

COMBINATORIAL APPROACH OF SEAGRASS EXTRACTS WITH ANTIBIOTICS AGAINST *CANDIDA ALBICANS*

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

by

GAYATHRI KUMARI. R (40770013)



DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO AND CHEMICAL ENGINEERING

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

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **GAYATHRI KUMARI. R (40770013)** who carried out the project "**COMBINATORIAL APPROACH OF SEAGRASS EXTRACTS WITH ANTIBIOTICS AGAINST CANDIDA ALBICANS**" under my supervision from September 2023 to March 2023.

Internal Guide

Dr. S. JAYASHREE

EXTERNAL GUIDE 29/04/23

Dr. D. JEYAPRAGASH

Head of the Department

Dr. V. RAMESH KUMAR

Submitted for Viva-voce Examination held on 12/5/23_____

Internal Examiner

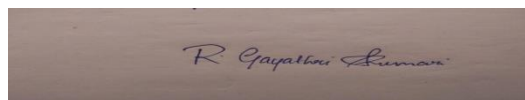
External Examiner

DECLARATION

I, **Gayathri Kumari. R**, hereby declare that the Project Report entitled **“COMBINATORIAL APPROACH OF SEAGRASS EXTRACTS WITH ANTIBIOTICS AGAINST *CANDIDA ALBICANS*”** done by me under the guidance of **Dr. S. Jayashree** (Internal) at **DEPARTMENT OF BIOTECHNOLOGY**, Sathyabama Institute of Science and Technology, Chennai and **Dr. D. JEYAPRAGASH** (External) **CENTRE FOR OCEAN RESEARCH**, Sathyabama Institute of Science and Technology, is submitted in partial fulfilment of the requirements for the award of Bachelor of Science degree in Biotechnology.

DATE: 12/5/23

PLACE: CHENNAI

A rectangular box containing a handwritten signature in cursive script, which reads "R. Gayathri Kumari".

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to **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., Ph.D.**, Head of the 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 Guide **Dr. S. JAYASHREE, Ph.D.**, Department of Biotechnology for her valuable guidance, suggestions and constant encouragement for the successful completion of my project work.

I would like to express my deepest appreciation to **Dr. D. JEYAPRAGASH, Ph.D.**, Scientist B, Centre for Ocean Research, Research and Development who supported me throughout my project work with his valuable guidance, brilliance in practical suggestions and constant encouragement 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.

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

Antibiotics are widely used to treat the infectious diseases caused by pathogenic organisms. On a larger scale, long term or continuous use of antibiotics in various fields had led to evolution of multidrug resistant organism. The search for new drugs against multidrug resistant pathogens is a major challenge and *C. albicans* is among the third opportunistic fungal pathogen which constantly develops the resistance against different antibiotics. Development of antimicrobial resistance has shifted the focus of research towards synergistic effect. Hence, the present study aims to determine the synergistic antifungal effect of ethyl acetate extracts of *H. pinifolia* in combination with Flucanazole, Itracanazole, Amphotericin B, Levofloxacin and Ofloxacin. The bioactive potential of *H. pinifolia* crude extracts (Methanol, Chloroform and Ethyl acetate) was assessed against *Candida albicans* and the results were interpreted with zone of inhibition and biofilm degradation. Among the crude extracts of *H. pinifolia*, ethyl acetate extract showed maximum zone of inhibition (8mm) as compared to the positive control respectively. Column fractionation of ethyl acetate was performed and the obtained eleven fractions (F1 – F11) were tested for antifungal and antibiofilm assays. It was found that fraction 4 and 8 exhibited the maximum inhibition of *C. albicans* growth and biofilm inhibition which paved a way to isolate the active constituent of those active fractions. Thin Layer Chromatography (TLC), High performance Liquid Chromatography (HPLC), and Nuclear Magnetic Resonance (NMR) analysis confirmed the presence of Rosmarinic acid and Vanillic acid as active compound which was taken up for MIC determination. MIC values of Rosmarinic acid (62.5 mg/ml) and Vanillic acid (125 mg/ml) was determined and the synergistic effect with active compounds and available antibiotics (except Amphotericin B), were performed. with different concentrations in combination with both the bioactive compounds (RA and VA). Synergistic and antagonistic effects were analysed and reported. Thus, we propose that extracts of the *Halodule pinifolia* could be of relevance in combination therapy and as a source of resistance modifying agents that could be useful as treatment options for persistent antifungal resistance.

Keywords: antifungal resistance, *Halodule pinifolia*, *Candida albicans*, Rosmarinic acid, Vanillic acid, synergistic effect.

TABLE OF CONTENTS

S.NO.	CONTENTS	PAGE NO.
	List of Figures	iv
	List of Tables	v
	List of Abbreviations	vi
1	INTRODUCTION	6
	1.1 Antimicrobial resistance	6
	1.2 Fungal species	6
	1.3 Mechanism of antimicrobial resistance	7
	1.4 Antifungal agents	7
	1.5 <i>Candida</i> species and pathogenicity	7
	1.6 Mechanism of pathogenicity in <i>Candida albicans</i>	8
	1.7 Biofilms	8
	1.8 Plant based natural treatment	9
	1.9 Secondary metabolites	9
	1.10 Marine based medicinal plants	10
	1.11 Seagrass	10
	1.12 Seagrass active metabolites	11
	1.13 <i>Halodule pinifolia</i>	11
	1.14 Combinatorial approach	12

	1.15 Combination of plant extract with antibiotics	13
2	LITERATURE SURVEY	14
	2.1. Evolution of antimicrobial resistant organisms	14
	2.2. Mechanism of antimicrobial resistance	14
	2.3. <i>Mechanism of antimicrobial resistance in Candida albicans</i>	15
	2.4. Natural source of treatment	15
	2.5. Seagrass <i>Halodule pinifolia</i>	16
	2.6 Metabolites of <i>Halodule pinifolia</i>	16
	2.7 Antimicrobial activity of plant extracts on <i>C. albicans</i>	17
	2.8 Approach to novel medicine	17
	2.9 Combinatorial approach	18
3	AIM AND OBJECTIVES	19
4	MATERIALS AND METHODS	20
	4.1. Sample collection	20
	4.2. Solvent Extraction	20
	4.3. Bacterial Strains and Inoculum preparations	21
	4.4. Anti-fungal activity of <i>H. pinifolia</i> crude extracts	22
	4.5. Anti-biofilm activity of <i>H. pinifolia</i> crude extracts	22
	4.6. Column fractionation of <i>H. pinifolia</i> Extracts	23
	4.7. Anti-fungal and Anti-biofilm activity of column fractions	23
	4.8. Thin layer chromatography (TLC)	23
	4.9. HPLC and NMR ANALYSIS	24

	4.10. Minimum Inhibitory Concentration (MIC) of Rosmarinic acid and Vanillic acid	24
	4.11. Synergistic effect	25
5	RESULTS AND DISCUSSION	27
	Results	27
	5.1 Antifungal and Antibiofilm activity of <i>H. pinifolia</i> crude extracts.	27
	5.2 Column fractionation and determination of active fractionations.	28
	5.3 Thin Layer Chromatography, High performance Liquid Chromatography and NMR analysis.	30
	5.4 Minimum Inhibitory Concentration (MIC)	33
	5.5 Synergistic effect	34
	Discussion	35
6	SUMMARY AND CONCLUSION	40
7	REFERENCES	42

List of Figures:

FIGURE NO.	FIGURE NAME	PAGE NO
4.1	<i>Halodule pinifolia</i> .	20
4.2	Solvent extraction of seagrass chloroform, ethyl acetate, methanol.	21
4.3	<i>Candida albicans</i> strain.	21
4.4	Broth culture of <i>Candida albicans</i> .	22
5.1.1	Antifungal activity of <i>H. pinifolia</i> crude extracts.	27
5.1.2	Antibiofilm activity of <i>H. pinifolia</i> crude extracts.	28
5.2.1	Column fractionation of ethyl acetate crude extract.	29
5.2.2	Antifungal activity of F1-F11.	29
5.2.3	Antibiofilm activity of fractions (F1-F11) obtained along with Ofloxacin as positive control.	30
5.3.1	Thin Layer Chromatography.	30
5.3.2	HPLC analysis of Rosmarinic acid.	31
5.3.3	HPLC analysis of Vanillic acid.	31
5.3.4	NMR analysis of Rosmarinic acid.	32
5.3.5	NMR analysis of Vanillic acid.	32
5.4.2	Minimum Inhibitory Concentration (MIC) of Vanillic acid.	33
5.5	Synergistic effect of different combinations of Rosmarinic acid and Vanillic acid with antibiotics against <i>candida albicans</i> .	34

LIST OF TABLES

TABLE NO.	CONTENT	PAGE NO.
1	Different combinations of bioactive compounds and antibiotics.	26
2	Zone of inhibition of <i>H. pinifolia</i> crude extract	28

LIST OF ABBREVIATIONS

ABBREVIATIONS	FULL FORM
AMR	Antimicrobial resistance
TLC	Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
NMR	Nuclear Magnetic Resonance
MIC	Minimum Inhibitory Concentration
RA	Rosmarinic acid
VA	Vanillic acid
HIV	Human immunodeficiency virus
AIDS	Acquired Immune Deficiency Syndrome
EMA	European Medicines Agency
FDA	Food and Drug Administration
WHO	World Health Organization
LOH	Loss Of Heterozygosity
MeOH	Methanol
Chl	chloroform
EtAc	ethyl acetate
µg/ mL	microgram per millilitre
mg/mL	milligram per millilitre
µl	Microlitre

Mm	Millimetre
SSC	Suspension Cultured Cells
GC-MS	Gas Chromatography - Mass Spectrometry
KEGG	Kyoto Encyclopaedia of Genes and Genomes
OD	Optical Density

CHAPTER-1: INTRODUCTION

1.2 Antimicrobial resistance

Antimicrobial resistance has been a major concern worldwide due to multidrug resistant pathogens causing severe diseases which requires novel drugs to be developed for an effective treatment (Lozano and Torres 2017). Infections caused by these multidrug resistance organisms has led to severe illness which lacks proper treatment. These healthcare related infections have gained high mortality rates in recent years and the rise has been directly correlated with antimicrobial resistance which makes the antibiotics restrictive to be used against the infections caused by multidrug resistant organisms (Tacconelli *et al.*, 2014). Drug resistance mostly affects the health of million people worldwide in both developed and developing nations which leads to excessive financial crisis among the societies (Ventola 2015). High mortality rates also lead to economic burden as in the United States, it was estimated over 20 billion dollars were spent per year for the treatment (Cosgrove 2006; DiazGranados *et al.*, 2005). Centre for disease control and prevention in US estimated that about 23000 people die annually due to antimicrobial resistant organisms (CDCP 2013).

1.2 Fungal species

Fungi among the pathogenic microorganisms are a large group of about 250000 species out of which 300 species has been reported to have clinically pathogenic effect on humans (Guarro *et al.*, 1997). As per the reports, 90% of fungal infections are caused by *Aspergillus*, *Candida*, *Cladosporium*, *Epidermophyton*, *Microsporum* and *Trichophyton* spp (Dasgupta 1998; Pathak 1998) and the major systemic mycoses by *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Fusarium* sp., *Histoplasma capsulatum* and *Pneumocystis carinii*. Among these species, *Candida albicans* has been the most isolated culture with frequency of ten-fold increase in candidiasis over recent years. (Beck-Sague and Jarvis 1993).

3. Mechanism of Antifungal resistance

Theoretically, fungal cells can produce enzymes for defence mechanisms which can degrade or inactivate the antimycotic agents to prevent their actions. Unlike bacterial transfer of resistant genes horizontally, fungal pathogens have been reported with the mechanism of vertical transmission of mutations in fungal genome. There is no report of plasmid-mediated resistance in fungi (White 1997; Kurtz 1998). They acquire resistance by synthesis of enzymes that will degrade the active parts of antibiotics, drug efflux, modifying antibiotic binding sites, and biofilm formation (Munita 2016).

4. Antifungal agents

Among the usage of different antibiotics, antifungal drugs are also the significant group of drugs to treat the mycotic diseases, to which the pathogens have gained the resistance. The need for effective drugs against the resistant pathogenic fungi has major challenge in health care sectors especially with the opportunistic infections in immunosuppressed host cells (Bevan *et al.*, 1995). These pathogenic fungi not just target the immunosuppressed patients with weaker immune system by AIDS, aggressive cancer chemotherapy, or drugs aimed at foiling rejection of transplanted organs but also when the pathogenic fungi are resistant to antifungal agents. (McGinnis and Rinaldi 1991; Odds 1993). Antifungal agents have been used widely in agriculture, veterinary medicine and in human medicines (Misato and Yamaguchi 1977; Vandamme 1984). There are five major classes of antifungal drugs currently in use to treat the mycotic infections. These are the polyene antibiotics, the azole derivatives, the allylamines and thiocarbamates, the morpholines and the nucleoside analogues (Georgopapadakou and Walsh 1996).

5. *Candida* species and pathogenicity

The vulnerable pathogens among the *Candida* genus along with the yeasts are the *Candida albicans*, *Cryptococcus neoformans*, and the mold *Aspergillus fumigatus*.

Amongst the pathogenic *Candida* species *C. albicans* followed by *C. tropicalis* (Cannon et al. 1995) are the most virulent and common fungal human pathogen in humans to cause significant rates of mortality and morbidity (Fraser et al., 1997; Pittet et al., 1995). Clinical infections caused by these fungi ranges from superficial mucosal infections such as oropharyngeal candidiasis, to systemic infections causing life threatening effects by entering the bloodstreams such as disseminated candidiasis, *cryptococcal meningitis*, and invasive *aspergillosis* (Ghannoum and Rice 1999). *Candida* species has been reported commensal to about 75% of healthy individuals (Ghannoum et al., 2010) which are opportunistic to compromised patients with immune system (Scully et al., 1994). Candidiasis can be treated using antifungal drugs including polyene derivatives like amphotericin B, nystatin (mycostatin), and the azoles such as According to the clinical reports, it was estimated that about 5-10% of the patients with AIDS have oropharyngeal infections with *Candida* which has been difficult to treat with available drugs such as flucanazole. (Ben-Yaacov et al., 1995; Phoebe et al., 2001).

6. Mechanism of pathogenicity in *Candida albicans*

Candida albicans usually colonizes gastrointestinal tract and are found on mucosal surfaces establishing themselves part of normal microflora (Millsop and Fazel 2016). But they become opportunistic with immunocompromised state of host cell and environmental conditions switching from commensal to pathogenic due to morphological variations from yeast to filamentous hyphal forms. (Berman and Sudbery 2002). They attach to the abiotic surfaces due to adhesion of yeast cells to substratum and subsequent hyphae formation (Miller and Johnson 2002; Daniels et al., 2006). In most cases, *C. albicans* exhibit biofilms on host cells and are the causes of most of the biofilm related infections (Douglas 2002).

7. Biofilms

Biofilms are the aggregation of microbial population of single or different species attached over biotic or abiotic surfaces by a self-produced extracellular polymeric matrix (Sharma et al., 2019). Interaction between these biofilms is a precursor to increased drug tolerance, immune evasion, and virulence, which results in

increased mortality rates (Todd and Peters 2019). According to National institutes of Health (NIH), it is recommended that about 60% of human infections are caused due to biofilm formation on human mucosa (Costerton *et al.*, 1999). The initial attachment and subsequent maturation of these biofilms has underlying mechanisms of resistance which includes restricted penetration, heterogeneous metabolic activity, and expression of certain genes conferring enhanced resistance to antibiotics. This has led the scientific communities to identify such compounds with potential antibiofilm activities imperative to combat pathogenesis of resistant pathogens (Papenfert and Bassier 2016), with cost effective and potent antimicrobial agents as alternative treatments to treat infections caused by these multidrug resistant strains (Aslam *et al.*, 2018).

8. Plant based natured treatment

Since overuse of antibiotics also has some adverse effects, medicinal plants are considered for the extraction of biologically active compounds that has antimicrobial properties in herbal medicines. Worldwide medicinal plants are used for antibacterial, antiviral and antifungal activities (Caceres *et al.*, 1990). Estimation of medicinal activities of plant extracts to ensure efficacy and safety of dosage formulations, efficacy and pharmacokinetic profile of the new drug (Tanaka *et al.*, 2006). They have been used for curing infections related to urinary tract, gastrointestinal tract, vaginitis, and skin infections. (Caceres *et al.*, 1990).

9. Secondary metabolites

Flavones, flavonoids and flavanols are phenolic structures with one carbonyl group. They are synthesized by plants in response to microbial infection (Dixon *et al.*, 1983) and are often found effective in vitro as antimicrobial substance against a wide array of microorganisms (Bennet and Wallsgrove 1994). Tannins are polymeric phenolic substances possessing the astringent property. These compounds are soluble in water, alcohol and acetone and give precipitates with proteins (Basri and Fan 2005). Coumarins are phenolic substances made of fused benzene and α -pyrone rings (O'Kennedy and Thornes 1997). They have a characteristic odour and several of them have antimicrobial properties.

Plants produce aromatic secondary metabolites, most of which are phenols or their oxygen-substituted derivatives (Geissman 1963). Important subclasses of plant derivative compounds include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins. These secondary metabolites show antimicrobial effect and serves as plant defence mechanisms against pathogenic microorganisms (Scalbert 1991).

10. Marine based medicinal plants

Marine natural products have gained the attention of scientists worldwide past 5 decades for their exceptional medicinal benefits. Marine derived natural products are vast which exhibit medicinal properties and so marine organisms are a major source of new bioactive compounds that has variable characteristic features compared to terrestrial natural organisms and their derived products (Blunt *et al.*, 2018). Isolation of Several chemically unique compounds from marine organisms that exhibit different biological activities have been under investigation for development of new antimicrobial agents (Ghareeb *et al.*, 2020). Although they are mostly anticancer agents, a number of viral infections, chronic pain relievers, and hypertriglyceridemia drugs have also been approved. However, there is no report of any drugs being isolated from seagrasses to be approved by the FDA or EMA since now. According to the World Health Organization (WHO), medicinal plants could be the best source for obtaining a variety of drugs, as they produce several bioactive compounds with known therapeutic properties. Many plant extracts exhibit effective antibacterial activity towards different tested bacterial isolates therapeutically as indicated by their MIC values.

11. Seagrass

Seagrasses are one of the true marine flowering plants that belong to the group of angiosperms, which have 72 species worldwide, divided into four families (Zosteraceae, Hydrocharitaceae, Posidoniaceae, and Cymodoceaceae) (Short *et al.*, 2016, Danaraj *et al.*, 2021). These are one of the valuable sources of bioactive compounds for the treatment of infectious human diseases. Among the eukaryotic

organisms, seagrasses are one of the species that is found in shallow water areas of temperate, subtropical, and tropical seas, except in polar regions. Seagrass meadows are found in shallow coastal areas worldwide, with distribution ranging from 50–90 m in depth (Mehari Ghebretinsae *et al.*, 2019). Seagrass meadows can store carbon, improve water quality, provide food and habitat to many marine organisms, and act as biological indicators (Unsworth *et al.*, 2014).

1.12 Seagrass active metabolites

Seagrasses produce certain active secondary metabolites that behave as antimicrobial agents to control infectious diseases (Mani *et al.*, 2012), due to the exhibition of defence mechanisms under stress conditions. These active metabolites include polyphenols, terpenoids, and halogenated compounds, are produced by several species of the seagrass reported to have anticancer (antitumor), antifungal, anti-inflammatory, antimicrobial, antiviral, antidiabetic, antimalarial, antioxidant, anti-aging, and cytotoxic properties (Kim *et al.*, 2021). Solvent Extracts of *C. rotundata*, *C. serrulata*, *E. acoroides*, *H. pinifolia*, *H. ovalis*, *H. uninervis*, *H. stipulacea*, *S. filiforme*, *S. isoetifolium*, and *T. hemprichii* were found to inhibit the growth of test bacteria. (Iyapparaj *et al.*, 2014). Seagrasses are also been used since ancient period as folk medicine for a variety of treatments including fevers, stomach problems, muscle pain, wounds, and skin diseases; they are also used as a remedy for stings of different kinds of rays and also act as tranquilizers for babies (Grignon-Dubois *et al.*, 2015).

1.13 *Halodule pinifolia*

Halodule pinifolia is a seagrass species in the genus *Halodule*. It is found in shallow sea waters and grows in sandy or muddy sand substrates from upper littoral to subtidal areas. Seagrass *H. pinifolia*, has been detected with some active metabolites that has potent antibacterial activity against human pathogens than other seagrass species such as *C. serrulata* and *H. ovalis* (Kannan *et al.*, 2010, Kannan *et al.*, 2012, Kannan *et al.*, 2013). It also has other therapeutic activities including anti-viral (Hamdy, *et al.*, 2012) anti-oxidant (Jeyapragash *et al.*, 2016),

anti-inflammatory (Hua *et al.*, 2006), anti-diabetic and vasoprotective (Gokce *et al.*, 2008), insecticidal (Mani *et al.*, 2012), larvicidal and antifeedent (Ali *et al.*, 2012).

1.14 Combinatorial approach

Since antibiotics alone aren't a sustainable solution, medicinal plants are taken as an alternative to effectively treat the infectious diseases by enhancing the antibiotics which are ineffective on multidrug resistant organisms (Mishra *et al.*, 2012). When antibiotics are combined with plant extract for synergistic antimicrobial property, the bacteria which is already resistant to the antibiotic will exhibit almost the least zone of inhibition with same concentration. Over dosage of antibiotic and extract concentration in combination can be used to check antimicrobial susceptibility. (Eze *et al.*, 2013, Fadila *et al.*, 2016, Abdul, 2019). Moreover, a combinational approach that allows synergism between plant extracts and conventional antibiotics is notably the most effective method to prevent antibacterial resistance. Reported evidences are available for the enhancement of conventional antibiotics synergistically with plant-derived compounds (Cheesman *et al.*, 2017). Therefore, the potentiality of plant compounds to make conventional antibiotics effective again in the treatment of microbial infections on a larger scale may significantly impact global health in overcoming resistant pathogenic microorganisms (Van Vuuren *et al.*, 2011).

The term "synergy" indicates that the combination of two or more drugs significantly results with an enhanced effect that is probably greater than sum of their individual components (Van Vuuren *et al.*, 2011; Levinson *et al.*, 2002). The combinatorial approach of two antimicrobial agents can also be classified, such as additive, noninteractive, and antagonistic. An "additive" effect is observed when substances combined together will have a positive impact by increasing the efficacy, although not to the synergism level. Secondly, for "non-interactive" (or indifferent) combinations, the individual substances used in combination exhibit neither additive nor antagonistic effect. Finally, "Antagonism" is when a combination of antimicrobial agents produces an overall effect which is lesser than the sum of their individual component effects (i.e., both the drugs are reducing the efficacy of each other) (Tallarida 2006).

Recently, Combinatorial approach has gained a tremendous increase worldwide in the field of treating the infectious diseases. According to the WHO, combinational therapy is mostly preferred over monotherapy in most of the life-threatening infectious diseases such as malaria, tuberculosis, and HIV/AIDS due to its ability to have multiple target sites and exhibit resistance against the present microbial treatments. (WHO. Global Tuberculosis Report 2016).

1.15 Combination of plant extract with antibiotics

Antimicrobial natural plant product in combination with antibiotic drugs have become a research aspect due to several factors, which includes cost effective analysis of compounds and efficient bioavailability of synergistic drug. In effort to discover a new drug which requires years of research and testing, application of combinatorial approach to restore the effects of existing drugs with reduced resistance is considered to be an efficient medical treatment. This way of enhancing the drugs can help reach clinical trials on a faster pace at a low developmental cost as the bioactive compound has already been through extensive clinical evaluations.

Additionally, there are certain advantages of synergistic interactions including increased efficiency, reduced side effects, increased stability and bioavailability, and the requirement of lower doses in comparison to synthetic alternatives (Inui T *et al.*, 2007). Combinations of bioactive compounds and antibiotic combinations not just enhance the overall antimicrobial activity but also modifies the resistance mechanisms or modulating targets. Synergistic effect manifests such outcome due to the adverse effects of crude plant extracts in damaging the cytoplasmic membrane of resistant bacteria which results in loss of intracellular components.

There is a very limited available literature over the recent years which proposed the combinatorial strategies to achieve synergistic effect in enhancement of antimicrobial properties of ineffective antibiotics using plant extracts to overcome multidrug resistant organisms at lowest MIC values (Mathew Gideon *et al.* 2023). So, this research has been performed in order to evaluate the combinatorial approach of synergism, additive or antagonism between Seagrass *Halodule pinifolia* and the commercially available antibiotics in aim to combat antimicrobial resistance.

Chapter-2: Literature survey

1. Evolution of antimicrobial resistant organisms

Antimicrobial resistant organisms were once restricted only to hospitals but are now found everywhere, (Lozano and Torres 2017) ranging from humans to animals which is due to the large-scale production and misuse of antibiotics in agriculture, aquaculture, horticulture clinical and veterinary medicine. (Maciá *et al.*, 2005). Long term use of these antibiotics can also cause adverse effects on host cells including hypersensitivity, immunosuppression and allergic reactions, depletion of beneficial gut and mucosal microorganisms. (Idose 1968). The rapid emergence of these multidrug resistant pathogens has led to inadequacy of information on evolutionary mechanisms of microbial ecosystem. (Aminov RI 2009). Thus, it's very important to understand the mechanism behind the antimicrobial resistance pathogens and their evolution. Initially antimicrobial resistance is considered since ancient periods with the expected outcomes of the interaction between many organisms with their surrounding environment. This is because many naturally occurring plants produce antimicrobial compounds as of which the pathogens have evolved mechanisms to overcome their antimicrobial activity.

2. Mechanism of antimicrobial resistance

Further, Antibiotics were developed with the mechanism of inhibiting the synthesis of proteins at ribosomal level and altering the biochemical reactions such as acetylation, phosphorylation, and adenylation. (Wilson 2014). In general, antimicrobial resistance due to intrinsic mutations alter the effects of antibiotics via following mechanisms such as (i) structural modifications of antimicrobial target which reduces the affinity of drugs to bind effectively and have a cause, (ii) modifications which leads to reduced uptake of drugs, (iii) changes in metabolic pathways by modulating the regulatory networks, and (iv) activating the efflux mechanisms to exclude the antimicrobial compounds (Munita 2016).

3. Mechanism of antimicrobial resistance in *Candida albicans*

Candida albicans is a commensal fungus of the human gastrointestinal tract and a prevalent opportunistic pathogen. To examine diversity within this species, extensive genomic and phenotypic analyses were performed on 21 clinical *C. albicans* isolates. Genomic variation was evident in the form of polymorphisms, copy number variation chromosomal inversions, subtelomeric hypervariation, loss of heterozygosity (LOH), and whole or partial chromosome aneuploidies. All 21 strains were diploid, although karyotypic changes were present in eight of the 21 isolates, with multiple strains being trisomic for chromosome 4 or Chromosome 7. Aneuploid strains exhibited a general fitness defect relative to euploid strains when grown under replete conditions. All strains were also heterozygous, yet multiple, distinct LOH tracts were present in each isolate. Higher overall levels of genome heterozygosity correlated with faster growth rates, consistent with increased overall fitness. Genes with the highest rates of amino acid substitutions included many cell wall proteins, implicating fast evolving changes in cell adhesion and host interactions. One clinical isolate, P94015, presented several striking properties including a novel cellular phenotype, an inability to filament, drug resistance, and decreased virulence. Several of these properties were shown to be due to a homozygous nonsense mutation in the EFG1 gene. Furthermore, loss of EFG1 function resulted in increased fitness of P94015 in a commensal model of infection. Based on their analysis, it was analysed that there are intra-species genetic and phenotypic differences in *C. albicans* and delineates a natural mutation that alters the balance between commensalism and pathogenicity (Hirakawa *et al.*, 2015).

4. Natural source of treatment

Naturally plant based materials that have been used since ancient periods as herbal medicines are taken into research for their antimicrobial properties to treat infectious diseases (Abreu *et al.*, 2012, Anand *et al.*, 2019). Medicinal plants refer to the herbal plants which possess some active ingredients and secondary metabolites with biological activity (Doughari *et al.*, 2019; Rungsung *et al.*, 2015; Sofowora *et al.*,

1980) are the valuable source of alternative antimicrobials against the pathogenic organisms. They play an important role as herbal medicines which has been considered as the traditional medical treatment in most of the rural and hilly areas since ancient period. They have been tested for biological, antimicrobial, and hypoglycemic activity (Atta, *et al.*, 1998; Glombitza, *et al.*, 1994; Vats *et al.*, 2002).

2.5 seagrass *Halodule pinifolia*

A study performed on the phytochemical and antimicrobial potential of different extract of *Halodule pinifolia* revealed many potential groups of secondary metabolites. Amongst the different species of seagrasses, *Halodule pinifolia*, which is usually found on coastal areas in Thanjavur was selected for bioactive potential. They used agar diffusion method to test the seagrass extracts' resistance to *E. coli*, *B. subtilis*, *A. niger*, and *C. albicans*. Aqueous extracts of *Halodule pinifolia* was phytochemically screened and was detected with reducing sugars, alkaloids, saponins, phenolic compounds, flavonoids, and carbohydrates. Accordingly, they concluded that *Halodule pinifolia* expressed broad spectrum of antimicrobial properties that may be effective antioxidant for biological systems that are vulnerable to free radical-mediated reactions (Mary *et al.*, 2022).

2.6 Metabolites of *Halodule pinifolia*

A report on comparative metabolomics analysis of wild and suspension cultured cells (SCC) of seagrass *Halodule pinifolia* (Miki) Hartog of Cymodoceaceae family was performed to investigate the systematic identification and characterization of metabolic changes in wild and SCC of *H. pinifolia* using GC–MS based metabolomics approach. With an extensive and careful analysis, it was identified to express 98 metabolites in wild and 125 metabolites in SCC. Their relative abundance was determined and the Identified metabolites were mapped to general biochemical pathways and these metabolites spanned 77 primary and secondary metabolism pathways in wild, while 73 metabolism pathways in SCC. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis of integration data demonstrated that wild and SCC showed profound biochemical changes including glutathione metabolism, glutamate metabolism, phenylalanine and tyrosine metabolism in wild, while SCC caused significant variations in citric acid

metabolism, glycolysis, gluconeogenesis, oxidative phosphorylation related pathways respectively. Random forest analysis showed top 15 metabolites and their correct classification. The findings reported in this study provide baseline information on *H. pinifolia* metabolism in the marine and artificial environments. Moreover, metabolomics data obtained from *H. pinifolia* may be used to compare the metabolites associated with stress response which would facilitate further dissection mechanism of stress tolerance of other seagrass species in the marine environments (Danaraj *et al.*, 2020).

7. Antimicrobial activity of plant extracts on *C. albicans*

Plants as sources of medicinal compounds have continued to play a predominant role in the maintenance of human health since ancient times. Even though several effective antifungal agents are available for oral candida infections, the failure is not uncommon because isolates of *Candida albicans* may exhibit resistance to the drug during therapy. This research was conducted to evaluate the antimicrobial effects of few plant extracts on *Candida albicans*. They also identified an alternative, inexpensive, simple, and effective method of preventing and controlling *Candida albicans*. Stock solutions of plant extracts were inoculated on petri plates containing species of *Candida albicans* and incubated at $25 \pm 2^\circ\text{C}$ for 72 h. They concluded that the Alcoholic curry leaves showed the maximum zone of inhibition on *Candida albicans* followed by aqueous tea leaves. The other plant extracts like alcoholic onion leaves, alcoholic tea leaves, alcoholic onion bulb, alcoholic aloe vera, and alcoholic mint leaves also inhibited the growth of *Candida albicans* but to a lesser extent. This study reveals few medicinal plants as an alternative medicine to the field of dentistry which can be used adjunct to conventional therapy of oral candidiasis (Doddanna *et al.*, 2013).

8. Approach to novel medicine

Discovery of novel medicines has declined due to multidrug resistant microorganisms causing severe infections and diseases acquiring antimicrobial resistance. (Olga *et al.*, 2020). Other factors such as Mutation, Gene Transfer, Inappropriate Use and Diagnostics, and prescription of antibiotics to undiagnosed

patient are the significant ways in which antimicrobial resistance happens faster. (Diseases, National Institute of Allergy and Infectious 2011). Identifying novel methods to develop active compounds for selectivity, efficacy, and safety for a clinical trial candidate remains a scientific challenge (Yerevan State Medical University [Online] 2017). In most developing countries, medicinal plants are under research to analyse bioactive and inactive substances that are still in use to treat various diseases, these bioactive substances comprise of one-fourth of the active ingredients in most prescribed drugs (Heinrich *et al.*, 2010).

2.9 Combinatorial approach

An approach to utilize the Combinations of selected phytochemicals (reserpine, pyrrolidine, quinine, morin and quercetin) with antibiotics (ciprofloxacin, tetracycline and erythromycin) was tested on the prevention and control of *Staphylococcus aureus* biofilms. The phytochemicals were also studied for their ability to avoid antibiotic adaptation and to inhibit antibiotic efflux pumps. They detected that Morin, pyrrolidine and quercetin at subinhibitory concentrations had significant effects in biofilm prevention and/or control when applied alone and combined with antibiotics. Thus, they evaluated that the combination of antibiotics and phytochemicals showed synergism especially against biofilms of NorA overexpressing strain *S. aureus* SA1199B. This strain when grown using subinhibitory concentrations of ciprofloxacin developed increased tolerance to this antibiotic. However, this was successfully reversed by quinine and morin. In addition, reserpine and quercetin showed significant efflux pump inhibition. Conclusively, their overall results demonstrate the role of phytochemicals in co-therapies to promote more efficient treatments and decrease antimicrobial resistance to antibiotics, with substantial effects against *S. aureus* in both planktonic and biofilm states (Abreu *et al.*, 2016).

A recent study reported that multiple *Salvia* spp. and *Matricaria recutita* exhibited synergistic effects with oxacillin, greatly enhancing its efficacy against the resistant pathogen (Chovanova R *et al.*, 2013). The exact mechanism for the resultant effect of synergism is still unclear but it has been stated according to the studies that it was due to the modifications in the structures of resistant bacteria. The plant

extracts in combination with oxacillin, potentially caused significant disruption of cell membrane resulting in cell death (Chovanova R *et al.*, 2013).

Chapter – 3: AIM AND OBJECTIVES

AIM:

Though Seagrass *Halodule pinifolia* been reported for the antimicrobial, antioxidant, antimalarial, anti-inflammatory, antifungal activity, however, there is no human ortholog reported from seagrasses against harmful pathogens and hence, the following objectives was delineated.

OBJECTIVES:

1. To perform solvent extraction from *Halodule pinifolia* using methanol, ethyl acetate and chloroform.
2. To fractionate the active crude extract of extract *Halodule pinifolia* using column chromatography.
3. To assess antifungal and antibiofilm activity of different fractions against *C. albicans*.
4. To identify the bioactive fractions and isolate the active compound using TLC.
5. To determine the MIC values of bioactive compounds using antibiofilm activity.
6. To evaluate the synergistic effect of seagrass active constituent with antibiotics of different concentrations against *C. albicans* using combinatorial approach.

Chapter- 4: MATERIALS AND METHODS

4.1 Sample collection

Seagrass *Halodule pinifolia* was collected during low tide from Vellar estuary; Parangipettai, Tamil Nadu and the collected sample were washed with native water to remove the epiphytes and extraneous matter. The samples were transported to the Halophyte Micropropagation Lab, Sathyabama Institute of Science and Technology and washed thrice with distilled water. It was shade dried and stored at room temperature for future use (Danaraj *et al.*, 2020).



Figure 4.1: *Halodule pinifolia*

4.2 Solvent Extraction

Seagrass *H. pinifolia* (5 gm) was ground to a fine powder using mortar and pestle, and the extraction was done using 3 different solvents each of 25 mL, including methanol (MeOH), chloroform (Chl), and ethyl acetate (EtAc). It was then incubated in a waterbath for about 2 hours at 40° C. After incubation the extracts were filtered using Whatmann No. 1 filter paper and concentrated by rotary

evaporator. The final yield of the extracts (100 µg/ mL) was dissolved in DMSO (20%) and stored at 4 °C for further analysis (Danaraj *et al.*, 2020).



Figure 4.2: Solvent extraction of seagrass chloroform, ethyl acetate, methanol.

4.3 Bacterial Strains and Inoculum preparations

The strain *C. albicans* were obtained from the Jawaharlal Nehru Institute of Post-graduate Medical Education and Research (JIPMER), Puducherry. The strain was sub-cultured on freshly prepared sterile Sabouraud dextrose broth and incubated at 37° C for 24 hours. After incubation, the turbidity was adjusted to 0.5 on the Mc Farland scale (1.5×10^8 cells/mL). This fungal suspension was used throughout the experiments (Marie 2005).



Figure 4.3: Candida albicans strain

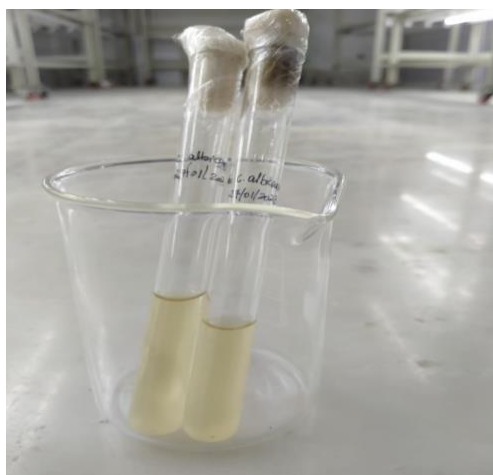


Figure 4.4: Broth culture of *Candida albicans*

4. Anti-fungal activity of *H. pinifolia* crude extracts

Different solvent extracts of *H. pinifolia* was assayed for antifungal potential against *C. albicans* by well diffusion assay. The fungal suspension of *C. albicans* was swabbed on Mueller-Hinton agar plates and incubated for 48 hours at 37° C. Following 20 µl of *H. pinifolia* crude extracts (MeOH, EtAc and Chl; 100 µg/ mL) were added to each well and oxofloxacin (75 µg/ mL) was used as positive controls respectively. The plates were then 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 (Hassawi *et al.*, 2006, Ahmad, *et al.*, 2001).

5. Anti-biofilm activity of *H. pinifolia* crude extracts

To determine the effects of crude *H. pinifolia* extracts on *C. albicans* biofilm, MHB broth (3ml) along with the fungal suspension (20 µl) were added to the six well plates in the presence and absence of crude extracts (100 mg/ ml). Ofloxacin and Levofloxacin (50 mg/ ml) was used as positive control. Wells containing MHB broth and fungal suspension were used as negative control, while the wells with only MHB 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 (Vijayakumar *et al.*, 2018, Danaraj *et al.*, 2020).

6. Column fractionation of *H. pinifolia* Extracts

The crude EtAc extract of *H. pinifolia* which exhibited the potential inhibition against *C. albicans* 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 Ethyl acetate 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 Ethyl acetate 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, EtAc was added to the top of the silica slurry to 1cm height. Dried extracts of EtAc were 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 *C. albicans*.

7. Anti-fungal and Anti-biofilm activity of column fractions

The anti-fungal and anti-biofilm activity of column fractions was performed following the methodology mention in section 3.4. & 3.5. The fractions which showed the potential inhibition of *C. albicans* biofilm was taken up for further analysis.

8. Thin layer chromatography (TLC)

The active constituents exhibited activity against *C. albicans* 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 (2.5:2:0.5). 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 was confirmed by HPLC following the structural elucidation by ^1H and ^{13}C NMR.

4.9 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; 4.6×250 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}C and ^1H NMR according to the method of (Mitra *et al.*, 2006).

4.10 Minimum Inhibitory Concentration (MIC) of Rosmarinic acid and Vanillic acid

The active compounds isolated from *H. pinifolia* were identified as Rosmarinic acid and Vanillic acid and the minimum inhibitory concentration of active constituents were 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 RA and VA (500 - 0.97 mg/ 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.

4.11 Synergistic effect

The combined effect of RA and VA with commercially available antibiotics against *C. albicans* was examined. Different antibiotics were used after performing antimicrobial susceptibility test against *C. albicans* (Daniel *et al.*, 2022) and the following commercially available antibiotics were reported to inhibit the pathogen growth were used for synergistic effect and the zone of inhibition was measured with different concentrations. For this study, Ofloxacin, Levofloxacin, Amphotericin B, Flucanazole and Itracanazole were used for the combinations with RA and VA (MIC) at different concentrations were tabulated in Table 1. Using a 24 well-plate, antibiofilm assay was carried out. Inoculum of 1.5×10^8 cells was diluted from overnight culture and 20 μ l of it was added to 2ml of LB broth except control respectively. The bioactive compounds with different combinations were then added in the wells with column 1-23 consequently. MIC values of Rosmarinic acid (62.5 mg/ml) and Vanillic acid (125mg/ml) was selected for different combinations of synergism (Table 1).

Table 1: Different combinations of bioactive compounds and antibiotics.

S.NO.	Rosmarinic Acid (mg/ml)	Vanillic acid (mg/ml)	Ofloxacin (µg/ml)	Levofloxacin (µg/ml)	Itracanazole (µg/ml)	Flucanazole (µg/ml)
1	62.5	-	50	-	-	-
2	62.5	-	-	50	-	-
3	62.5	-	-	-	800	-
4	62.5	-	-	-	-	1000
5	31.25	-	25	-	-	-
6	31.25	-	-	25	-	-
7	31.25	-	-	-	400	-
8	31.25	-	-	-	-	500
9	-	125	50	-	-	-
10	-	125	-	50	-	-
11	-	125	-	-	800	-
12	-	125	-	-	-	1000
13	-	62.5	25	-	-	-
14	-	62.5	-	25	-	-
15	-	62.5	-	-	400	-
16	-	62.5	-	-	-	500
17	62.5	125	-	-	-	-
18	62.5 + 31.25	125 + 31.25	-	-	-	-
19	31.25	62.5	-	-	-	-
20	31.25	62.5	25	-	-	-
21	31.25	62.5	-	25	-	-
22	31.25	62.5	-	-	400	-
23	31.25	62.5	-	-	-	500

Chapter-5: RESULTS AND DISCUSSION

RESULTS

5.1 Antifungal and Antibiofilm activity of *H. pinifolia* crude extracts.

Different solvent extracts (methanol, chloroform, and ethyl acetate) of *H. pinifolia* was performed with antifungal and antibiofilm activity against *C. albicans*. The results showed that ethyl acetate extract exhibited the maximum zone of inhibition against *C. albicans* as compared to the positive controls respectively (**Figure 5.1.1**).

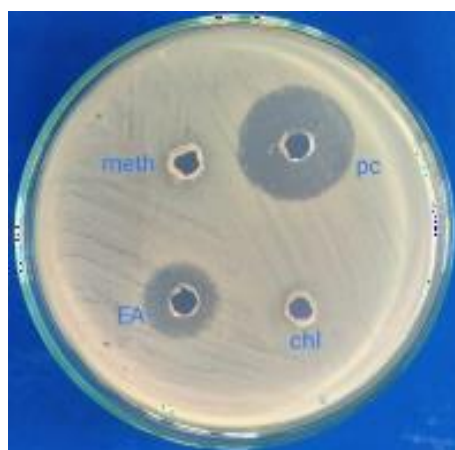


Figure 5.1.1: Antifungal activity of *H. pinifolia* crude extracts.

Antifungal activity of different solvent extracts of *H. pinifolia* was assayed against *C. albicans* by well diffusion assay. Oxofloxacin (75 µg/ mL) was used as positive control. Triplicates were performed and the mean zone of inhibition was recorded as follows:

Table 2: Zone of inhibition of *H. pinifolia* crude extract

EXTRACTS	Concentration of extracts ($\mu\text{g/ mL}$)	MEAN ZONE OF INHIBITION (mm)
Positive control (ofloxacin)	75	15
Methanol	100	0
Chloroform	100	0
Ethyl acetate	100	8

For antibiofilm activity, Control was loaded only with MHB broth and acted as blank. Ofloxacin and levofloxacin (50 mg/ ml) was used as positive control along with MHB broth and culture. Biomass of biofilm was quantified by measuring the OD at 570 nm.

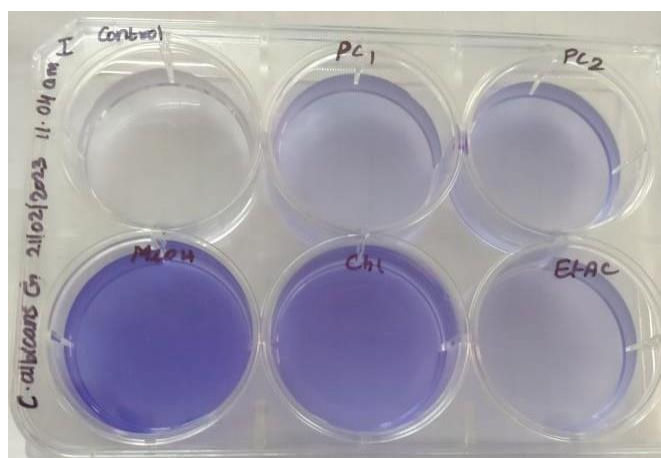


Figure 5.1.2: Antibiofilm activity of *H. pinifolia* crude extracts.

5.2 Column fractionation and determination of active fractionations.

Column fractionation was performed to separate the active metabolites of ethyl acetate crude extract, which showed the maximum zone of inhibition in antifungal activity and antibiofilm activity. Eleven fractions were collected based on the polarity of solute to the solvent and was further taken for antifungal and antibiofilm activity to determine the fraction with antimicrobial active compounds.

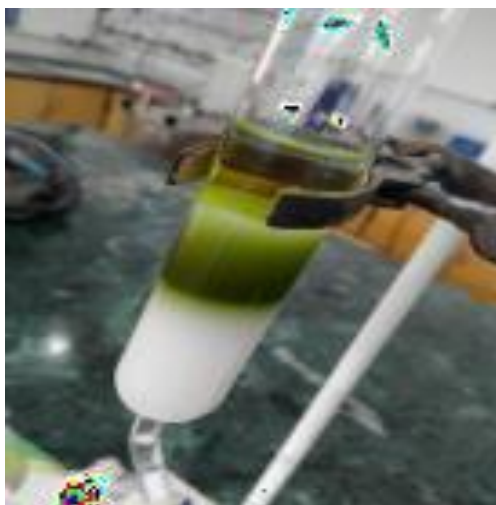


Figure 5.2.1: Column fractionation of ethyl acetate crude extract

Antifungal activity of all the eleven (F1 – F11) fractions were carried out by well diffusion method. Each well was loaded with 20 μ l of the fraction and was incubated for 24h at 37°C. The mean zone of inhibition was recorded for each fraction and was used to determine the fraction with maximum zone of inhibition.

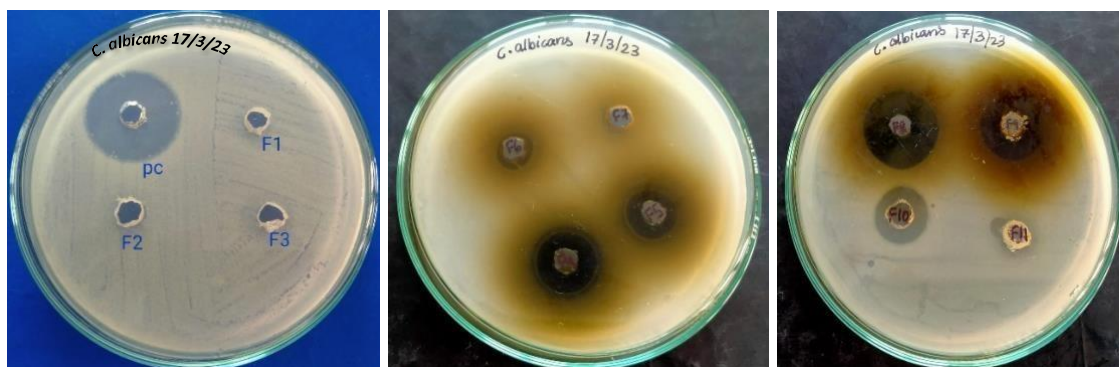


Figure 5.2.2: Antifungal activity of F1-F11 along with Ofloxacin.

Antibiofilm activity of the fractions was determined by biofilm inhibition assay which was carried out using MHB broth and an inoculation of 20 μ l overnight culture. Fractions ranging from F1 – F11 were added and the plate was incubated at 37°C for 24h. Control was used as blank without the culture and positive control was added with Ofloxacin along with the culture.

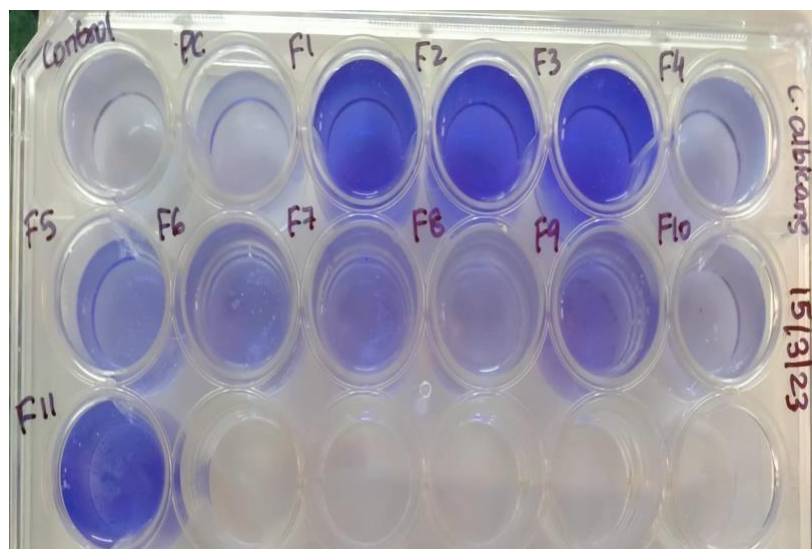


Figure 5.2.3: Antibiofilm activity of Fractions (F1-F11) obtained along with Ofloxacin as positive control.

5.3 Thin Layer Chromatography, High performance Liquid Chromatography and NMR analysis.

Fraction 4 and 8 showed the maximum zone of inhibition and biofilm inhibition in Antifungal and Antibiofilm assays respectively. Preparative TLC was used to isolate the active compounds present in these fractions of ethyl acetate extract that was carried out using Mobile phase of methanol: chloroform: glacial acetic acid (2.5:2:0.5) to separate the bands and identify the individual compounds.

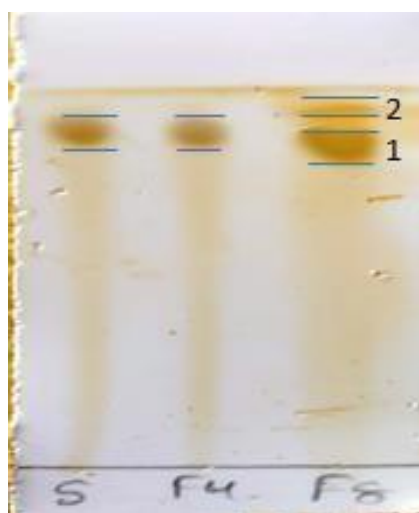


Figure 5.3.1: Thin Layer Chromatography

The compound isolated from the TLC were analysed for purity check with the help of standards using HPLC. The chromatogram showed that the active constituents isolated from *H. pinifolia* were 99% pure (Figure: 5.3.2 and Figure:5.3.3) and hence further analysed for structural elucidation by NMR. The ^1H and ^{13}C NMR results revealed that the active constituents that inhibited *C. albicans* growth and biofilm were found to be Rosmarinic acid and Vanillic acid (Figure 5.3.4 and Figure 5.3.5).

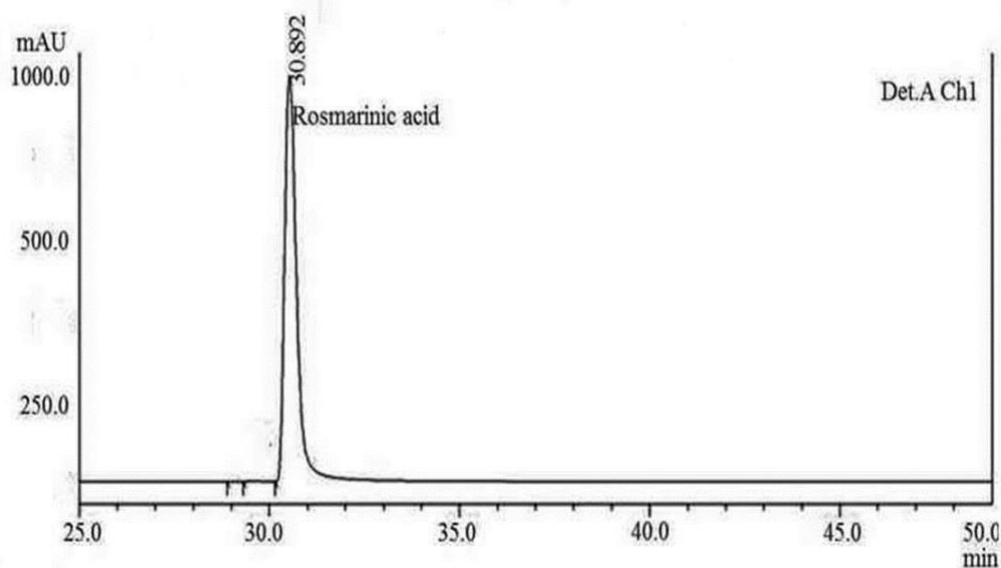


Figure 5.3.2: HPLC analysis of Rosmarinic acid.

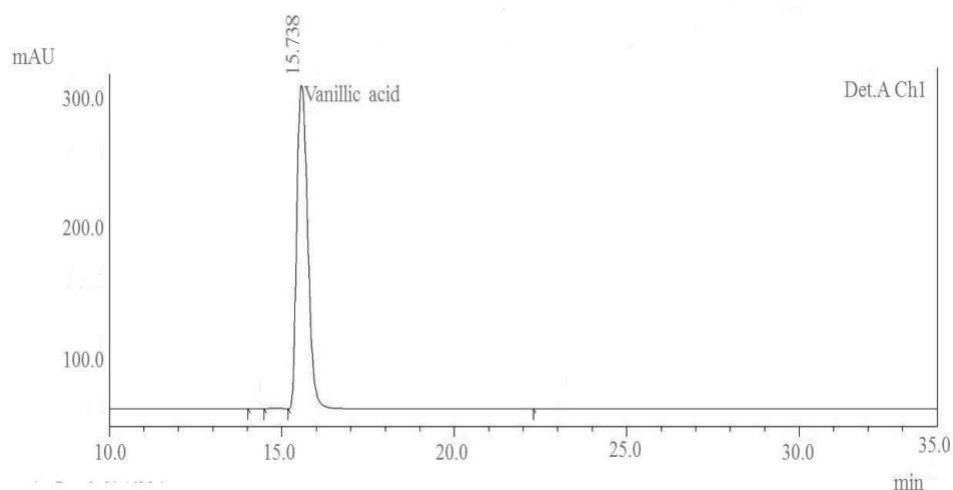


Figure 5.3.3: HPLC analysis of Vanillic acid.

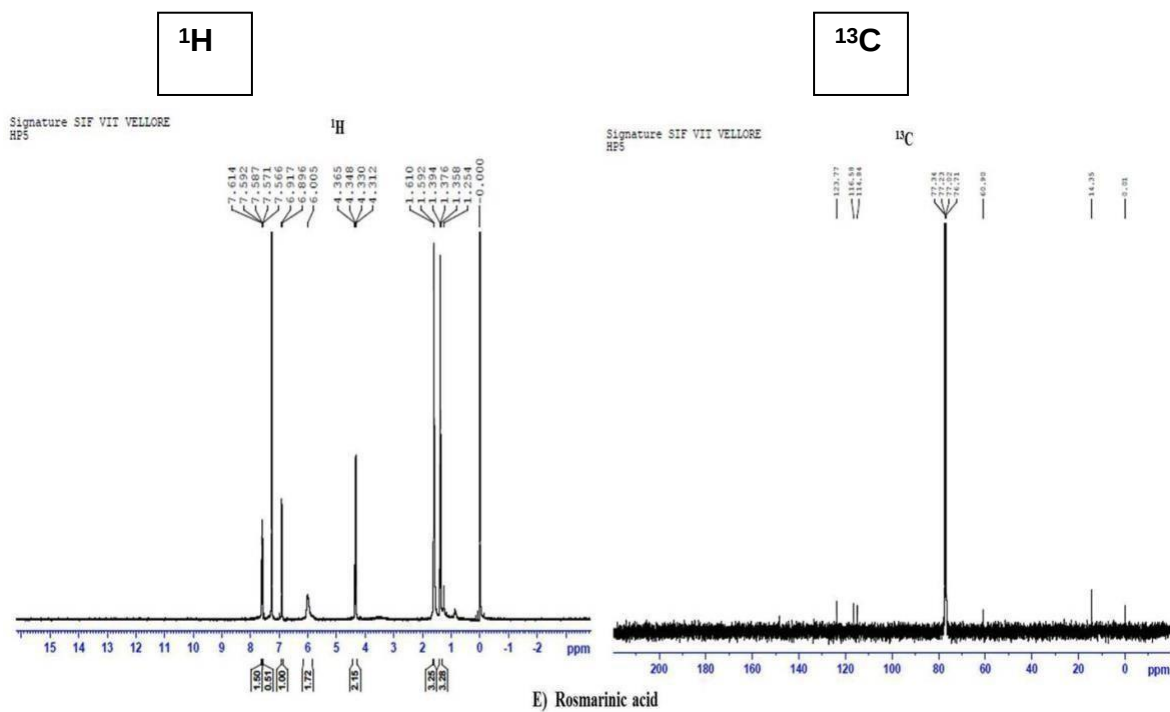


Figure 5.3.4: NMR analysis of Rosmarinic acid.

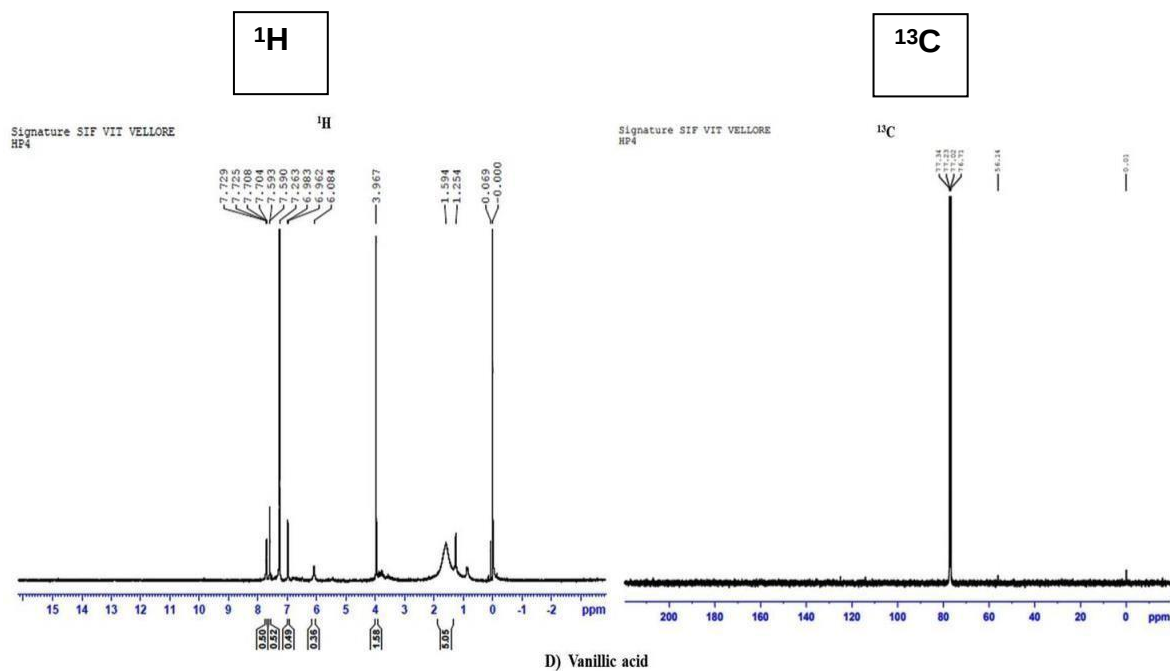


Figure 5.3.5: NMR analysis of Vanillic acid.

5.4 Minimum Inhibitory Concentration (MIC)

Further on identification and confirmation of Rosmarinic acid and Vanillic acid with the commercially available standards respectively, they were taken for the determination of MIC using gradient concentrations ranging from (500-0.97mg/ml) individually.

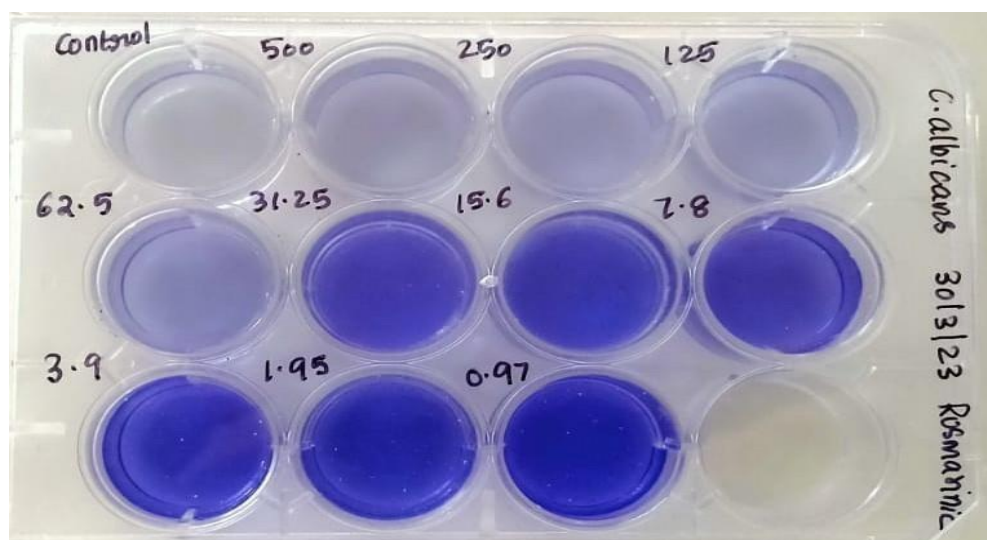


Figure 5.4.1: Minimum Inhibitory Concentration (MIC) of Rosmarinic acid.

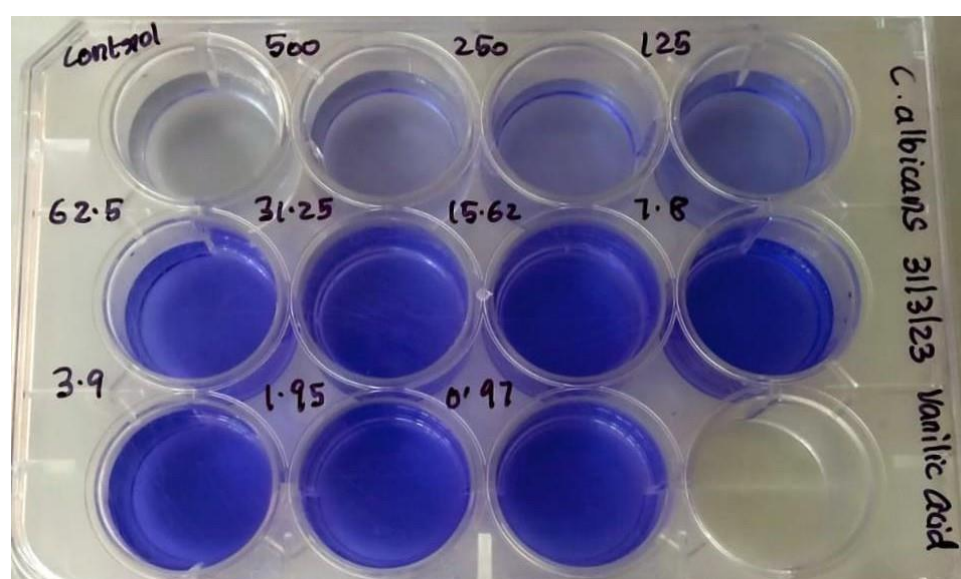


Figure 5.4.2: Minimum Inhibitory Concentration (MIC) of Vanillic acid.

5.5 Synergistic effect

- ❖ MIC values of both Rosmarinic acid and Vanillic acid was used in combination with each other, with and without antibiotics. Half the values of MICs were also used to see the effect of bioactive compounds with antibiotics.
- ❖ Rosmarinic acid and Vanillic acid showed better antimicrobial activity in combination with Levofloxacin and Ofloxacin.
- ❖ Fraction 11- Though Itraconazole showed some inhibitory activity in combination with Vanillic acid (MIC), it had no results with half the MIC values of both antibiotic and Vanillic acid.
- ❖ Combination of both Rosmarinic acid and Vanillic acid showed better inhibition with their MICs and their half the concentrations without antibiotics.

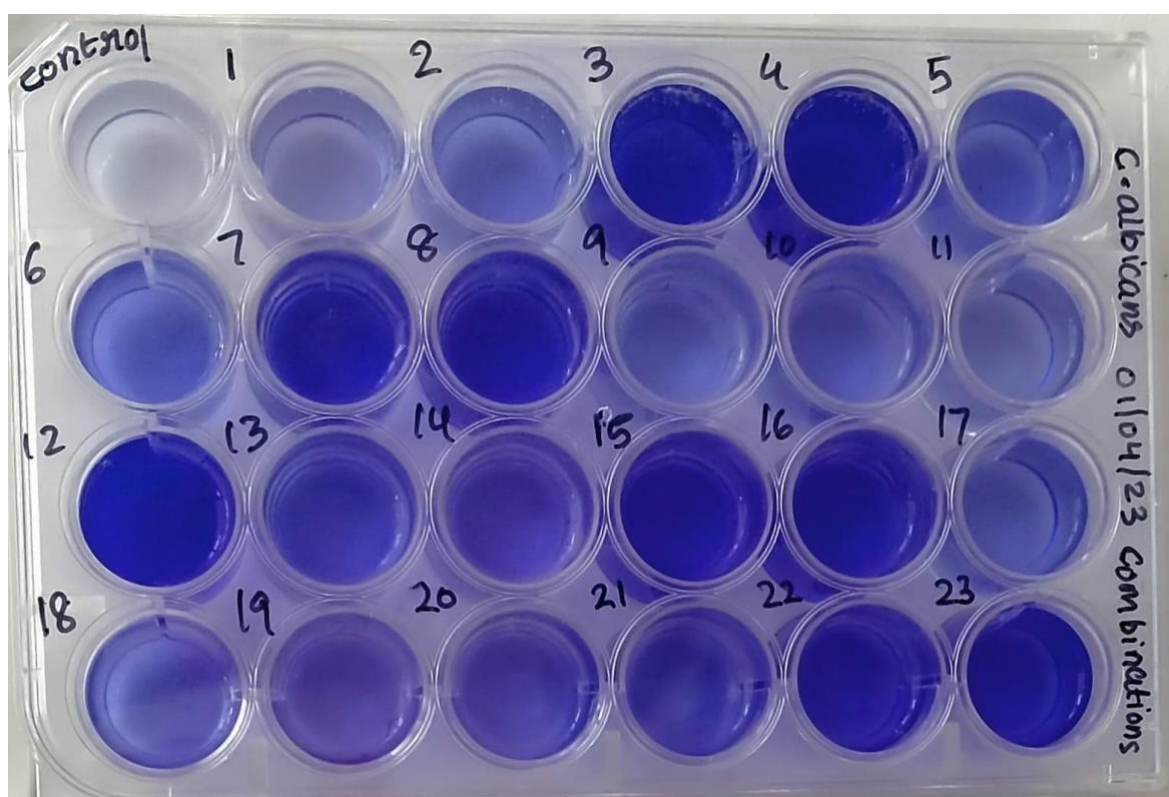


Figure 5: Synergistic effect of different combinations of Rosmarinic acid and Vanillic acid with antibiotics against *candida albicans*.

DISCUSSION

Antibiotic resistance mechanism

The overexpression of the efflux pumps contributes only partially to fluconazole resistance in such isolates and high-level resistance is usually achieved when multiple different mechanisms are combined (Franz *et al.*, 1998a; Morschhäuser 2002; Perea *et al.*, 2001; Sanglard *et al.*, 1998; White 1997). Upregulation of CDR1, CDR2, and MDR1 has also been observed in fluconazole-resistant strains that were generated in vitro by sequential passages of susceptible strains in the presence of fluconazole (Albertson *et al.*, 1996; Cowen *et al.*, 2000; Kohli *et al.*, 2002; Marr *et al.*, 2001; Riggle and Kumamoto, 2006; Yan *et al.*, 2008).

Sea grass bioactive compounds:

Marine angiosperms produce a diverse range of secondary metabolites with unique and wide range of structural characteristics differing from terrestrial plants that have the potential to be developed into effective and potent drugs for a number of diseases. As they are available on a larger scale, they provide an unlimited sources of sample collection and extraction. 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 these 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).

Seagrass *Halodule pinifolia*

The response, adaptation and underlying tolerance mechanism of tropical seagrass *Halodule pinifolia* upon temperature stress (24, 29, 37, and 45 °C) and the effect of temperature stress on net photosynthesis ($\Delta F/F'm$) and dark respiration (F_v/F_m) has been investigated in the literature studies. The results of GC-MS based omics approach showed heat stress could cause large metabolite variations with respect to sugar, amino acids and organic acids and the first report of occurrence of three thermo-protective metabolites such as trehalose (sugar), glycine betaine (amino

acid) and methyl vinyl ketone (organic acid) were profiled from *H. pinifolia* (45 °C) was reported (Jeyapragash *et al.*, 2021). In contrast to this research, Comparative metabolomics analysis of wild and suspension cultured cells (SCC) of seagrass *Halodule pinifolia* (Miki) Hartog of Cymodoceaceae family was also studied. The GC-MS data identified the total metabolites present in both wild and SCC. The results reported were biochemical pathways of the mapped metabolite, the top 15 metabolites and also novel compounds in *H. pinifolia* were identified (Jeyapragash *et al.*, 2020).

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 Rosmarinic acid and Vanillic acid showed an effective inhibition against *C. albicans* in which MIC was showed at concentration of 62.5 mg/mL and 125 mg/mL. This study reveals that seagrass can act as a good source for the purpose of drug discovery and in which the Rosmarinic acid and Vanillic acid derived from *H. pinifolia* may act as active fractionate against the growth of *C. albicans* (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).

Synergistic effect

Synergism of plant and antibiotics

This study was done aiming at finding some antibacterial agents that may be mixed with pharmaceuticals or combined with some antibiotics to inhibit the growth of the virulent pathogenic microorganism. In this connection, (Yang *et al.*, 2010) stated

that, in recent years, human pathogenic microorganisms have developed multiple drug resistance and caused nosocomial infections. Moreover, it was suggested that, plant extract from *Halodule pinifolia* can be further developed into antibiotic medicine due to their proven antifungal activity. In the present study and on testing the sensitivity of different fungal isolates to anti-biotics, it was found that they greatly differ, which might be due to continuous genetic recombination of microorganisms (Saleh 2000). The objective of testing plant extracts for potentials of synergy with antibiotics is to investigate if combinations of such extracts with antibiotics can bring about positive changes in the susceptibility of the test strains, thus necessitating the use of strains resistant to the test antibiotics.

Novel approach of antimicrobial agents

The antimicrobial and resistance modifying potentials of naturally occurring flavonoids and polyphenolic compounds have been reported in other studies, such as (Cushnie and Lamb 2005; Sato *et al.*, 2004). This would suggest that the synergy with antibiotics observed in this study could be attributable to such compounds. Combinatorial antibiotic therapy to control infections is a viable approach, but will not be effective for a long period of time because of the possible alteration in the susceptibility of bacteria (Rochon-Edouard *et al.*, 2000). Therefore, the development of new classes of antimicrobial compounds is of significant importance. One possible approach is to determine whether bioactive compounds from natural products and traditional medicinal plants, which have strong bactericidal activity against pathogenic microorganisms, either show synergistic interaction with antibiotics or enhance the susceptibility level of resistant strains to antibiotics.

Secondary metabolites

There are two main factors that greatly affect the fungal sensitivity to medicinal plant extracts. Concerning solvent effect (Al-Fatimi *et al.*, 2010) concluded that both methanol and ethyl acetate solvent extract showed strong activities against most human pathogenic fungi. In contrast, ethyl acetate extract showed broader and stronger antibacterial activities than the methanol. On the contrary, water extract did not show any biological activity.

Flavonoids and essential oils greatly affect the pathogenic microorganism where, (Chandrashekar and Kulkarni 2011) stated that, the ethyl acetate extract of *H.*

pinifolia leaves was flavones type compounds that inhibited *C. albicans* showing inhibitory zone. The antimicrobial studies show that these isolated compounds successfully inhibited *P. aeruginosa*, *K. pneumonia*, *S. aureus*, *Alcaligenes*, *E. coli*, *C. albicans* and *A. niger*. In this concern, (Kovac and Kocsis 2011) found that the antibacterial activity of the essential oils (thyme, clove, cinnamon, thymol, carvacrol, eugenol and cinnamic aldehyde) against *C. albicans* could be related to their most abundant components but the effect of the minor component should also be taken into consideration.

Our data agree with the reported literature and verify the antibacterial activity of the plant extract obtained from *H. pinifolia* contribute to the total quantity and quality of different compounds and finally to their increased antibacterial activity. Mixing plant extracts with different commercial antibiotics enhanced and synergized their antibacterial effect. In this regard, (Liu *et al.*, 2011) stated that many clinical isolates of *C. albicans* are resistant to numerous antimicrobials including fluoroquinolones (FQs). Flavonoids such as biochanin A (BCA) are naturally present in fruits, vegetables and can be combined with FQs to produce a powerful antimicrobial agent. The possible synergistic action between the antimicrobial agents BCA and ciprofloxacin (CPFX) when used in combination, the inhibitory concentration index (FICI) data showed that there was synergy in all 12 of the 12 *S. aurei* tested (Khalil and Li 2011).

Thoroughly, surveying the data analyses synergistic action of plant extract mixtures upon different groups of antibiotics, we can summarize that the aminoglycoside antibiotics (Ofloxacin and Levofloxacin) were synergized. The mode of action of these glycoside antibiotics are the inhibition of amino acid polymerization (Aronson 2010). Also, the β -lactam antibiotics (cefotaxime, spectinomycin GD) were synergized. The mode of action of these antibiotics is the binding to and inhibition of fungal enzyme located on the cell membrane that is involved in fungal cell wall synthesis (chitin), inhibition of cell growth, cell death and lysis. Ciprofloxacin (fluoroquinolone) antibiotics, tetracyclines (that inhibit ribosomal protein synthesis and vancomycin (inhibit fungal cell wall synthesis) were also synergized. Further studies to elucidate the detailed and specific mode of action of these plant extracts upon pathogenic microorganisms will have to be done.

According to their study they concluded that a persistent fungal infection, particularly in immunocompromised hosts, requires aggressive empiric therapy with at least two antimicrobial agents (Song *et al.*, 2003). Empiric combination antimicrobial therapy is usually applied to expand antifungal spectrum and reduce the selection of resistant mutants during treatment. In addition, combinations of agents that exhibit synergy or partial synergy could potentially improve the outcome for patients with difficult to treat infections (Song *et al.*, 2003).

Chapter 6: SUMMARY AND CONCLUSION

Antimicrobial resistance is of a major concern worldwide due to ineffectiveness of commercially available antibiotics which were once used to treat the infections caused by these organisms. Likewise, Fungal species such as *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Fusarium* sp., *Histoplasma capsulatum* and *Pneumocystis carinii* are gaining resistance against the antifungal agents including Flucanazole, itraconazole, amphotericin B, which were once used to cure the infections caused by these organisms. It is because of extensive usage of Antifungal agents in agriculture, veterinary medicine and in human medicines. Amongst the pathogenic *Candida* species *C. albicans* are the most virulent and common fungal human pathogen in humans to cause significant rates of mortality and morbidity. *C. albicans* are the opportunistic fungal pathogen which targets the mucosal surfaces of gastrointestinal tracts and immunosuppressed patients.

Medicinal plants which have been used since ancient period with antimicrobial properties are examined to treat such fungal infections. One of the most important marine angiosperms is Seagrass *H. pinifolia* which is detected with several active metabolites to have inhibit the growth of many microorganisms due to its antimicrobial properties. Thus, to overcome the antifungal resistance of *C. albicans* we have come up with a novel approach of **synergism** which is defined as the interaction of two or more drugs to have a greater combined effect compared to the sum of the effects seen when each drug is used individually. This is achieved by isolating the Bioactive compounds (Rosmarinic acid and Vanillic acid) from seagrass *H. pinifolia* which was effective against *C. albicans* and combine them with commercially available antibiotics which are reported to inhibit the growth of *C. albicans*. MIC values of antibiotics, Rosmarinic acid and Vanillic acid with different concentrations was used to analyse and carry out the combinatorial approach to combat the resistance of *C. albicans*.

Accordingly, we propose that the ethyl acetate extract from *H. pinifolia* showed potentials of synergy in combination with some antibiotics against reference strains

of pathogenic organisms that often-present problems of drug resistance. The detection of synergy between crude extract of *H.pinifolia* and antibiotics demonstrates the potential of this plant as a source of antibiotic resistance modifying compounds. It is necessary to carry out a bioassay guided fractionation of the ethyl acetate extract of this plant in a bid to isolate and identify the compounds responsible for the synergistic activity with antibiotics. An elucidation of the mechanisms of action of these compounds must be followed by toxicity and *in vivo* tests to determine the therapeutic applicability of such compounds in combination therapy. These are subjects of on-going investigation in our research group.

References

1. A.E. Mani, V. Bharathi, J. Patterson, Antibacterial activity and preliminary phytochemical analysis of seagrass *Cymodocea rotundata*, *Int. J. Microbiol. Res.* 3 (2) (2012) 99–103.
2. A.H.A. Hamdy, W.S.A. Mettwally, M.A. El Fotouh, B. Rodriguez, A.I. El-Dewany, S. A.A. El-Toumy, M.A.A. Hussein, Bioactive phenolic compounds from the Egyptian Red Sea seagrass *Thalassodendron ciliatum*, *Z. Naturforsch. C Biosci.* 67 (2012) 291–296.
3. Abdul, H.; Maleeha, S.; Syeda, Z. B.; Arshad, I.; Alam, K.; Dildar, K.; Samiullah, K.; Haji, K.; Aamer, A. S.; Fariha, H.; Safia, A.; Malik, B. A comprehensive investigation on the synergistic antibacterial activities of *Jatropha curcas* pressed cake and seed oil in combination with antibiotics. *AMB Expr.* 2019, 9.
4. Abreu AC, McBain AJ, Simões M. Plants as sources of new antimicrobials and resistance-modifying agents. *Nat Prod Rep* 2012;29:1007-21.
5. Abreu AC, Saavedra MJ, Simões LC, Simões M. Combinatorial approaches with selected phytochemicals to increase antibiotic efficacy against *Staphylococcus aureus* biofilms. *Biofouling.* 2016 Oct 20;32(9):1103-14.
6. Afifi FU, Abu-Irmaileh B. Herbal medicine in Jordan with special emphasis on less commonly used medicinal herbs. *Journal of Ethnopharmacology.* 2000 Sep 1;72(1-2):101-10.
7. Ahmad I, Beg AZ. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *Journal of ethnopharmacology.* 2001 Feb 1;74(2):113-23.
8. Aminov RI. The role of antibiotics and antibiotic resistance in nature. *Environmental microbiology.* 2009 Dec;11(12):2970-88.
9. Anand U, Jacobo-Herrera N, Altemimi A, Lakhssassi N. A comprehensive review on medicinal plants as antimicrobial therapeutics: potential avenues of biocompatible drug discovery. *Metabolites.* 2019 Nov 1;9(11):258.
10. Aslam B, Wang W, Arshad MI, Khurshid M, Muzammil S, Rasool MH, Nisar MA, Alvi RF, Aslam MA, Qamar MU, Salamat MK. Antibiotic resistance: a rundown of a global crisis. *Infection and drug resistance.* 2018;11:1645.
11. Atta-ur-Rahman and M.I. Choudhary. 1995. Diterpenoid and steroidal alkaloids. *Nat. Prod. Rep.* 12:361–379.

12. Basri DF, Fan SH (2005). The potential of aqueous and acetone extracts of galls of *Quercus infectoria* as antibacterial agents. *Indian J. Pharmacol.* 37(1): 26-29.
13. Beck-Sague´, C.M., W.R. Jarvis, and the National Nosocomial Infection Surveillance System. 1993. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980–1990. *J. Infect. Dis.* 167:1247–1251.
14. Bennett RN, Wallsgrove RM (1994). Secondary metabolites in plant defense mechanisms. *Phytother. Res.* 127: 617-633.
15. Ben-Yaacov R, Becker JM, Oppenheim JM, Oppenheim A, Goldway M, Schmidt R, Jiang W, Clifford J, Koltin Y (1995) Multi drug resistance in *Candida albicans*. *J Cell Biochem* 19B:146
16. Ben-Yaacov, R., Knoller, S., Caldwell, G.A., Becker, J.M., Koltin, Y., 1994. *Candida albicans* gene encoding resistance to benomyl and methotrexate is a multidrug resistance gene. *Antimicrobial Agents and Chemotherapy* 38, 648–652.
17. Berman, J., Sudbery, P.E., 2002. *Candida albicans*: a molecular revolution built on lessons from budding yeast. *Nat. Rev. Genet.* 3, 918–931.
<https://doi.org/10.1038/nrg948>.
18. Bevan P, Ryder H, Shaw I (1995) Identifying small-molecule lead compounds: The screening approach to drug discovery. *Trends Biotechnol* 13:115–121
19. Blunt, J.W.; Carroll, A.R.; Copp, B.R.; Davis, R.A.; Keyzers, R.A.; Prinsep, M.R. Marine Natural Products. *Nat. Prod. Rep.* 2018, 35, 8–53.
20. Brantner A, Grein E. Antibacterial activity of plant extracts used externally in traditional medicine. *Journal of ethnopharmacology.* 1994 Aug 1;44(1):35-40.
21. Caceres A, Cano O, Samayoa B, Aguilar L. Plants used in Guatemala for the treatment of gastrointestinal disorders. 1. Screening of 84 plants against enterobacteria. *Journal of Ethnopharmacology.* 1990 Aug 1;30(1):55-73.
22. Cannon RD, Holmes AR, Mason AB, Monk BC. Oral *Candida*: clearance, colonization, or candidiasis?. *Journal of dental research.* 1995 May;74(5):1152-61.
23. Cheesman MJ, Ilanko A, Blonk B, Cock IE. Developing new antimicrobial therapies: are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution?. *Pharmacognosy reviews.* 2017 Jul;11(22):57.
24. Chovanova R, Mikulašova M, Vaverkova S. *In vitro* antibacterial and antibiotic resistance modifying effect of bioactive plant extracts on methicillin-resistant *Staphylococcus epidermidis*. *Int J Microbiol* 2013;2013:760969.

25. Cosgrove SE. 2006. The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay and healthcare costs. *Clin Infect Dis* 42(Suppl 2):S82–S89.
26. Coste AT, Turner V, Ischer F, Morschhäuser J, Forche A, Selmecki A, Berman J, Bille J, Sanglard D. 2006. A mutation in Tac1p, a transcription factor regulating CDR1 and CDR2, is coupled with loss of heterozygosity at Chromosome 5 to mediate antifungal resistance in *Candida albicans*. *Genetics* 172: 2139–2156.
27. Costerton JW. Introduction to biofilm. *Int J Antimicrob Agents*. 1999;11:217–21.
28. Danaraj J, Mariasingarayan Y, Ayyappan S, Karuppiah V. Seagrass *Halodule pinifolia* active constituent 4-methoxybenzoic acid (4-MBA) inhibits quorum sensing mediated virulence production of *Pseudomonas aeruginosa*. *Microbial Pathogenesis*. 2020 Oct 1;147:104392.
29. 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.
30. Daniel, M. W.; Meghan, A. W.; Carey-Ann, D. B. Stop waiting for tomorrow: Disk diffusion performed on early growth is an accurate method for antimicrobial susceptibility testing with reduced turnaround time. *Journal of Clinical Microbiology*. 2022, 60, 5.
31. Daniels, K.J., Srikantha, T., Lockhart, S.R., Pujol, C., Soll, D.R., 2006. Opaque cells signal white cells to form biofilms in *Candida albicans*. *EMBO J.* 25, 2240–2252. <https://doi.org/10.3410/f.1032148.374112>.
32. Dasgupta F (1998) Antifungal agents – past, present and future possibilities. In: Varma RS, Khan IK, Singh AP (eds) *Antifungal agents past, present, future prospect*. National Academy of Chemistry and Biology, Lucknow, pp 29–54
33. Davis, J., 1994. Inactivation of antibiotic and the dissemination of resistance genes. *Science* 264, 375–382.
34. Diaz Granados CA, Zimmer SM, Klein M, Jernigan JA. 2005. Comparison of mortality associated with vancomycin-resistant and vancomycin-susceptible enterococcal blood-stream infections: a meta-analysis. *Clin Infect Dis* 41:327–333.
35. Dixon RA, Dey PM, Lamb CJ (1983). Phytoalexins: enzymology and molecular biology. *Adv. Enzymol.* 55: 1-69.

36. Doddanna SJ, Patel S, Sundarrao MA, Veerabhadrapa RS. Antimicrobial activity of plant extracts on *Candida albicans*: An in vitro study. *Indian Journal of Dental Research*. 2013 Jul 1;24(4):401.
37. Doughari JH (2006). Antimicrobial activity of *Tamarindus indica* Linn. *Trop. J. Pharm. Res*. 5(2): 597-603.
38. Doughari JH. Hand washing-its role in preventing water, foodborne and healthcare-associated diseases. *CABI Reviews*. 2019 Aug 28(2019):1-0.
39. Du, W., H. Chen, S. Xiao, W. Tang, and G. Shi. 2017. New insight on antimicrobial therapy adjustment strategies for Gram-negative bacterial infection. A cohort study. *Medicine* 96:e6439.
40. Dunkel N, Blass J, Rogers PD, Morschhäuser J. 2008. Mutations in the multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of MDR1 overexpression in fluconazole-resistant *Candida albicans* strains. *Mol Microbiol* 69: 827–840.
41. Eze, E. A.; Oruche, N. E.; Eze, C. N. Interaction of the extracts of three medicinal plants with antibiotics against some antibiotic-resistant bacteria. *Academic Journals*. 2013, 8, 1360-1367.
42. Fadila, M.; Tajelmolk, A. Evaluation of antibacterial activity and synergistic effect between antibiotic and the essential oils of some medicinal plants. *Asian Pac. J. Trop. Biomed*. 2016, 6, 32-37.
43. Fraser VJ, Jones M, Dunkel J, Storer S, Medoff G, Dunagan WC. *Candidaemia* in a tertiary care hospital: epidemiology, risk factors, and predictors of mortality. *Clin Infect Dis* 1997; 15: 414-21.
44. Geissman, T.A. 1963. Flavonoid compounds, tannins, lignins and related compounds, p.265. In M. Florkin and E.H. Stotz (ed.), *Pyrrole pigments, isoprenoid compounds and phenolic plant constituents*, vol.9. Elsevier, New York, N.Y.
45. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, Gillevet PM. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS pathogens*. 2010 Jan 8;6(1):e1000713.
46. Ghannoum, M.A., Rice, L.B., 1999. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clinical Microbiology Reviews* 12, 501–517.

47. Ghareeb, M.A.; Tammam, M.A.; El-Demerdash, A.; Atanasov, A.G. Insights about Clinically Approved and Preclinically Investigated Marine Natural Products. *Curr. Res. Biotechnol.* 2020, 2, 88–102.
48. Gideon M, Ladan Z. Synergistic combinatorial strategy for combating Antimicrobial Resistance (AMR) in clinical bacteria by combining antibiotics with plant extracts. *Fine Chemical Engineering.* 2023 Jan 17:1-2.
49. Glombitza KW, Mahran GH, Mirhom YW, Michel KG, Motawi TK. Hypoglycemic and antihyperglycemic effects of *Zizyphus spina-christi* in rats. *Planta medica.* 1994 Jun;60(03):244-7.
50. Gokce, M.Z. Haznedaroglu, Evaluation of antidiabetic, antioxidant and vasoprotective effects of *Posidonia oceanica* extract, *J. Ethnopharmacol.* 115 (2008) 122–130.
51. Grignon-Dubois, M.; Rezzonico, B. Phenolic Fingerprint of the Seagrass *Posidonia oceanica* from Four Locations in the Mediterranean Sea: First Evidence for the Large Predominance of Chicoric Acid. *Bot. Mar.* 2015, 58, 379–391.
52. Guarro J, Llop C, Aguilar C, Pujol I (1997) Comparison of in vitro antifungal susceptibilities of conidia and hyphae of filamentous fungi. *Antimicrob Agents Chemother* 41:2760–2762.
53. Hall MJ, Middleton RF, Westmacott D. The fractional inhibitory concentration (FIC) index as a measure of synergy. *J Antimicrob Chemother* 1983;11:427-33.
54. Hassawi D, Kharma A. Antimicrobial activity of some medicinal plants against *Candida albicans*. *J. Biol. Sci.* 2006;6(1):109-14.
55. Hayat S, Sabri AN. Screening for antibiofilm and antioxidant potential of turmeric (*Curcuma longa*) extracts. *Pak J Pharm Sci.* 2016;29(4):1163-70.
56. Heinrich, M. Ethnopharmacology in the 21st century-grand challenges. *Frontiers in Pharmacology.* 2010, 1.
57. Hirakawa MP, Martinez DA, Sakthikumar S, Anderson MZ, Berlin A, Gujja S, Zeng Q, Zisson E, Wang JM, Greenberg JM, Berman J. Genetic and phenotypic intra-species variation in *Candida albicans*. *Genome research.* 2015 Mar 1;25(3):413-25.
58. Hua, H.Y. Hsu, Y.C. Su, I.F. Lin, S.S. Yang, Y.M. Chen, *et al.*, Study on the antiinflammatory activity of methanol extract from Seagrass *Zostera japonica*, *J. Agric. Food Chem.* 54 (2) (2006) 306–311.

59. Inui T, Wang Y, Deng S, Smith DC, Franzblau SG, Pauli GF. Counter-current chromatography based analysis of synergy in an anti-tuberculosis ethnobotanical. *J Chromatogr A* 2007;1151:211-5.
60. Iyapparaj, P.; Revathi, P.; Ramasubburayan, R.; Prakash, S.; Palavesam, A.; Immanuel, G.; Anantharaman, P.; Sautreau, A. Hellio, C. Antifouling and Toxic Properties of the Bioactive Metabolites from the Seagrasses *Syringodium isoetifolium* and *Cymodocea serrulata*. *Ecotoxicol. Environ. Saf.* 2014, 103, 54–60.
61. Jeyapragash, P. Subhashini, S. Raja, G. Abirami, T. Thangaradjou, Seagrass as a Potential Source of Natural Antioxidants. *Free Radicals and Antioxidants*, 2016.
62. Kannan R.R.R, R. Arumugam, M. Grignon Dubois, P. Anantharaman, Antioxidant activity of seagrasses of the Mandapam coast, India, *Pharmaceut. Biol.* 50 (2) (2012) 182–187.
63. Kannan R.R.R, 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.
64. Kannan R.R.R., R. Arumugam, P. Anantharaman, Antibacterial potential of three Seagrasses against Human pathogens, *Asian Pac. J. Tropical Med.* 11 (2010) 890–893
65. Kim, D.H.; Mahomoodally, M.F.; Sadeer, N.B.; Seok, P.G.; Zengin, G.; Palaniveloo, K.; Khalil, A.A.; Rauf, A.; Rengasamy, K.R. Nutritional and Bioactive Potential of Seagrasses: A Review. *S. Afr. J. Bot.* 2021, 137, 216–227.
66. Kurtz MB (1998) New antifungal drug targets: a vision for the future. *ASM News* 64:31–35
67. Levinson W, Jawetz E. *Medical Microbiology and Immunology: Examination and Board Review*. NY, USA: Lange Medical Books/McGraw-Hill; 2002.
68. Lozano, C., and C. Torres. 2107. [Update on antibiotic resistance in Gram positives]. *Enferm. Infecc. Microbiol. Clin.* 35:2–8.
69. 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.
70. M.S. Ali, S. Ravikumar, J.M. Beula, Bioactivity of seagrass against the dengue fever mosquito *Aedes aegyoti* larvae, *Asian Pac. J. Tropical Biomed.* 2 (7) (2012) 570–573.
71. Maciá, M.D.,Blanquer,D.,Togores,B.,Sauleda,J.,Pérez, J.L., and Oliver,A. (2005) Hypermutation is a keyfactor in development of multiple-antimicrobial resistance in

Pseudomonas aeruginosa strains causing chronic lung infections. *Antimicrob Agents Chemother* 49: 3382–3386.

72. Mani, A.E.; Bharathi, V.; Patterson, J.; Devadason, S. Antibacterial Activity and Preliminary Phytochemical Analysis of Sea Grass *Cymodocea Rotundata*. *Int. J. Microbiol. Res.* 2012, 3, 99–103.
73. Marie, B. C. (2005): Manual of antimicrobial susceptibility testing, ASM press, Washington, D.C.
74. Marine Pharmacology. Available online: <https://www.marinepharmacology.org/> (accessed on 2 May 2022).
75. Martin MV. The use of fluconazole and itraconazole in the treatment of *Candida albicans* infections: a review. *Journal of Antimicrobial Chemotherapy.* 1999 Oct 1;44(4):429-37.
76. Mary S, Bharathidasan R, Ramamurthy V. Phytochemical Screening and Antimicrobial Potential of *Halodule pinifolia*. *Asian Journal of Research in Biochemistry.* 2022 Dec 2;11(2):18-22.
77. McGinnis MR, Rinaldi MG (1991) Antifungal drugs: mechanisms of action, drug resistance, susceptibility testing and assays of activity in biological fluids. In: Lorain V (ed) *Antibiotics in laboratory medicine*. Williams and Wilkins, Baltimore, pp 198–257
78. 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.
79. Miller, M.G., Johnson, A.D., 2002. White-opaque switching in *Candida albicans* is controlled by mating-type locus homeodomain proteins and allows efficient mating. *Cell* 110, 293–302. <https://doi.org/10.3410/f.1008675.109957>.
80. Millsop, J.W., Fazel, N., 2016. Oral candidiasis. *Clin. Dermatol.* 34, 487–494. <https://doi.org/10.1016/j.clindermatol.2016.02.022>.
81. Misato TK, Yamaguchi I (1977) Use of antibiotics in agriculture. *Adv Appl Microbiol* 21:53–88
82. Mishra RP, Oviedo-Orta E, Prachi P, Rappuoli R, Bagnoli F. Vaccines and antibiotic resistance. *Curr Opin Microbiol* 2012;15:596-602
83. Munita JM, Arias CA. Mechanisms of antibiotic resistance. Virulence mechanisms of bacterial pathogens. 2016 Jun 22:481-511.

84. Ncube NS, Afolayan AJ and Okoh AI (2008). Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. *African Journal of Biotechnology*, 7(12): 1797-1806.
85. O'Kennedy R, Thornes RD (1997). Coumarins: biology, applications and mode of action. (eds), John Wiley & Sons, Inc., New York, N.Y.
86. Odds FC (1993) Resistance of yeasts to azole-derivative antifungals. *J Antimicrob Chemother* 22:473–481
87. Olga, P.; Blasco, L.; Bleriot, I.; Fernandez-Garcia, L.; Bardanca, G. M.; Ambroa, A.; López, M.; Bou, G.; Tomás, M. Strategies to combat multidrug-resistant and persistent infectious diseases. *Antibiotics*. 2020, 9, 65.
88. Pathak AN (1998) Antifungal agents to manage mycotic infections. In: Varma R S, Khan IK, Singh AP (eds) Antifungal agents past, present, future prospect. National Academy of Chemistry and Biology, Lucknow, pp 149–186
89. Phoebe CH, Combie J, Albert FG, Tran KV, Cabrera J, Correia HJ, Guo Y, Lindermuth J, Rauert N, Galbraith W, Selitrennikoff CP (2001) Extremophilic organisms as an unexplored source of antifungal compounds. *J Antibiot* 54:56–65
90. Pittet D, Wenzel RP. Nosocomial bloodstream infections. Secular trends in rates, mortality, and contribution to total hospital deaths. *Arch Intern Med* 1995; 155: 1177-84.

REFERENCES

91. Rungsung W, Ratha KK, Dutta S, Dixit AK, Hazra J. Secondary metabolites of plants in drugs discovery. *World Journal of Pharmaceutical Research*. 2015 May 6;4(7):604-13.
92. Scalbert, A. 1991. Antimicrobial properties of tannins. *Phytochemistry* 30: 3875–3883.
93. Scully C, Ei-Kabir M, Samaranayake LP. Candida and oral candidosis: a review. *Critical Reviews in Oral Biology & Medicine*. 1994 May;5(2):125-57.
94. Sharma D, Misba L, Khan AU. Antibiotics versus biofilm: an emerging battleground in microbial communities. *Antimicrobial Resistance & Infection Control*. 2019;8(1):76.
95. 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.

96. Sofowora A. Research on medicinal plants and traditional medicine in Africa. *The Journal of Alternative and Complementary Medicine*. 1996 Sep 1;2(3):365-72.
97. Sofowora A. The present status of knowledge of the plants used in traditional medicine in western Africa: a medical approach and a chemical evaluation. *Journal of Ethnopharmacology*. 1980 Jun 1;2(2):109-18.
98. Srivastava J, Chandra H, Nautiyal AR, Kalra SJ. Antimicrobial resistance (AMR) and 5 plant-derived antimicrobials (PDAMs) as an alternative drug line to control infections. *3 Biotech* 2014;4:451-60.
99. Sun,Y.,J.Ye,Y.Hou,H.Chen,J.Cao,andT.Zhou. 2107. Predation efficacy of *Bdellovibrio bacteriovorus* on multidrug-resistant clinical pathogens and their corresponding biofilms. *Jpn. J. Infect. Dis.* 70:485–489.
100. Tacconelli, E., M.A. Cataldo, S.J. Dancer, G. De Angelis, M. Falcone, U. Frank, G. Kahlmeter, A. Pan, N. Petrosillo, J. Rodriguez-Bano, N. Singh, M. Venditti, D.S. Yokoe, and B. Cookson. 2014. ESCMID guidelines for the management of the infection control measures to reduce transmission of multidrug-resistant Gram-negative bacteria in hospitalized patients. *Clin. Microbiol. Infect.* 20: 1–55.
101. Taguri, T.; Tanaka, T.; Kouno, I. Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. *Biol. Pharm. Bull.* 2006, 29, 2226-2235.
102. Tallarida RJ. An overview of drug combination analysis with isobolograms. *J Pharmacol Exp Ther* 2006;319:1-7.
103. Tanaka JCA, da Silva CC, de Oliveira AJB, Nakamura CV (2006). Dias Filho BP Antibacterial activity of indole alkaloids from *Aspidosperma ramiflorum*. *Braz. J. Med. Biol. Res.* 39(3): 387-391.
104. Tkacz JS (1992) Glucan biosynthesis in fungi and its inhibition. In: Sutcliffe J, Georgopapadakou NH (eds) *Emerging targets in antibacterial and antifungal chemotherapy*. Chapman and Hall, New York, pp 495–523
105. Todd OA, Fidel Jr PL, Harro JM, Hilliard JJ, Tkaczyk C, Sellman BR, Noverr MC, Peters BM. *Candida albicans* augments *Staphylococcus aureus* virulence by engaging the staphylococcal agr quorum sensing system. *MBio*. 2019 Jun 25;10(3):e00910-19.
106. Todd OA, Peters BM. *Candida albicans* and *Staphylococcus aureus* pathogenicity and polymicrobial interactions: lessons beyond Koch's postulates. *Journal of Fungi*. 2019 Sep 4;5(3):81.

107. U.S. Centers for Disease Control and Prevention. *Antibiotic Resistance Threats Report*. Department of Health and Human Services, CDC. Atlanta, GA. Antibiotic Resistance Coordination and Strategy Unit. 2019.
108. Unsworth, R.; van Keulen, M.; Coles, R. Seagrass Meadows in a Globally Changing Environment. *Mar. Pollut. Bull.* 2014, 83, 383–386.
109. Van Vuuren S, Viljoen A. Plant-based antimicrobial studies – Methods and approaches to study the interaction between natural products. *Planta Med* 2011;77:1168-82.
110. Vandamme EJ (1984) Antibiotic search and production: an overview. In: Vandamme EJ (ed) *Biotechnology of industrial antibiotics*, vol 22. Dekker, New York, pp 3–31
111. Vats V, Grover JK, Rath SS. Evaluation of anti-hyperglycemic and hypoglycemic effect of *Trigonella foenum-graecum* Linn, *Ocimum sanctum* Linn and *Pterocarpus marsupium* Linn in normal and alloxanized diabetic rats. *Journal of ethnopharmacology*. 2002 Feb 1;79(1):95-100.
112. Velasco García, Wendy Johana. "Efecto potencia inhibitorio de compuestos naturales sobre quorum sensing en *Pseudomonas aeruginosa*." (2022).
113. Ventola, C.L. 2015. The antibiotic resistance crisis: part 1: causes and threats. *P T* 40:277–283.
114. White TC. 1997. The presence of an R467K amino acid substitution and loss of allelic variation correlate with anazole-resistant lanosterol 14 α demethylase in *Candida albicans*. *Antimicrob Agents Chemother* 41: 1488–1494.
115. WHO. Global Tuberculosis Report 2016. World Health Organization; 2016. Available from: http://www.who.int/tb/publications/global_report/en/. [Last cited on 2017 Feb 10].
116. Wilson DN. Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nature Reviews Microbiology*. 2014 Jan;12(1):35-48.
117. Yerevan State Medical University [Online]. 2017. https://www.ysmubooks.am/uploads/Ph_Ch_textbook.pdf (accessed Aug 16, 2022).