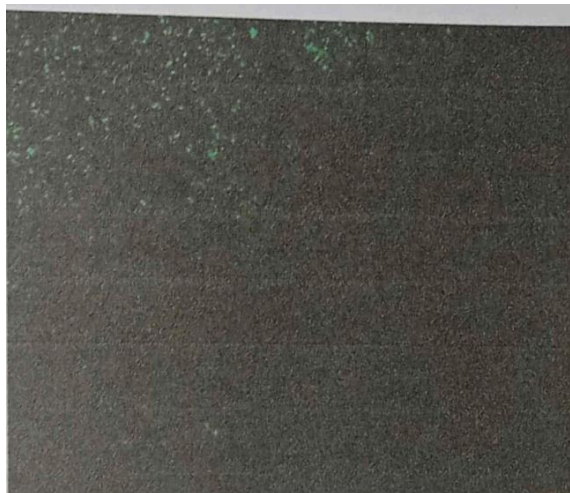


Figure :10(b)

DISCUSSION

Biofilm resistance to antimicrobial agents is a major worldwide health issue. Therefore, a successful reduction of surface colonization can be a potential strategy for the management of unwashed devices and work surface. In this instance, the use of plant-derived extracts to modulate biofilm genesis and dispersion may be a viable alternative. The present study is the first report describing the antibiofilm efficacy of non-lethal concentrations of *H. pinifolia* and methanol extracts in counteracting microbial biofilms, highlighting the possibility that the selected seagrass species act as an extracellular signal mediating their biofilm activities. *E. coli* and *S. aureus* were chosen as model systems for bacterial infections, respectively. *E. coli* biofilms are found to be the major causative agent of many intestinal infections, for recurrent urinary tract infections, and it is also responsible for indwelling medical device-related infectivity. *S. aureus* is one of the bacterial species causing disease in humans. *MRSA* remains a concerning hospitals where it can attack those most vulnerable older adults and people with weakened immune systems. *MRSA* staph infections start as swollen, painful red bumps that might resemble pimples or spider bites. The bioactive properties of the seagrass species selected in this work are well known, and have been reported in detail by several authors. However, until now attention has mainly focused on the antimicrobial activity of seagrass extracts, which, through disk diffusion assays, were investigated not in their capacity as biofilm-forming microorganisms but in their planktonic state, reported the antibacterial activity of *H. pinifolia* extracts, obtained using different solvents, against different microbial strains, recording maximum antibacterial activity by the ethanol extract of *H. pinifolia*. Instead, **Choi et al.,2008**. Reported the antimicrobial properties of *Zostera marina* methanol extract and its organic solvent fractions on three human skin pathogens (*Staphylococcus aureus*, *S. epidermidis* and *C. albicans*), **Natrah et al.,2022** Reported the antibacterial properties of methanol extracts of *MRSA* acoroides and other seagrass and seaweed species on different aquaculture pathogens

monas hydrophila, Vibrio alginolyticus (Aeromonas and others). In contrast to Parahaemolyticus V. alginolyticus, the best of our knowledge, no studies have investigated the antibacterial activity of *Halodule pinifolia* at different concentrations against bacterial strains (E. coli) and MRSA biofilms. At this end, methanol extracts obtained from seagrass species *Halodule pinifolia* leaves) were screened for their ability to modulate biofilm formation in killing cells. Methanol was used as the extractive solvent, having been previously reported as the most effective solvent to obtain high concentrations of active compounds with antibacterial activity from seagrasses, compared to other extracts. The aim of this study was to investigate the effect of seagrass extracts on cell adhesion to surfaces, the first step of biofilm formation, microtiter-based assays were performed. The results revealed excellent anti-adhesion activity for *H. pinifolia* leaf extract, bacterial coverage up to 61% at 0.01 and 10 mg/l, respectively. Therefore, 0.01 mg/l and 10 mg/l *H. pinifolia* leaf extract were chosen as the best non-biocidal concentrations for and MRSA respectively, and were used in the subsequent studies. These concentrations significantly decreased the number of adhered cells on a hydrophobic surface, more than on the hydrophilic one. Previous studies had highlighted the preference for hydrophobic surfaces, these reporting a decreased adhesion. In the present study the anti-adhesion activity of the seagrass extracts was dose-dependent, but the highest concentrations did not correspond to those with the best performance. Indeed, several studies have reported a weak activity of the compounds at low and high concentrations, and excellent activity at intermediate concentrations. The assessment of the antibiofilm effect in the adhesion phase microplate-based biofilm assays are the most suitable. This result confirms the anti-adhesion activity observed in microtiter assays. It has been well known that the antibiofilm activity of plant extracts is closely linked with the content of secondary metabolites, such as phenols and flavonoids, which represent the total amount of phenolic compounds in a plant extract.

Researcher discovered that the mechanism of action behind the performance of caffeic acid. These compounds have shown antibiofilm properties at non-lethal concentrations. Reduced biofilm formation of methicillin-sensitive *S. aureus* significantly reported that sub-lethal concentrations of plant extracts inhibit *E. coli* and *S. aureus*. Seagrasses have long been used as a folk medicine for a numerous dial purposes, e.g., for the treatment of skin diseases, wounds, muscle stomach problems and tranquilizers for babies. In addition, seagrass *H. pinifolia* reported to exhibit a potential antibacterial and antioxidant activities against different harmful pathogens. 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 OS-mediated virulence transcript levels and biofilm inhibition of *MRSA*. Hence, the present study was explored to understand the potential of *H. pinifolia* on regulating the OS-mediated virulence mechanism of Methicillin-resistant *Staphylococcus aureus*.

5.7 ANTI-BIOFILM POTENTIAL AND CHARACTERIZATION OF ACTIVE COMPOUNDS FROM *H. PINIFOLIA*

Methanol (MeOH) solvent extracts of *H. pinifolia* were used for determining the Methicillin-resistant *Staphylococcus aureus* biofilm inhibition and the preliminary study revealed that the methanolic extract of *H. pinifolia* (250 µg/ml) exhibited a promising anti-biofilm activity of *S. aureus* when compared to the control respectively. In most cases, *S. aureus* show resistance towards commercial antibiotics 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 *S. aureus* 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 interact with the nearby cells with chemical signals. In the present study, methanolic extract of *H. pinifolia* (250 µg/mL) found to inhibit the *S. aureus*

adhesion on the surface of the microtiter wells. It was reported that the methanolic extract of *Halophila* was found to be more effective against *S. aureus*. Balasubramanian et al. (2018) reported that the water soluble phenolic extract of *H. pinifolia* exhibited antibacterial activity against *S. aureus*. Which was in agreement with the present study. Further, the active compounds from the methanolic extract were determined by column fractionation. The two methanolic fractions showed potential biofilm inhibition of about 96% as compared to other fractions and hence the active compounds present in the fraction 8 and 9. In the active compounds caffeic acid (59.18%) at maximum concentration were profiled from the fraction 8 and 9 of *H. pinifolia* was further purified by HPLC and structurally confirmed by NMR.

5.8 EFFECT OF CAFFEIC ACID ON BIOFILM MATRIX COMPONENTS AND QUORUM-REGULATORY MECHANISM

The biofilm formation which in turn produces antibiotic resistance mechanism by 3D complex biofilm architecture formation regulated by QS system. The biofilm formation; thereby decrease in antibiotic resistance mechanism will occur. The lead compound caffeic acid at MIC concentration inhibited the production to 83% in *S. aureus*. Similar findings reported with chest honey and its extract inhibited the biofilm formation up to 61% of about 69% and *H. pinifolia* extracts of about 73% respectively. The result found that interference of caffeic acid with quorum sensing repressed the transcript levels and down regulated the virulence production, thereby prevents the biofilm formation in *S. aureus*. The study suggests that Caffeic acid might have directly impacted the biofilm formation by *S. aureus* by disturbing the AHL mediated QS-system. Amaral and Molnar reported the possible role of various secondary and natural compounds as inhibitors of QS mechanism in enteric microorganisms.

CHAPTER-6

SUMMARY AND CONCLUSION

The results of the study suggest that seagrass components might be used as an antipathogenic agent. These and their active compounds prevent microbial diseases. As it regulates the signaling in current Phytomedicine to produce the bioactive component from caffeoyl-OH-mediated virulence, caffeoyl-OH may be marketed as an anti-infective agent for *S. aureus* and other harmful microbes. Furthermore, Caffeic acid may be marketed as a successful treatment for *S. aureus* infections as a result of molecular docking and animal experimentation research. Moreover, finding and studying the anti-QS agents or active components from different seagrass species against hazardous diseases opens the door for the development of marine-based drugs that may effectively decrease virulence and show their value against drug-resistant bacteria. *H. pinifolia* leaf extract proved to be the most promising extract among those tested. Indeed, the selected non-lethal concentrations of *H. pinifolia* leaf extract were found to exert an antibiofilm effect on MRSA biofilm in the first phase of biofilm genesis, opening up the possibility of developing preventive strategies to hinder the adhesion of microbial cells to surfaces. The leaf extract also affected the dispersion and maturation steps in Methicillin resistant *staphylococcus aureus* respectively, suggesting an important role in cell signaling processes. In order to identify the active components and better understand the mechanisms of action of this seagrass species, the leaf extract is now being researched. Determining the precise medication/plant ratio at which the interaction between the plant extract and the antimicrobial agent is at its most powerful, the mechanism of, which is fundamental to development of pharmacological agents to treat diseases by *S. aureus* using medicinal plants. Our results revealed that the use of plant extracts *Halodule pinifolia* could be useful in fighting emerging drug-resistance problem and in vivo experiments are needed to confirm Methicillin resistant *staphylococcus aureus* protection using these Caffeic acid.

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