

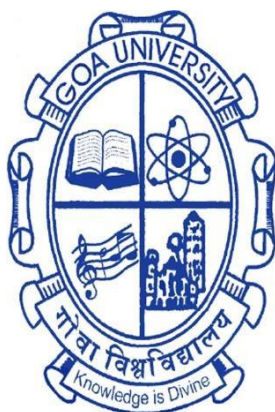
# Bioactive Cationic Peptides from Mangrove Plants & Marine Microbes

A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE OF

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in  
**Biotechnology**

IN THE **School of Biological Sciences and Biotechnology**

**GOA UNIVERSITY**



by

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**April 2024**

## **DECLARATION**

I, Pingal G. Naik, hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any other University or Institution for the award of any research degree.

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## **CERTIFICATE**

I hereby certify that the above Declaration of the candidate, Pingal G. Naik, is true and that the work was carried out under my supervision.

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Dedicated to  
My Family

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# **CHAPTER 1:**

## **General Introduction**

### **&**

## **Review of Literature**

## 1.1 Introduction

Bioactive peptides can be defined as specific protein fragments that have a positive impact on the functioning or conditions of living beings, thereby improving their health (Korhonen & Pihlanto 2003). Traditionally, small molecule drugs with a molecular mass of 500 Da or less have been used as medicine. In the 20<sup>th</sup> century, peptides have gained importance as a relatively novel class of therapeutics (Sewald & Jakubke, 2009). Their beneficial effects (Fig. 1.1) include properties such as antimicrobial (Reddy et al., 2004; Rajanbabu & Chen 2011), antioxidant (Sarmadi & Ismail 2010), antithrombotic (Wang & Ng 1999), anti-hypertensive (Erdmann et al. 2008), and immunomodulatory activities (St Georgiev 1990; Gauthier et al. 2006). The activity of peptides depends on their structure and amino acid composition (Karami & Akbari-adergani, 2019).

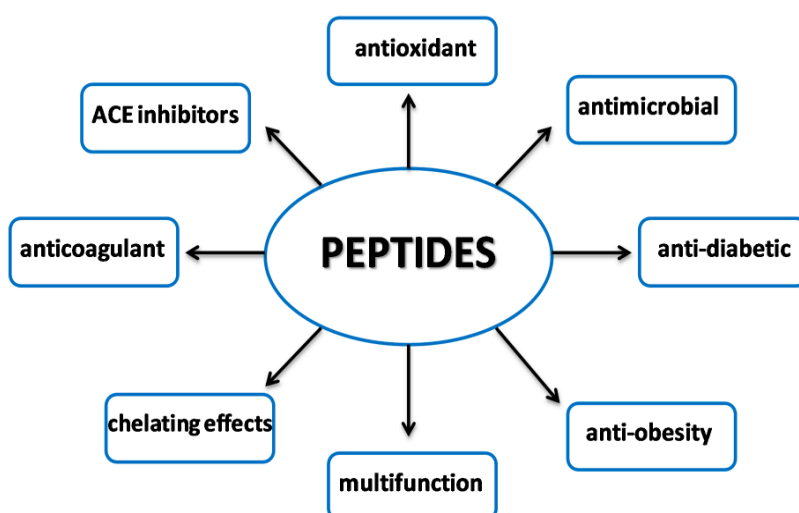


**Fig 1.1:** Properties of bioactive peptides. [adapted from Karami & Akbari-adergani, 2019]

The present study is on antimicrobial peptides, more specifically antimicrobial cationic peptides, and their potential bioactivities. A brief history of conventional antibiotics and the advantage of cationic peptides over conventional antibiotics in tackling antibiotic resistance is documented below.

### 1.1.1. Antibiotics –A brief history

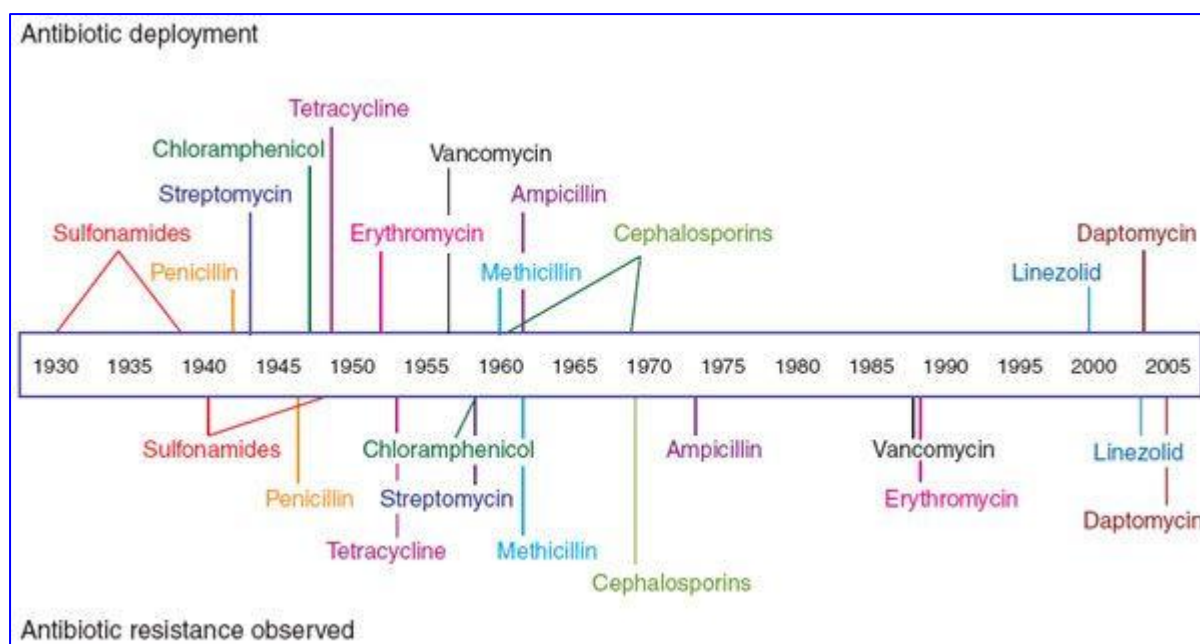
Antibiotics have been used since millennia to treat infections that posed a threat to human health. Paul Ehrlich, in the year 1907, discovered a chemical known as arsphenamine which

was used as an effective treatment for syphilis, caused by the spirochete *Treponema pallidum* (Williams, 2009). These synthetic compounds containing a sulfonamide group inhibited the enzyme dihydropteroate synthetase involved in folate biosynthesis, thereby killing the pathogens. Later these sulfonamides were used to treat clinical infections and hence became the first known antibiotic. Although the term antibiotic was not used at that point of time, Ehrlich referred to his discovery as 'chemotherapy'. The first commercially available sulpha drug was 'Prontocil', for which work Gerhard Domagk received the Nobel prize in the year 1939 (Otten, 1986).

Although there were drugs available for treatment of bacterial infections, the term 'antibiotics' did not come into existence until the discovery of 'Penicillin'. Selman Waksman, the Ukrainian-American inventor and microbiologist was the first to use the term 'antibiotics' in the year 1943. The Nobel prize of 1945 was awarded jointly to Sir Alexander Fleming for his discovery of penicillin in 1928, along with Howard Florey and Ernst Boris Chain for its mass production (Chain et al., 2005). It was a path-breaking discovery of the first true antibiotic which was used in the treatment of streptococcal and chlamydial infections (Aminov, 2010) which has paved the way for a new era in modern medicine. The search for new antibiotics continued and most of the antibiotics being used today were discovered within the short period between the 1950s and 1970s, (Fig. 1.2), and could treat majority of the infections including tuberculosis, cholera, malaria, etc.

### **1.1.2. Antibiotic resistance (AMR)**

While antibiotics became widely available for the treatment of various diseases, their excessive use has led to the emergence of antimicrobial resistance in the pathogens (Neu, 1992). It became a serious problem (as summarized in Fig. 1.2), to the extent that in the year 2018, the WHO listed antimicrobial resistance among the top 10 global threats.



**Timeline of antibiotic discovery and the evolution of antibiotic resistance.** The year above the timeline depicts the discovery of the antibiotic and the year below the timeline depicts the resistance to each antibiotic.

**Fig. 1.2:** History of the discovery of various antibiotics and antibiotic resistance observed [adapted from Clatworthy et al. 2007]

As per the WHO, some of the causes of antimicrobial resistance are 1) overprescribing of antibiotics, 2) patients not completing their treatment course, 3) overuse of antibiotics in livestock and fish farming, 4) poor infection control in hospitals and clinics, 5) lack of hygiene and poor sanitation, and 6) lack of new antibiotics being developed. Listed below are some classes of antibiotics, their target of action and the mode of resistance shown by the pathogens.

**Table 1.1.** Modes of action and resistance mechanisms of commonly used antibiotics [adapted from Davies & Davies, 2010].

| Antibiotic class                  | Example(s)                                                                                         | Target                     | Mode(s) of resistance  |
|-----------------------------------|----------------------------------------------------------------------------------------------------|----------------------------|------------------------|
| <b><math>\beta</math>-Lactams</b> | Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam) | Peptidoglycan biosynthesis | Efflux, altered target |

|                          |                                         |                            |                                                                                                |
|--------------------------|-----------------------------------------|----------------------------|------------------------------------------------------------------------------------------------|
| <b>Aminoglycosides</b>   | Gentamicin, streptomycin, spectinomycin | Translation                | Phosphorylation, acetylation, nucleotidylation, efflux, altered target                         |
| <b>Glycopeptides</b>     | Vancomycin, teicoplanin                 | Peptidoglycan biosynthesis | Reprogramming peptidoglycan biosynthesis                                                       |
| <b>Tetracyclines</b>     | Minocycline, tigecycline                | Translation                | Monooxygenation, efflux, altered target                                                        |
| <b>Macrolides</b>        | Erythromycin, azithromycin              | Translation                | Hydrolysis, glycosylation, phosphorylation, efflux, altered target                             |
| <b>Lincosamides</b>      | Clindamycin                             | Translation                | Nucleotidylation, efflux, altered target                                                       |
| <b>Streptogramins</b>    | Synercid                                | Translation                | C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target |
| <b>Oxazolidinones</b>    | Linezolid                               | Translation                | Efflux, altered target                                                                         |
| <b>Phenicols</b>         | Chloramphenicol                         | Translation                | Acetylation, efflux, altered target                                                            |
| <b>Quinolones</b>        | Ciprofloxacin                           | DNA replication            | Acetylation, efflux, altered target                                                            |
| <b>Pyrimidines</b>       | Trimethoprim                            | C1 metabolism              | Efflux, altered target                                                                         |
| <b>Sulfonamides</b>      | Sulfamethoxazole                        | C1 metabolism              | Efflux, altered target                                                                         |
| <b>Rifamycins</b>        | Rifampin                                | Transcription              | ADP-ribosylation, efflux, altered target                                                       |
| <b>Lipopeptides</b>      | Daptomycin                              | Cell membrane              | Altered target                                                                                 |
| <b>Cationic peptides</b> | Colistin                                | Cell membrane              | Altered target, efflux                                                                         |

Infections due to bacterial pathogens resistant to antimicrobial drugs currently claim 7 lakh lives globally each year, a number projected to soar to 10 million deaths annually by 2050. This surge could cost the global economy a staggering \$100 trillion due to decreased productivity. Moreover, the COVID-19 pandemic might worsen AMR, a US multicenter study having found that 72% of COVID-19 patients received unnecessary antibiotics, potentially fuelling the rise of antimicrobial resistance (Strathdee et al., 2020).

In India, antibiotic-resistant bacterial infections are being increasingly reported among neonates. A review of bloodstream infections among neonates and children born between 2000 and 2015 showed that the most common pathogens isolated were *S. aureus* and *Klebsiella* sp.

(Dharmapalan et al., 2017). In this study 50% of the *S. aureus* isolates were methicillin resistant *S. aureus* (MRSA), and 63% of *Klebsiella* sp. were third generation cephalosporin resistant. In a study conducted between 2011 and 2014 in three neonatal intensive care units in New Delhi, India, *Acinetobacter* sp. and *Klebsiella* sp. were found to be the two most frequent organisms isolated in neonatal sepsis cases (Chaurasia et al., 2019). Of these, being resistant to three or more antibiotic classes, 82% of the *Acinetobacter* sp. and 54% of the *Klebsiella* sp. were labelled as multidrug resistant. However, the most concerning issue is that 78% of *Acinetobacter* sp. and 35% of the *Klebsiella* sp. were carbapenem resistant. Detailed information on the study of antimicrobial resistant infections in different parts of India and their resistance percentage is presented in Table 1.2.

**Table 1.2:** Antibiotic resistance development rates of various organisms in India [adapted from Kumar et al., 2013]

| Location (year published)                         | Isolates                                                   | Organism                                                                                                                                    | Resistance rate (%)                                                                                                                                                                                                                                           |
|---------------------------------------------------|------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| MVIDH, Delhi (2007)                               | 9858 stool samples                                         | <i>V. cholera</i> 01                                                                                                                        | 96 to furazolidone, Cotrimoxazole and nalidixic acid.                                                                                                                                                                                                         |
| Kolkata (2007)                                    | 284 clinical isolates                                      | <i>metallo-beta-lactamase (MBL) producing bacteria</i>                                                                                      | 43.3 were resistant to at least seven antibiotics (ampicillin, amoxicillin, cephalixin, ciprofloxacin, cotrimaxazole, erythromycin, gentamycin)                                                                                                               |
| Lucknow (2007)                                    | 2995 blood samples                                         | <i>Klebsiellaspp.</i>                                                                                                                       | ESBL producing <i>Klebsiellaspp.</i> were 98.28 resistant to ampicillin, ticarcillin and piperacillin. Monobactam and cephalosporin resistance was also higher (>60%).                                                                                        |
| Puducherry (2008)                                 | 261 clinical isolates                                      | <i>Staphylococcus isolates</i>                                                                                                              | 72.34 of <i>staphylococcus aureus</i> resistant to oxacillin                                                                                                                                                                                                  |
| Nagpur (2009)                                     | 1300 nasopharyngeal swabs from school children             | MRSA                                                                                                                                        | 4.16                                                                                                                                                                                                                                                          |
| CMC Vellore, Various centres across India (2010)  | 176 clinical specimens                                     | <i>P. aeruginosa</i>                                                                                                                        | Among the 61 <i>P. aeruginosa</i> isolates, resistance to carbapenem was 42.6.                                                                                                                                                                                |
| Puducherry (2010)                                 | 31 clinical samples                                        | <i>K. Pneumoniae</i>                                                                                                                        | 93.55 multiple drug resistant and ESBL producer                                                                                                                                                                                                               |
| Mangalore (2010)                                  | 83 CA-MRSA clinical isolates                               | Community-associated methicillin resistant <i>Staphylococcus aureus</i> (CA-MRSA) strains                                                   | 92.8% were resistant to penicillin, 31.32 to erythromycin.                                                                                                                                                                                                    |
| LokNayak Hospital, New Delhi (2010)               | 83 isolates from OPD cases of pyoderma                     | CA-MRSA                                                                                                                                     | 9.6                                                                                                                                                                                                                                                           |
| Mangalore (2010)                                  | 180 clinical samples                                       | Enterococcal strains                                                                                                                        | 16.67 to 42.86 to aminoglycosides                                                                                                                                                                                                                             |
| Sikkim (2011)                                     | 291 clinical specimens 196 carrier screening nasal samples | MRSA                                                                                                                                        | 38.14 in clinical specimens 20.92 in nasal samples                                                                                                                                                                                                            |
| Tertiary trauma center of AIIMS, New Delhi (2011) | 3,984 clinical specimens                                   | <i>Gram Negative Pseudomonas Acinetobacter Klebsiella E. coli Enterobacter spp. Gram positive S.aureus Coagulase negative staphylococci</i> | Overall resistance of gram negative organisms were 50 against carbapenems, 66 aminoglycosides, 76 Fluoroquinolones, 88 third generation cephalosporins, 66 beta lactam-betalactamase inhibitor combinations 58 methicillin resistant 85 methicillin resistant |

Considering the current situation and the threat posed by AMR on health-care, the World Health Assembly (WHA) adopted the Global Action Plan (GAP) on AMR in 2015 (<http://www.who.int/antimicrobial-resistance/global-action-plan/en/>). Following this, many countries have formulated their own National Action Plans (NAPs). The Indian Ministry of Health and Family Welfare published the National Action Plan for containing AMR in April 2017 ([http://www.searo.who.int/india/topics/antimicrobial\\_resistance/nap\\_amr.pdf](http://www.searo.who.int/india/topics/antimicrobial_resistance/nap_amr.pdf)). It was submitted at the 70th WHA in Geneva in May 2017. The NAP on AMR (2017–2021) is involved in planning and implementation of strategies for curbing AMR in India.

The global threat posed by AMR has led researchers to search for new antibiotics. The two approaches used to tackle AMR were (i) modifying the existing conventional antibiotics by targeting the “resistance factors” and (ii) finding new antibiotics with novel modes of action (Setti & Micetich, 1998). It was observed that bacteria could easily develop resistance by the same mechanism to the analogs of existing antibiotics, which made the search for new class(es) of antibiotics with different target of action increasingly important.

Among the molecules under consideration, antimicrobial peptides have been receiving considerable attention. These peptides, which are encoded by genes (Martin et al., 1995), are widely distributed in nature (Gabay, 1994) and are integral components of the non-specific defence systems in both plants and animals (Boman, 1995).

### **1.1.3. What are Antimicrobial cationic peptides?**

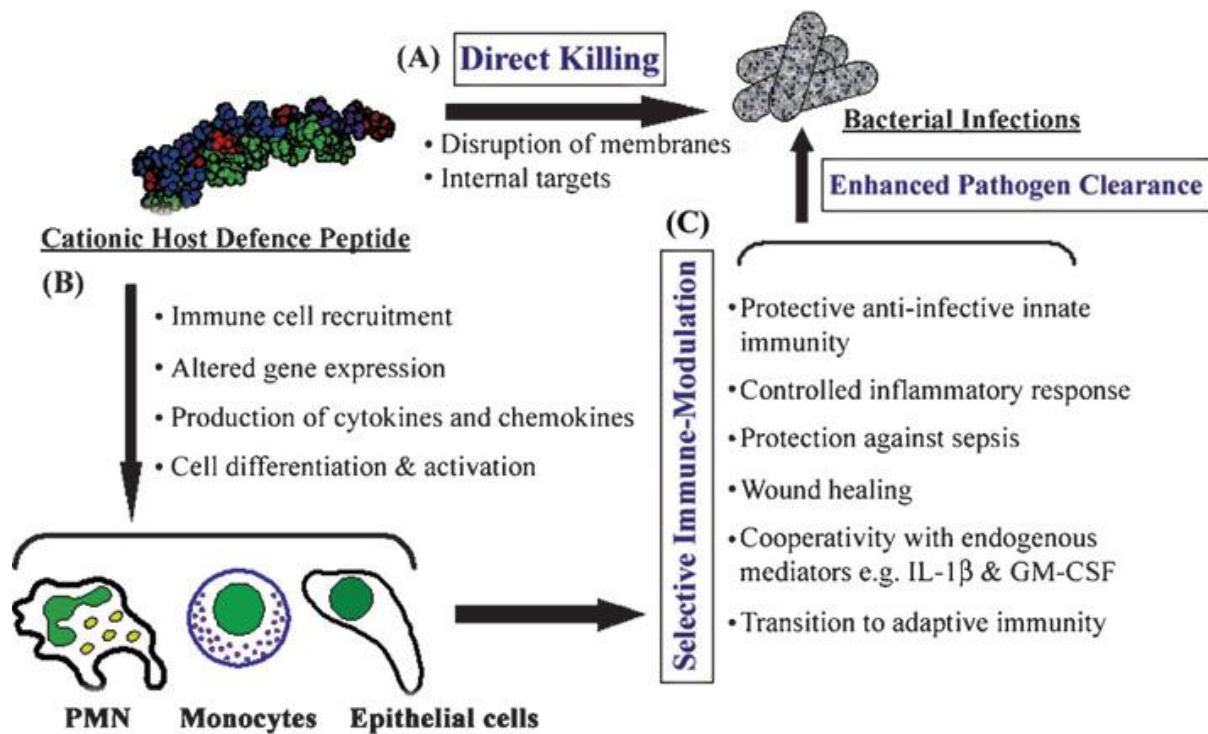
Antimicrobial Cationic Peptides (ACPs) are widespread natural antibiotics found in nearly all organisms, serving as a vital component of the innate immune system. They are positively charged peptides containing 12-50 amino acids and with a net charge of +2 to +7. The positive charge is due to the relative abundance of basic amino acid residues viz., arginine, lysine and histidine, over the acidic amino acids. Cationic peptides are amphipathic, possessing both hydrophilic and hydrophobic amino acids. They consist approximately of 50% hydrophobic residues, which helps them to interact with the membrane of bacteria and kill them (Hancock & Chappel, 1999; Zasloff, 2002; Reddy et al., 2004; Jenssen et al., 2006).

Cationic antimicrobial peptides exhibit a broad spectrum of activity, targeting various organisms including Gram-negative and Gram-positive bacteria, fungi, eukaryotic parasites,

and viruses (Huan et al., 2020). Cationic peptides have demonstrated the ability to inhibit the replication of enveloped viruses such as Influenza A, Vesicular stomatitis virus, and human immunodeficiency virus (Powers & Hancock, 2003; Jenssen et al., 2006). Importantly, cationic peptides are effective against antibiotic-resistant bacterial strains.

#### **1.1.4. Advantages of peptides over conventional antibiotics**

Antimicrobial peptides have diverse potential applications. They have been used as single antimicrobials or in combination with conventional antibiotics for a synergistic effect. They also show immunomodulatory and/or endotoxin-neutralizing properties (Zasloff, 2002). Although the potency of these antimicrobial peptides against the more susceptible pathogens is normally not as strong as that of certain conventional antibiotics, one of their major strengths lies in their ability to kill multi-drug-resistant bacteria at similar concentrations. Compared with conventional antibiotics, the killing of bacteria by peptides is extremely rapid and can involve multiple bacterial cellular targets (Fig. 1.3). Although interaction with the cytoplasmic membrane is obligatory and some peptides are able to perforate membranes at their minimum inhibition concentration (MIC), a number of peptides have been shown to translocate across the membrane and have an effect on cytoplasmic processes, including inhibition of macromolecular synthesis, particular enzymes or cell division, or the stimulation of autolysis. Minimal inhibitory concentrations and minimal bactericidal concentrations often coincide (less than a two-fold difference), indicating that killing is generally bactericidal, a highly desirable mode of action. Furthermore, peptides are not hindered by resistance development (Table 1.3).



Antimicrobial cationic peptides protect against pathogens by directly killing it or by selectively boosting host immune responses. The overall effect of host defence peptides is to promote optimal anti-infective efficacy and a balanced immunomodulatory response without exacerbated proinflammation. (a) Cationic peptides that are present exhibit direct antimicrobial properties either by disrupting the bacterial membrane or by targeting intracellular components of the pathogen. (b) cationic peptides regulate responses in host innate immune cells such as monocytes/macrophages, polymorphonuclear leukocytes, and epithelial cells. They modulate gene expression of the host cells, and induce production of chemokines and cytokines that promote immune cell recruitment to the site of infection and also influence cell differentiation and activation. (c) They induce innate immune responses, leading to protection against infection, selective suppression of pro-inflammatory responses ensuring protection against sepsis, induction of wound healing, and the subsequent initiation and polarisation of adaptive immunity.

**Fig. 1.3:** Anti-infective properties of cationic host defence peptides [adapted from Mookherjee & Hancock, 2007].

**Table 1.3:** Comparison of conventional antibiotics with cationic peptides [adapted from Marr, et al., 2006]

| A comparison of conventional antibiotics with cationic antimicrobial peptides. |                                                                                                                                                                                                                                                          |                                                                                                                                                                                                                                                                                                |
|--------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Property                                                                       | Conventional antibiotics                                                                                                                                                                                                                                 | Cationic antimicrobial peptides                                                                                                                                                                                                                                                                |
| Spectrum of activity                                                           | Bacterial infections (often selective)                                                                                                                                                                                                                   | Bacterial, fungal and viral infections; septicaemia; and/or inflammation                                                                                                                                                                                                                       |
| Uptake                                                                         | Specific mechanisms                                                                                                                                                                                                                                      | Relatively non-specific: based on charge. Self-promoted uptake                                                                                                                                                                                                                                 |
| Targets                                                                        | Usually one dominating target or class of targets (e.g. penicillin-binding proteins, topoisomerases, ribosomes)                                                                                                                                          | Relatively less specific (possibly multiple targets in any given cell)                                                                                                                                                                                                                         |
| Resistance rate and mechanism                                                  | Resistance development at frequencies of $10^{-7}$ to $10^{-10}$ , or after a few passages at sub-MIC. Resistance caused by mechanisms such as reduced uptake or increased efflux, chemical modification or degradation of antibiotic, or altered target | Resistance generally cannot be directly selected. Needs multiple passages on sub-MIC concentrations to induce resistance. Resistance caused by mechanisms such as an impermeable outer membrane or specific proteases (can be overcome by incorporating D-amino acids or backbone alterations) |
| Additional activities                                                          | No                                                                                                                                                                                                                                                       | Include anti-endotoxic and/or boosting of innate immunity                                                                                                                                                                                                                                      |
| Pharmacokinetics                                                               | Varies but once per week antimicrobials under development                                                                                                                                                                                                | Short systemic half-life owing to proteolytic degradation                                                                                                                                                                                                                                      |
| Toxicology                                                                     | Antibiotics tend to be one of the safest groups of pharmaceuticals                                                                                                                                                                                       | No known topical toxicities; systemic toxicity issues remain undefined                                                                                                                                                                                                                         |
| Manufacturing costs                                                            | Can be inexpensive (e.g. \$0.8 per gram for aminoglycosides)                                                                                                                                                                                             | Expensive (\$50–400 per gram)                                                                                                                                                                                                                                                                  |

### 1.1.5 Classification of AMPs

Nuclear magnetic resonance (NMR) has emerged a useful technique for studying the details of structures of most of the known antimicrobial peptides. Analysis of the three-dimensional structure of these peptides has led to the better understanding of their function. Based on the NMR structures of known peptides along with sequence analysis, AMPs are broadly classified into four groups (Peters, et al., 2010): (a)  $\alpha$ -helices (Gesell et al., 1997) (b)  $\beta$ -sheet molecules (Jin et al., 2005) (c) extended molecules (Rozek et al., 1986) (d) loops due to a single disulphide bond (Mandard et al., 1998).

#### 1.1.5.1 $\alpha$ -Helical peptides

Peptides of the  $\alpha$ -helical class are characterized by their  $\alpha$ -helical conformation, and often contain a slight bend in the centre of the molecule. In one study, this bending was found critical for selectivity by suppressing the haemolytic activity (Zhang et al., 1999; Lyu et al., 2023). The  $\alpha$ -helical magainins are representative of this structural class. Isolated from the skin of the African clawed frog *Xenopus laevis*, magainin 1 and 2 are 23 residues in length and possess modest antimicrobial activities (e.g., MIC of 50  $\mu\text{g/mL}$  for *E. coli*) (Zasloff et al., 1988). The structure of magainin 2 has been determined by NMR in the presence of

dodecylphosphocholine and sodium dodecyl sulfate micelles. The peptide adopts an amphipathic  $\alpha$ -helical conformation with a slight bend centered at residues 12 and 13 (Gesell et al., 1997). The antimicrobial mechanism of magainin has been proposed to involve selective permeabilization of bacterial membranes leading to disruption of the membrane potential (Bechinger et al., 2020).

#### **1.1.5.2 $\beta$ – Sheet peptides**

This class of peptides is characterized by the presence of an antiparallel  $\beta$ -sheet, generally stabilized by disulfide bonds. Larger peptides within this family may also contain minor helical segments. Perhaps the best characterized  $\beta$ -sheet peptides are the small 17–18 residue tachyplesins. Although the structure and *in vitro* activity of the tachyplesins are well characterized, the exact mechanism of antimicrobial activity remains poorly understood. Additional studies involving the related  $\beta$ -sheet peptide polyphemusin I, have demonstrated that these peptides are effective at inducing lipid flip-flop and undergoing membrane translocation but do not cause substantial calcein release from model membrane systems (Zhang et al., 2001). This suggests that these peptides disrupt lipid organization leading to the translocation of peptide molecules across the bilayer but do not form long-lived pores or channels. Currently, several  $\beta$ -sheet antimicrobial peptides (AMPs) have been identified, such as tachyplesin and thanatin, whose structure was elucidated using NMR.

#### **1.1.5.3 Extended Peptides**

The Extended class of peptides lacks classical secondary structures, generally due to their high proline and/or glycine contents. Indeed, these peptides form their final structures not through inter-residue hydrogen bonds but by hydrogen bonds and Van der Waals interactions with membrane lipids. These peptides are generally rich in regular amino acids such as proline and tryptophan. Histatin, a peptide isolated from human saliva is rich in histidine residues and is active against *C. albicans* (Xu et al., 1991; Oyston et al., 2009). While cathelicidins are proline-rich peptides and have irregular structures, indolicidins and tritripticin (Lawyer et al., 1996) are rich in tryptophan. Bactenecins Bac-5 and Bac-7, like cathelicidins, are proline-rich (Gennaro et al., 1989; Rotem et al., 2009) while the peptide PR-39 is rich in arginine residues. The antimicrobial mechanism of indolicidin has yet to be unambiguously identified; it possesses reasonable antimicrobial activity (MIC of 10 g/mL against *E. coli*) but does not have

a high affinity for lipopolysaccharides when compared to other peptides such as the  $\beta$ -hairpin tachyplesins. It was first hypothesized that indolicidin acts by disrupting the cytoplasmic membrane by voltage-induced channel formation driven by membrane potential (Falla & Karunaratne, 1996).

#### **1.1.5.4 Loop Peptides**

This class of peptides is characterized by their loop structure imparted by the presence of a single bond. The only member of the loop family of peptides with an available high resolution structure is thanatin. Thanatin is a 21-residue loop peptide isolated from the spined soldier bug, *Podisus maculiventris* (Fehlbaum et al., 1996). The solution structure of thanatin has been determined by  $^1\text{H}$  NMR and is that of an anti-parallel  $\beta$ -sheet, formed by residues 8–21 stabilized by the single disulfide bond between residues 11 and 18. Thanatin possesses reasonable antimicrobial activity against Gram-negative and Gram-positive bacteria as well as fungi (Dash et al., 2021) and is comparable in activity to members of the  $\beta$ -sheet family of peptides.

#### **1.1.6 Mode of action of antimicrobial peptides**

The membrane-active properties of such peptides, predicted by their physicochemical characteristics, have been corroborated by model studies demonstrating that antimicrobial peptides induce leakage of artificial liposomes (Matsuzaki, 1999; Wu et al., 1999). The positively charged antimicrobial peptide binds to the negatively charged bacterial phospholipid membrane by means of an electrostatic force until a threshold concentration has been reached. Upon binding, antimicrobial peptides adjust their structure to penetrate or degrade cell membranes. It should be noted that structural transitions occur in peptides on passing from aqueous medium to the lipid medium of the membrane. In the case of Gram-negative bacteria, it has been suggested that the peptides interact with and cross both cell envelope membranes, killing cells by a multihit mechanism that involves action on more than one anionic target (Eisenberg, 1984). Several models have been proposed to describe the molecular events taking place during the peptide induced leakage of the target cell, but direct experimental evidence is still lacking. The most common models (Peravali, et al., 2013; Huan et al., 2020) are described below in more detail.

#### **1.1.6.1 The Barrel-Stave Model**

The peptide helices form a bundle in the membrane with a central lumen, much like a barrel composed of helical peptides as the staves (Yang et al., 2001). The nonpolar side chains face the hydrophobic fatty acid tails at the inside of the phospholipid bilayer and the hydrophilic side-chains are pointed inward into the water-filled pore. Progressive recruitment of additional peptide monomers leads to a steadily increasing pore size. Leakage of intracellular components through these pores subsequently leads to cell death. Peptides that act *via* this mechanism should presumably kill bacteria below the experimentally observed micromolar concentrations, becoming lethal once they penetrate into the phospholipid membrane of the target cell. This mechanism is well explained by analyzing a lantibiotic peptide, nisin.

#### **1.1.6.2 Carpet Model**

Membrane interaction of more amphipathic peptides would rather occur according to the so-called Carpet model (Shai, 1999). In the ‘carpet model’, peptides accumulate on the bilayer surface (Pouny & Shai, 1992). This model explains the activity of antimicrobial peptides such as ovispirin that orientate parallel (‘in-plane’) to the membrane surface (Bechinger, 1999). In this model, the microbial cell membrane is fully covered by a carpet-like cluster of peptides. When a critical concentration is reached, the membrane collapses and in a short span of time, worm holes are formed all over the membrane, leading to lysis of the microbial cell. The Carpet model has been proposed as the mechanism of action of magainins.

#### **1.1.6.3 Toroidal-Pore Model**

In the ‘Toroidal-pore model’ antimicrobial peptide helices insert into the membrane and induce the lipid monolayers to bend continuously through the pore so that the water core is lined by both the inserted peptides and the lipid head groups (Matsuzaki et al., 1996). This type of transmembrane pore is induced by magainins, protegrins and melittin. In forming a toroidal pore, the polar faces of the peptides associate with the polar head groups of the lipids. The lipids in these openings then tilt from the lamellar normal and connect the two leaflets of the membrane, forming a continuous bend from the top to the bottom in the fashion of a toroidal hole; the pore is lined by both the peptides and the lipid head groups, which are likely to screen and mask cationic peptide charges (Won et al., 2009).

#### **1.1.6.4 Ion Channel Formation**

Besides membrane perturbing activities, AMPs also possess the ability to form ion-channels. Linear polycationic helical peptides (dermaseptins, cecropins, magainins and alamethicin) form pores or channels that can be assayed by conductance studies in planar lipid bilayers (Winans et al., 1999). This ability to form transbilayer ion channels is correlated to the helical hydrophilic and hydrophobic components of the peptide. Alamethicin is one of the best-studied models with regard to its channel-forming properties. When incorporated into planar lipid bilayers under applied voltage, it displays unique conductance properties characterized by high voltage dependence of microscopic current voltage curves and multistate single-channel behaviour.

#### **1.1.7 Antimicrobial peptides from plants**

Plant AMPs exhibit a diverse range of structural motifs that contribute to their antimicrobial activity and functional versatility. Below is a description of the structural classification of plant AMPs.

##### **1.1.7.1 Defensins:**

Defensins, a category of cysteine-rich plant AMPs, are characterized by their typical cysteine-stabilized  $\alpha$ -helix and  $\beta$ -sheet (CS $\alpha\beta$ ) structure marked by the presence of six to eight cysteine residues involved in disulfide bonds. This structural feature grants them stability and robustness, enabling them to disrupt microbial membranes effectively, thus displaying broad-spectrum antimicrobial activity against bacteria, fungi, and other pathogens. Within the plant kingdom, defensins constitute a significant superfamily of AMPs, extensively distributed across various plant species. Among these, the *Arabidopsis thaliana* genome has been extensively studied, revealing plant defensins as the most prominent and abundant among membrane-soluble plant AMPs (Graham et al., 2008).

The initial discovery of plant defensins occurred through the isolation of a 5 kDa peptide from wheat and barley, characterized by four disulfide bonds (Bruix et al., 1993). Similar characteristics are shared with  $\alpha$ -thionins,  $\beta$ -thionins, and  $\gamma$ -thionins. Despite initial classification,  $\gamma$ -thionins were later reclassified as plant defensins due to their sequence, structural, and functional similarities with mammalian and insect defensins (Broekaert et al.,

1995; Mendez et al., 1990; Pelegrini & Franco, 2005). Plant defensins, being a vital class of AMPs, exhibit highly conserved structural frameworks found across most plant species (Taylor et al., 2008). While their overall structures remain conserved, their amino acid sequences vary significantly, except for the conserved cysteine residues forming disulfide bonds and certain other residues (Parisi et al., 2019).

Typically, the three-dimensional structure of plant defensins comprises three antiparallel  $\beta$ -sheets and a parallel  $\alpha$ -helix aligned with the  $\beta$ -sheets. These peptides are stabilized by four disulfide bonds, including the cysteine-stable C $\alpha$  $\beta$  motif (García-Olmedo et al., 1998). Plant defensins manifest various biological functions such as inhibiting microbial growth, suppressing  $\alpha$ -amylase and trypsin activity, influencing self-incompatibility, mediating responses to abiotic stress, acting as epigenetic regulators, and modulating the redox state of ascorbic acid (Carvalho & Gomes, 2011; Fujimura et al., 2003; Gao et al., 2000; Sitaram, 2006; Terras et al., 1995; Wijaya et al., 2000).

#### **1.1.7.2 Thionins:**

Thionins, small peptides abundant in cysteine and renowned for their potent antimicrobial characteristics, typically consist of 45-48 amino acid residues and harbour multiple disulfide bonds. These peptides often adopt a ring-like structure owing to disulfide bonds linking the N- and C-termini, thus facilitating their categorization as cyclic peptides (Hughes et al., 2000). Despite this, they do not precisely fit the cyclic peptide definition as the cysteines forming disulfide bonds are not immediately positioned at the peptide ends (Stec, 2006). Thionins were initially discovered in wheat and barley seeds, noted for their amphipathic nature and small molecular size (~5 kDa), containing 6 or 8 cysteine residues forming 3 or 4 disulfide bonds. They have been identified across various plant species (Hughes et al., 2000).

Initially named for their impacts on a broad spectrum including bacteria, fungi, animal and plant cells and insect larvae, thionins have been extensively studied (Ebrahim-Nesbat et al., 1989; Evans et al., 1989; Fernandez et al., 1972; Schmidt et al., 2019; Taveira et al., 2014).

#### **1.1.7.3 Hevein-like Peptides:**

Hevein-like peptides, classified as plant AMPs, bear resemblance in structure to hevein, a chitin-binding protein commonly found in rubber plants. These peptides possess a chitin-

binding domain, allowing them to engage with chitin present in fungal cell walls and thereby exert antifungal effects. Additionally, hevein-like peptides may demonstrate antibacterial properties by disrupting microbial membranes.

Originally discovered in the latex of the rubber tree *Hevea brasiliensis*, hevein-like peptides were identified as the predominant protein component with notable antifungal activity observed *in vitro* (Archer et al., 1960; Van Parijs et al., 1991). Structurally, hevein-like peptides feature a conserved chitin-binding domain characterized by the amino acid sequence SXFGY/SXYGY, where X denotes any amino acid residue (Beintema, 1994; Jiménez-Barbero et al., 2006; Kini et al., 2015). The hevein domain comprises an antiparallel  $\beta$ -sheet and a short  $\alpha$ -helix, with the scaffold stabilized by 3–5 disulfide bonds (Porto et al., 2012). Analogous to hevein, hevein-like peptides exhibit the ability to impede the growth of chitin-containing fungi, thereby safeguarding plants against various fungal pathogens (Beintema, 1994; Van Parijs et al., 1991).

#### **1.1.7.4 Knottins:**

Knottins represent a category of plant peptides characterized by a unique knotted configuration stabilized by disulfide bonds. This distinctive structure often confers compactness and stability to these peptides, setting them apart with their intricate knot arrangement. Knottins exhibit a wide array of biological functionalities, encompassing antimicrobial, insecticidal, and antifungal properties, thus rendering them highly promising for various biotechnological applications.

Plant knottins consist of roughly 30 amino acids and belong to a superfamily that includes inhibitors targeting  $\alpha$ -amylase, carboxypeptidase, and trypsin families, alongside cyclic peptides (Le Nguyen et al., 1990). Generally, knottin-type peptides rank among the smallest in size within the spectrum of plant AMPs. They demonstrate versatile target binding capabilities and multifaceted biological roles, such as bolstering resistance against biotic and abiotic stresses, stimulating root growth, serving as signaling molecules, and fostering symbiotic interactions. Moreover, they exhibit antimicrobial activity against bacteria, fungi and viruses, along with cytotoxic, insecticidal and anti-HIV properties (Aboye et al., 2015; Hwang et al., 2010a,; Pallaghy et al., 1994).

The characteristic structure of knottins typically involves conserved disulfide bonds formed among multiple pairs of cysteines, resulting in a cystine knot configuration. Notably, the cystine motifs of plant knottins may vary across different subfamilies. Interestingly, certain functionally unrelated protein families in diverse organisms share similar knottin structures. In the plant kingdom, proteins such as defensins and protease inhibitors also exhibit cystine motifs akin to those observed in knottin-type peptides (Gelly et al., 2004). Although plant defensins also feature a cystine-knotting motif, they distinguish themselves from knottin-like peptides based on their cysteine spacing (Tam et al., 2015).

#### **1.1.7.5 $\alpha$ -Hairpinins:**

$\alpha$ -Hairpinins are a subset of plant AMPs characterized by their adoption of a hairpin-like secondary structure, featuring a loop region linking two antiparallel  $\alpha$ -helices. This structural configuration, comprising two antiparallel  $\alpha$ -helices stabilized by two disulfide bonds, contributes to the peptide's stability and firmness, thereby enhancing its antimicrobial effectiveness.  $\alpha$ -Hairpinins typically exert their antimicrobial effects by targeting microbial membranes or intracellular components.

Within the realm of plant defence peptides,  $\alpha$ -hairpinins stand out as a class rich in Lys/Arg residues and distinguished by unique Cys motifs, facilitating the formation of the characteristic helix-loop-helix secondary structure (Slavokhotova & Rogozhin, 2020). This helix-loop-helix configuration, stabilized by disulfide bonds, underscores the peptide's tertiary structure (Sousa et al., 2016). Notably,  $\alpha$ -hairpinins exhibit a spectrum of biological activities, including antimicrobial, trypsin-inactivating, and ribosome-inactivating properties (Slavokhotova & Rogozhin, 2020; Tam et al., 2015).

One prominent example within this AMP family is MBP-1, a 33-amino acid peptide isolated from maize kernels. MBP-1 has demonstrated the ability to inhibit spore germination and mycelial elongation in various phytopathogenic fungi and bacteria (Duvick et al., 1992).

#### **1.1.7.6 Lipid transfer proteins:**

Lipid transfer proteins (LTPs) are a category of small, cysteine-rich peptides found in plants, playing crucial roles in lipid transport and the maintenance of membrane integrity. Certain

LTPs exhibit antimicrobial properties by disrupting microbial membranes or interfering with lipid metabolism in pathogens, thereby aiding in plant defence against microbial infections.

These cationic peptides typically weigh between 7 and 10 kDa and are characterized by the presence of eight cysteine residues (Yeats & Rose, 2008). The three-dimensional structure of these peptides reveals presence of eight cysteines, forming four disulfide bonds. These bonds stabilize a compact tertiary structure consisting of four flexible loop-linked helices with hydrophobic cavities, which include lipid-binding sites. While the eight cysteines remain constant, variations are observed in the pairs of cysteine motifs forming disulfide bonds (Carvalho & Gomes, 2007).

The LTPs display the capability to bind a variety of lipids, such as fatty acids, phospholipids, prostaglandin B2, haemolytic derivatives and acyl coenzyme A (Carvalho & Gomes, 2007; Liu et al., 2015; Sels et al., 2008). They are categorized into two main groups based on their molecular weight: LTP1s (9 kDa) or LTP2s (7 kDa). These peptides have been observed to reversibly bind and transport hydrophobic molecules (Carvalho & Gomes, 2007).

Furthermore, in addition to their role in inhibiting the growth of fungi and bacteria, LTPs actively contribute to plant defence mechanisms (Blein et al., 2002; Bogdanov et al., 2015, 2016; Souza et al., 2018).

#### **1.1.7.7 Snakins:**

Snakins represent a class of plant AMPs notable for their coiled or snaking structural configuration, potentially enhancing their antimicrobial efficacy. These peptides commonly harbour conserved motifs that interact with microbial membranes or intracellular targets, leading to their antimicrobial effects.

Within the plant AMP category, snakins stand out with 12 cysteine residues, the highest among AMPs, resulting in numerous disulfide bonds. The first identified snakin peptide, snakin-1, was isolated from potato tubers (*Solanum tuberosum*), sharing sequence motifs with snake venoms (Segura et al., 1999). These peptides are recognized for their cysteine-rich nature (~19% Cys), consisting of approximately 63 amino acid residues and six disulfide bonds, contrasting with

other AMPs that typically have two to four disulfide bonds (Segura et al., 1999; Tavares et al., 2008).

Snakins may be expressed constitutively or induced by various stressors, such as biotic or abiotic factors, in different plant organs like roots, stems, leaves, flowers, and seeds. Silencing Snakin-1 in potatoes has been shown to impact cell division, primary metabolism, and cell wall composition, leading to alterations in potato height, leaf size, and leaf morphology (Nahirñak et al., 2012). Notably, both snakin-1 and snakin-2 have demonstrated inhibitory effects on fungal growth (*e.g.*, *Magnaporthe grisea*, *Fusarium solani*, and *Botrytis cinerea*) and bacterial proliferation (*e.g.*, *Dickeya dadantii*, *Ralstonia solanacearum* and *Sinorhizobium meliloti*) (Berrocal-Lobo et al., 2002).

#### **1.1.7.8 Cyclotides:**

Cyclotides represent a class of plant peptides characterized by their cyclic backbone and a conserved cystine knot motif, which confers exceptional stability and resistance to proteolytic degradation. These peptides exhibit a wide array of biological activities, encompassing antimicrobial, antiviral and insecticidal properties.

These long-chain cyclic peptides, typically comprising 28–37 amino acids, are prevalent among plant anionic AMPs, constituting a significant portion of this peptide family (Harris et al., 2009; Prabhu et al., 2014). Cyclotides feature a cyclic backbone composed of six loops, formed by six conserved cysteine residues arranged and crosslinked in a knotted manner, known as cyclic cystine knots, stabilized by three disulfide bonds (Fig. 2h) (Craig, 2009; Ireland et al., 2006; Pr nting et al., 2010). This cysteine junction arises from the first two disulfide bonds (Cys1-Cys4 and Cys2-Cys5) and their interconnected backbone, forming a ring penetrated by the third disulfide bond, Cys3-Cys6 (Ireland et al., 2010).

The unique cyclic cystine knot architecture imparts cyclotides with exceptional resistance to thermal and chemical denaturation, as well as proteolytic degradation, rendering them promising candidates for therapeutic applications (Mehta et al., 2020). These applications include antitumor (Lindholm et al., 2002), anti-HIV (Sangphukieo et al., 2015), insecticidal (Colgrave et al., 2008), and antibacterial potential (Pr nting et al., 2010).

### 1.1.8 Antimicrobial peptides from marine microorganisms

Bacteriocins are antimicrobial peptides or proteins produced by bacteria as a defence mechanism against closely related bacterial strains. These compounds exhibit a wide range of antibacterial activities, targeting specific bacterial species or strains. Marine microorganisms represent a promising source of novel antimicrobial compounds. However, compared to their terrestrial counterparts, marine-derived bacteriocins have received relatively limited attention. These bacteriocins, originating from lactic acid-producing bacteria such as *Lactobacillus* and *Carnobacterium*, as well as *Bacillus* sp., and, to a lesser extent, *Enterococcus*, *Proteus*, *Vibrio*, and *Pseudomonas*, have been predominantly reported (Table 1.4).

**Table 1.4:** The characteristics of bacteriocins isolated from marine bacteria [adapted from Andryukov et al., 2018].

| Producing Strain                                                          | Name of Bacteriocin            | Inhibits                                                                                                                                                   | MW, (kDa) | References                        |
|---------------------------------------------------------------------------|--------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|-----------------------------------|
| <b><i>Bacillus licheniformis</i>, strain BTHT8</b>                        | <i>P. putida</i> strain FStm2  | <i>S. aureus</i> , <i>E. coli</i> , <i>Enterobacter aerogenes</i> , <i>Serratia marcescens</i> , <i>Salmonella</i> spp.                                    | 32        | (Ahmad et al., 2013)              |
| <b><i>Lactobacillus fermentum</i>, strain SBS001</b>                      | <i>L. fermentum</i> SBS001     | <i>Klebsiella oxytoca</i> , <i>P. aeruginosa</i> , <i>E. coli</i>                                                                                          | 78        | (Singh et al., 2013)              |
| <b><i>Vibrio</i> spp. MMB2 BLIS*</b>                                      | Vibriocin AVP10                | <i>Aeromonas hydrophila</i>                                                                                                                                | 16        | (Selvendran & Michael Babu, 2013) |
| <b><i>Enterococcus faecalis</i></b>                                       | <i>E. faecalis</i> bacteriocin | <i>S. aureus</i> , <i>Bacillus subtilis</i>                                                                                                                | 94        | (Noga, 2010)                      |
| <b><i>Bacillus sonorensis</i>, strain MT93</b>                            | Sonorensin                     | <i>Listeria monocytogenes</i> , <i>S. aureus</i>                                                                                                           | 6         | (Chopra et al., 2014)             |
| <b><i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>, strain PSY2</b> | Bacteriocin PSY2               | <i>Arthrobacter</i> sp., <i>Acinetobacter</i> sp., <i>B. subtilis</i> , <i>E. coli</i> , <i>L. monocytogenes</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> | 10        | (Sarika et al., 2012)             |

(Note: BLIS\* denotes Bacteriocin-Like Inhibitory Substance)

Gram-negative bacteria-produced bacteriocins are categorized into four main classes: colicins, colicin-like bacteriocins (CLB), phage tail-like bacteriocins (PTLB), and microcins (Rather et al., 2017). The main classes and characteristics of bacteriocins isolated from Gram-negative and Gram-positive bacteria are outlined in Tables 1.5 and 1.6, respectively.

**Table 1.5:** Characteristics of the main classes of bacteriocins from Gram-negative bacteria [adapted from Andryukov et al., 2018].

| <b>Bacteriocins</b> | <b>Description of mechanisms</b>      | <b>MW, (kDa)</b> | <b>Producing bacteria</b>                                      | <b>Examples</b>                     | <b>References</b>                           |
|---------------------|---------------------------------------|------------------|----------------------------------------------------------------|-------------------------------------|---------------------------------------------|
| <b>Colicins</b>     | Pore-forming/nucleases                | 20–80            | <i>E. coli</i>                                                 | Colicin A, B, E2, E3                | (Hjelm et al., 2004; Riley & Wertz, 2002)   |
| <b>CLB</b>          | Pore-forming/nucleases                | 20–80            | <i>Klebsiella</i> , <i>P. aeruginosa</i> , <i>Hafnia alvei</i> | Klebicins, S-pyocins, and alveicins | (Bakkal et al., 2012)                       |
| <b>PTLB</b>         | Cause depolarization of cell membrane | >80              | <i>P. aeruginosa</i>                                           | F-pyocins, R-pyocins                | (Cintas et al., 2001; Laverty et al., 2011) |
| <b>Microcins</b>    | Post-translational modification       | <10              | <i>E. coli</i>                                                 | Microcin V                          | (Cotter et al., 2005)                       |

(Note: CLB stands for colicin-like bacteriocins; PTLB stands for phage tail-like bacteriocins)

**Table 1.6:** Characteristics of bacteriocins isolated from Gram-positive bacteria, [adapted from Andryukov et al., 2018].

| <b>Class</b> | <b>MW, kDa</b> | <b>Type</b> | <b>Characteristics of bacteriocins</b>           | <b>Producing bacteria</b>               | <b>References</b>                                                                            |
|--------------|----------------|-------------|--------------------------------------------------|-----------------------------------------|----------------------------------------------------------------------------------------------|
| <b>I</b>     | <5             | A           | Cationic, disrupt cell membranes                 | <i>Lactococcus lactis</i>               | (Annamalai et al., 2008; Bakkal et al., 2012; Deshmukh & Thorat, 2013; (Phelan et al., 2013) |
|              |                | B           | Neutral or anionic, globular                     | <i>E. faecium</i>                       |                                                                                              |
|              |                | C           | Synergetic activity of two active peptides       | <i>Bacillus subtilis</i>                |                                                                                              |
| <b>II</b>    | <10            | a           | Pediocin-like                                    | <i>Lactic acid bacteria</i>             | (Chopra et al., 2014; (Desriac et al., 2010)                                                 |
|              |                | b           | Two-peptide                                      | <i>L. garvieae</i>                      |                                                                                              |
|              |                | c           | Cyclic                                           | <i>B. sonorensis</i>                    |                                                                                              |
|              |                | d           | Sec-dependent, inactive                          |                                         |                                                                                              |
| <b>III</b>   | >30            | a           | Large, hydrophilic, thermolabile                 | <i>Lactobacillus helveticus</i>         | (Chopra et al., 2014)                                                                        |
| <b>IV</b>    | >60            |             | Complex, bearing lipid or carbohydrate fragments | <i>E. faecalis</i> , <i>S. simulans</i> | (Cotter et al., 2005)                                                                        |

#### 1.1.8.1 Marine *Bacillus* – potential hosts of novel antimicrobial peptides

Bacteriocins from *Bacillus* can be classified into three main classes based on their size, structure, and activity (Nes et al., 2007):

#### **1.1.8.1.1 Class I: Post-translationally modified peptides (Abriouel et al., 2011):**

This class includes bacteriocins that undergo post-translational modifications, such as the formation of lanthionine and  $\beta$ -methyl lanthionine residues.

Subclass I.1: Includes single-peptide, elongated lantibiotics like subtilin, ericin S, and ericin A.

Subclass I.2: Comprises other single-peptide lantibiotics such as sublancin 168, mersacidin, and paenibacillin.

Subclass I.3: Consists of two-peptide lantibiotics like haloduracin and lichenicidin.

Subclass I.4: Contains other post-translationally modified peptides like subtilosin A.

#### **1.1.8.1.2 Class II: Nonmodified peptides (Abriouel et al., 2011):**

This class encompasses small, linear peptides that are heat and pH stable.

Subclass II.1: Includes small, linear peptides like cerein 7A, cerein 7B, and lichenin.

Subclass II.2: Comprises Thuricin-like peptides such as Thurincin H, Thurincin S, and Thurincin 17.

Subclass II.3: Consists of other linear peptides like cereins and thuricin 439 .

#### **1.1.8.1.3 Class III: Large proteins (Abriouel et al., 2011) :**

Large proteins with phospholipase activity, such as megacins A-216 and A-19213 produced by *Bacillus megaterium* strains fall into this class.

These bacteriocins have molecular weights ranging from 4 to 30 kDa and exhibit specific enzymatic activities.

Table 1.7 presents information on bacteriocins and bacteriocin-like inhibitory substances (BLIS) produced by *Bacillus* species.

**Table 1.7: Bacteriocins and BLIS produced by *Bacillus* species [ adapted from Abriouel et al., 2011]**

| <i>Bacillus</i> species       | Source                     | Bacteriocin/BLIS | Molecular weight (kDa) | Special features                                          | Class         | Reference                    |
|-------------------------------|----------------------------|------------------|------------------------|-----------------------------------------------------------|---------------|------------------------------|
| <i>Bacillus cereus</i> group  | Food                       | Cerein GN105     | 9                      | Active against other <i>B. cereus</i> strains             | -             | Naclerio et al. (1993)       |
|                               | Soil                       | Cerein 7A        | 3.94                   | -                                                         | Subclass II.3 | Oscariz & Pisabarro (2000)   |
|                               | Soil                       | Cerein 7B        | 4.89                   | Sec-independent leader peptide with GG                    | Subclass II.3 | Oscariz et al. (2006)        |
|                               | Woodland                   | Cerein 8A        | 26                     | Aromatic amino acid residues                              | -             | Bizani & Brandelli (2002)    |
|                               | Aquatic plant roots        | Cerein MRX1      | 3.14                   | N-terminal: DWTCWSCLVCAACSVELL                            | Subclass II.2 | Sebei et al. (2007)          |
|                               | Deep-surface oil reservoir | Cereicidin       | -                      | Lantibiotic                                               | -             | Xiong et al. (2009)          |
| <i>Bacillus thuringiensis</i> | Culture collection         | Thuricin HD-2    | 4.95                   | Phospholipase A activity                                  | -             | Favret & Yousten (1989)      |
|                               | Culture collection         | Thuricin 439     | 2.92 and 2.80          | Two singly active peptides                                | Subclass II.3 | Ahern et al. (2003)          |
|                               | Soybean rot nodules        | Thuricin 17      | 3.16                   | N-terminal: DWTXWSXL                                      | Subclass II.2 | Gray et al. (2006a, b)       |
|                               | Honey                      | Thurincin H      | 3.14                   | Three structural genes, N-terminal DWTXWSXL               | Subclass II.2 | Lee et al. (2009b)           |
|                               | Culture collection         | Thuricin S       | 3.14                   | N-terminal: DWTXWSXL                                      | Subclass II.2 | Chehimi et al. (2007)        |
|                               | Soil litter                | Bacthuricin F4   | 3.16                   | N-terminal: DWTXWSXL                                      | Subclass II.2 | Kamoun et al. (2005)         |
|                               | Human fecal sample         | Thuricin CD      | 2.76 and 2.86          | Dimer of two peptides; active against <i>C. difficile</i> | -             | Hill et al. (2009)           |
|                               | Culture collection         | Entomocin 110    | 4.8                    | Active against <i>L. monocytogenes</i>                    | -             | Cherif et al. (2008)         |
|                               | Culture collection         | Entomocin 9      | 12.4                   | -                                                         | -             | Cherif et al. (2003)         |
|                               | Soil                       | Thuricin 7       | 11.6                   | -                                                         | -             | Cherif et al. (2001)         |
|                               | Culture collection         | Tochicin         | 10.5                   | -                                                         | -             | Paik et al. (1997)           |
|                               | Culture collection         | Morricin 269     | 10                     | Wide inhibitory spectrum                                  | -             | Barboza-Corona et al. (2007) |
|                               | Culture collection         | Kurstacin 287    | 10                     | Wide inhibitory spectrum                                  | -             | Barboza-Corona et al. (2007) |
|                               | Culture collection         | Kenyacin 404     | 10                     | Wide inhibitory spectrum                                  | -             | Barboza-Corona et al. (2007) |
|                               | Culture collection         | Entomocin 420    | 10                     | Wide inhibitory spectrum                                  | -             | Barboza-Corona et al. (2007) |
|                               | Culture collection         | Tolworthcin 524  | 10                     | Wide inhibitory spectrum                                  | -             | Barboza-Corona et al. (2007) |

|                                          |                                                  |                       |               |                                                                     |               |                                 |
|------------------------------------------|--------------------------------------------------|-----------------------|---------------|---------------------------------------------------------------------|---------------|---------------------------------|
| <b><i>Bacillus mycoides</i></b>          | Whey                                             | BLIS                  | -             | Active against <i>L. monocytogenes</i>                              | -             | Sharma & Gautam (2008)          |
| <b><i>Bacillus anthracis</i></b>         | Culture collection                               | Heterocycloanthracins | 9.62          | Subfamily of putative thiazole-containing heterocyclic bacteriocins | -             | Haft (2009)                     |
| <b><i>Bacillus</i></b>                   | Culture collection                               | Subtilin B            | 3.42          | Succinylated subtilin, A-lantibiotic, binds lipid II                | Subclass I.1  | Chan et al. (1993)              |
|                                          | Chinese herbs, dairy product, soil, wild strains | Subtilosin A          | 3.39          | Macrocyclic lantibiotic                                             | Subclass I.4  | Zheng et al. (1999)             |
|                                          | Chinese herbs, dairy product, soil, wild strains | Subtilosin A1         | 3.41          | Macrocyclic lantibiotic, variant                                    | Subclass I.4  | Huang et al. (2009)             |
|                                          | Culture collection                               | Mersacidin            | 1.82          | Tetracyclic, B-lantibiotic, binds lipid II                          | Subclass I.2  | Bierbaum et al. (1995)          |
|                                          | -                                                | Sublancin 168         | 3.88          | AII-lantibiotic, unusual lantibiotic                                | Subclass I.2  | Paik et al. (1998)              |
|                                          | Fermented soybeans                               | Betacin               | -             | Produced by a SPb lysogenic strain                                  | -             | Hemphill et al. (1980)          |
|                                          | -                                                | MJP1                  | 4.5           | Antibacterial and antifungal activity                               | -             | Yang & Chang (2007)             |
|                                          | Plant rhizosphere                                | Ericin S              | 3.44          | Lantibiotic; active against <i>Clavibacter</i>                      | Subclass I.1  | Stein et al. (2002a)            |
|                                          | Plant rhizosphere                                | Ericin A              | 2.98          | Lantibiotic                                                         | Subclass I.1  | Stein et al. (2002a)            |
|                                          | Chinese herbs                                    | Bac 14B               | 21            | Antibacterial and antifungal activity                               | -             | Hammami et al. (2009)           |
|                                          | Chinese herbs                                    | LFB112                | 6.3           | Activity against Gram-positive and Gram-negative bacteria           | -             | Xie et al. (2009)               |
| <b><i>Bacillus licheniformis</i></b>     | Buffalo rumen                                    | Lichenin              | 1.4           | N-terminal: ISLEICXIFHDN                                            | Subclass II.3 | Pattnaik et al. (2001)          |
|                                          | Dairy product                                    | Bacillocin 490        | 2             | -                                                                   | -             | Martirani et al. (2002)         |
|                                          | Culture collections                              | Lichenicidin (a, b)   | 3.25 and 3.02 | Two-peptide lantibiotic                                             | Subclass I.3  | Begley et al. (2009)            |
|                                          | Amazon basin                                     | BLIS P40              | -             | Active on clinical <i>Streptococcus</i> spp. isolates               | -             | Cladera-Olivera et al. (2004a)  |
|                                          | -                                                | BLIS ZJU12            | 3             | Antibacterial and antifungal activity                               | -             | Lili et al. (2006)              |
|                                          | -                                                | BLIS MKU3             | 1.5           | Antibacterial and antifungal activity                               | -             | Kayalvizhi & Gunasekaran (2008) |
|                                          | Soil                                             | Peptide A-12 C        | 0.77          | Antibacterial and antifungal activity                               | -             | Gálvez et al. (1993)            |
| <b><i>Bacillus amyloliquefaciens</i></b> | Yogurt beverage                                  | Subtilosin A          | 3.39          | Macrocyclic bacteriocin from <i>B. subtilis</i>                     | -             | Babasaki et al. (1985)          |
|                                          | Probiotic                                        | BLIS 5940             | -             | Active against <i>C. perfringens</i>                                | -             | Diaz (2007)                     |

|                                         |                     |                          |                     |                                                                                                     |               |                                             |
|-----------------------------------------|---------------------|--------------------------|---------------------|-----------------------------------------------------------------------------------------------------|---------------|---------------------------------------------|
|                                         | Probiotic           | BLIS RC-2                | -                   | Antibacterial and antifungal activity                                                               | -             | Yoshida et al. (2001)                       |
|                                         | Atlantic forest     | BLIS 5006                | 5                   | Broad spectrum                                                                                      | -             | Lisboa et al. (2006)                        |
| <b><i>Bacillus atrophaeus</i></b>       | Culture collection  | Subtilisin A             | 3.39                | Macrocyclic bacteriocin from <i>B. subtilis</i>                                                     | -             | Stein et al. (2004)                         |
|                                         |                     |                          |                     |                                                                                                     |               |                                             |
| <b><i>Bacillus firmus</i></b>           | Oil reservoir       | BLIS                     | -                   | Active against SRB                                                                                  | -             | Korenblum et al. (2005)                     |
| <b><i>Bacillus pumilus</i></b>          | Water               | Pumilicin 4              | 1.99                | Active against VRE and MRSA                                                                         | -             | Aunpad & NaBangchang (2007)                 |
| <b><i>Bacillus circulans</i></b>        | Poultry environment | SRCAM 1580               | 3.5                 | Pediocin-like bacteriocin; anti- <i>Campylobacter</i>                                               | Subclass II.1 | Svetoch et al. (2005)                       |
| <b><i>Bacillus clausii</i></b>          | Probiotic           | BLIS                     | -                   | Active against <i>C. difficile</i>                                                                  | -             | Urdaci et al. (2004)                        |
| <b><i>Bacillus coagulans</i></b>        | Animal feces        | Coagulin                 | 4.6                 | Pediocin-like bacteriocin                                                                           | Subclass II.1 | Le Marrec et al. (2000)                     |
| <b><i>Bacillus halodurans</i></b>       | Soil                | Haloduracin (A1, A2)     | 3.04 and 2.33       | Two-peptide-lantibiotic                                                                             | Subclass I.3  | McClerren et al. (2006)                     |
| <b><i>Bacillus lentus</i></b>           | Fermented food      | BLIS                     | -                   | Active on <i>L. monocytogenes</i> and <i>S. aureus</i> , Able to extend the shelf stability of milk | -             | Sharma et al. (2006), Sharma et al. (2009a) |
| <b><i>Bacillus megaterium</i></b>       | Soil                | Megacin A-216            | 32.85; 21.02, 11.85 | Phospholipase A activity                                                                            | Class III     | Kiss et al. (2008)                          |
|                                         |                     | Megacin A-19213          | 39                  | Phospholipase A activity                                                                            | Class III     | Von Tersch & Carlton (1983a)                |
|                                         |                     | Megacin BII              | -                   | Narrow spectrum                                                                                     | -             | Stahl (1989)                                |
|                                         |                     | Megacin C                | 150                 | Large protein                                                                                       | -             | Donoghue (1972)                             |
|                                         |                     | Megacin Cx               | 210                 | Large protein                                                                                       | -             | Brusilow & Nelson (1981)                    |
|                                         | Vegetable wastes    | Megacin 19               | 3.5–6.5             | Broad spectrum against food-spoilage bacteria                                                       | -             | Khalil et al. (2009b)                       |
|                                         | Soil                | Megacin 22               | 3.5–6.5             | Broad spectrum against food-spoilage bacteria                                                       | -             | Khalil et al. (2009a)                       |
| <b><i>Bacillus polyfermenticus</i></b>  | Probiotic           | Polyfermentacin SCD 14.3 | -                   | Active against <i>C. perfringens</i>                                                                | -             | Lee et al. (2001)                           |
| <b><i>Lysinibacillus sphaericus</i></b> | Mosquitoes          | BLIS                     | -                   | Chromosomal                                                                                         | -             | Cetinkaya et al. (2003)                     |
|                                         |                     | BLIS SOPB1               | -                   | Plasmidic, active against MRSA                                                                      | -             | Aunpad et al. (2007)                        |
| <b><i>Brevibacillus brevis</i></b>      | Soil                | Bacillocin Bb            | 10                  | Broad spectrum, potential medical or food uses                                                      | -             | Saleem et al. (2009)                        |

|                                       |                            |                   |       |                                                  |               |                        |
|---------------------------------------|----------------------------|-------------------|-------|--------------------------------------------------|---------------|------------------------|
|                                       | Wheat field                | Brevicin AF01     | -     | Active against MRSA                              | -             | Faheem et al. (2007)   |
|                                       | Kimchi                     | BLIS              | 4.5–6 | Active against yeasts and Gram-negative bacteria | -             | Hyung et al. (2001)    |
| <i>Geobacillus stearothermophilus</i> | -                          | Thermocin 10      | -     | Glycoprotein                                     | -             | Fikes et al. (1983)    |
|                                       |                            | Thermocin 93      | -     | -                                                | -             | Sharp et al. (1979)    |
|                                       | Oil wells                  | Thermocin 17      | 6.9   | Narrow spectrum                                  | -             | Pokusaeva et al., 2009 |
|                                       |                            | Thermocin 30      | 5.6   | Narrow spectrum                                  | -             | Pokusaeva et al., 2009 |
|                                       |                            | Thermocin 31      | 7.1   | Narrow spectrum                                  | -             | Pokusaeva et al., 2009 |
|                                       |                            | Thermocin 32A     | 7.2   | Narrow spectrum                                  | -             | Pokusaeva et al., 2009 |
| <i>Geobacillus thermoleovorans</i>    | Mud/water samples          | Thermoleovorin-S2 | 42    | Broad spectrum                                   | -             | Novotny & Perry (1992) |
|                                       |                            | Thermoleovorin-N9 | 36    | Broad spectrum                                   | -             | Novotny & Perry (1992) |
| <i>Paenibacillus polymyxa</i>         | Fermented sausages         | Polyxin 10        | -     | Broad spectrum                                   | -             | Piuri et al. (1998)    |
|                                       | Broiler chicken, crop      | SRCAM 37          | 3.5   | Pediocin-like bacteriocin; antiCampylobacter     | Subclass II.1 | Svetoch et al. (2005)  |
|                                       | Broiler chicken, intestine | SRCAM 602         | 3.5   | Pediocin-like bacteriocin; antiCampylobacter     | Subclass II.1 | Svetoch et al. (2005)  |
|                                       | Kimchi                     | Paenibacillin     | 2.98  | Lantibiotic                                      | Subclass I.2  | He et al. (2007)       |
| <i>Bacillus</i> sp.                   | Amazon basin               | BLIS P34          | 1.45  | Broad spectrum                                   |               | Motta et al. (2007a)   |
| <i>Bacillus</i> sp.                   | Rhizosphere                | BLIS              |       | Broad spectrum                                   |               | Sharma et al. (2009b)  |
| <i>Bacillus</i> sp.                   | Fish intestine             | BLIS P45          | 1.45  | Active against bacteria and yeasts               |               | Motta et al. (2004)    |

### 1.1.9 Scope of the present study

Over millions of years, marine organisms have evolved advanced defences to survive in microorganism-rich environments. The ocean's unmatched microbial diversity offers a vast genetic resource, with marine species surpassing terrestrial ones in biodiversity, biomass, and bioactive compound production (Wang et al., 2023). The antimicrobial peptides (AMPs) highlight the ancient immune adaptations of marine life.

The Antimicrobial Peptide Database (APD) reveals that animals contribute 78% of natural AMPs, bacteria 12%, plants 8%, fungi 1%, and protists/archaea collectively less than 0.5%. Marine AMPs remain underexplored compared to terrestrial sources (Li et al., 2021). This study focuses on mining mangrove plants and marine microbes for novel AMPs and characterizing the most potent for clinical applications.

Marine AMPs stand apart from terrestrial ones in several aspects (Wang et al., 2023):

1. **Amino acid composition:** Favor arginine and leucine for enhanced antimicrobial activity.
2. **Cysteine richness:** Form more stabilizing disulfide bonds.
3. **Post-translational modifications:** Undergo unique modifications such as dihydroxylysine.
4. **Higher-order structures:** Exhibit structures stabilized by disulfide bonds or rare amino acids.
5. **Ecological adaptations:** Reflect environmental influences, e.g., migratory patterns in Atlantic salmon.

The present study was basically undertaken with the following research objectives in mind:

1. Screening of cationic peptides from mangrove plants and marine microbes
2. Bioactivity tests of the extracts
3. Purification and characterization of selected cationic peptides
4. Confirmation of activity in purified compounds
5. Biotechnological applications of potent cationic peptides

# **CHAPTER 2:**

## **Isolation of Antimicrobial Cationic Peptides from Mangrove Plants**

## **2.1 Introduction**

Mangrove ecosystems have recently become one of the most studied areas in wetland research due to the peculiar ecological traits that they possess and their critical roles in supporting coastal environments. Large mangrove forests are commonly located in protected muddy shorelines, often forming at the mouths of river systems, especially in tropical and subtropical regions globally. Mangroves are small, shrub-like woody plants that are widespread in marshy and estuarine areas where conditions favor their growth. They can also be found growing in sandy and rocky coastal areas, coral reefs, and oceanic islands. Additionally, mangroves thrive along riverbanks and in coastal lagoons. Mangrove plants are salt-tolerant trees, often referred to as halophytes. Found mainly in the coastal intertidal zones of tropical and sub-tropical regions, their ecosystem provides a wide range of ecological and economic benefits (Singh et. al., 2020). A variety of marine organisms use these habitats as spawning, nursery and feeding grounds (Sirajudin et al., 2018). The rich biodiversity within mangrove forests provides a vast array of secondary metabolites, which by definition are chemical compounds not directly involved in the primary metabolic processes of an organism but often playing crucial roles in ecological interactions and defence mechanisms. These secondary metabolites have found diverse applications in pharmacy and medicine.

Covering approximately 15.2 million hectares worldwide (Kathiresan, 2018), mangroves represent less than 1% of the Earth's surface (Saenger, 2002) but are present along about 75% of the world's tropical coastlines. India, with a coastline of approximately 7517 km, including its island territories, is no exception. It has the fourth largest mangrove area in the world covering about 6,749 sq. km (Naskar & Mandal, 1999). The mangrove regions in India (69°E-89.5°E longitude and 7°N-23°N latitude) are made up of three distinct zones: (i) East coast habitat corresponding to a coastline of about 2700 km, (ii) West coast habitat corresponding to a coastline of about 3000 km, and (iii) Island Territories comprising of about 1817 km coastline (Singh et. al., 2012).

### **2.1.1 Mangroves of Goa**

Goa, situated in the central west coast of the Indian sub-continent, has a coastline of approximately 105 km, and is bordered by the Arabian Sea which extends from north to south. Seven major estuaries found along the Goa coast, viz., Terekhol, Chapora, Mandovi, Zuari, Sal,

Talpona and Galgibag, and also the Cumbharjua canal are studded with mangrove vegetation (Untawale et al., 1982). There exists an intricate network of creeks and backwaters.

As per an earlier estimate, the total area covered by the estuaries in Goa is approximately 12,000 ha (Untawale et al., 1982), of which the mangrove forests occupy ~2000 ha. The Mandovi-Zuari estuarine system harbours a wide variety of mangrove species. About 900 ha of mangroves are found along the Zuari estuary, 700 ha along the Mandovi estuary and 200 ha along the Cumbharjua canal. However, as on today, the total mangrove area has been estimated to be 28 sq. km (Halдар & Nazareth, 2018). The composition of mangroves varies at different locations, the predominant being species such as *Rhizophora mucronata*, *Sonneratia alba*, *Avicennia officinalis*, etc., while the other widely distributed species are *Rhizophora apiculata*, *Sonneratia caseolaris*, *Kandelia candel*, *Bruguiera gymnorhiza*, *Aegiceras corniculatum*, etc. (Saddhe et al., 2017).

### **2.1.2 Ecological uses of mangroves**

Mangroves play a crucial role in shoreline stabilization and the prevention of erosion, acting as a natural barrier against storms to minimize coastal damage and protect residents. Their intricate root systems provide them mechanical support and also trap the sediment, preventing it from reaching the sea and affecting coral health. These coastal forests serve as carbon sinks, sequestering significant amounts of carbon dioxide, thereby aiding in climate change mitigation (Dasgupta et al., 2010). In Goa, mangroves fulfil essential ecological functions by buffering the coastline, improving water quality through pollutant filtration and maintaining estuarine ecosystem health. They serve as vital habitats and nurseries for diverse marine and terrestrial species, including fish, birds, and reptiles. Economically, Goa's mangroves provide valuable resources such as timber, fuelwood, honey, and medicinal plants, besides supporting local livelihoods through activities such as fishing, crab farming, and eco-tourism (Saddhe et al., 2016).

### **2.1.3 Mangroves in traditional medicine**

Mangroves thrive in challenging environments characterized by low oxygen levels, high salinity, nutrient-poor soil, and abundant microorganisms and insects. To cope with these biotic and abiotic stressors, they have evolved a common strategy of osmotic adjustment. This involves storing inorganic salts, particularly NaCl, in their vacuoles, and accumulating organic

solutes in their cytoplasm. Additionally, mangroves have developed an impressive array of defense mechanisms, including the production of various chemicals, to protect themselves and adapt to their surroundings (Chandrakala et al., 2017; Kabera et al., 2014). They are recognized for their rich reservoir of bioactive compounds, including essential oils, limonoids, flavonoids, coumarins, terpenoids, glycosides, and alkaloids (Mitra et al., 2021). These molecules contribute to the diverse secondary metabolite profile of mangroves, which in turn accounts for their diverse bioactivities. Their unique chemical compositions and therapeutic properties have resulted in mangrove plants gaining interest as potential candidates for medicinal applications and prompting extensive investigation into their medicinal values, which have for long been traditionally acknowledged.

The people of Kutch and Saurashtra use the smoked dry leaves of *Avicennia officinalis* for relief from Asthma (Banerjee et al. 1989). The leaves and bark of *Acanthus ilicifolius*, a shrubby plant associated with the mangrove community, were found to be useful in treating nervous disorders (Banerjee et al., 1989; Kothari & Rao, 2002). The rhizome paste of another mangrove *Acrostichum aureum* was found useful in the treatment of boils (Naskar & Mandal, 1999). *Calophyllum inophyllum* has a wide variety of medicinal applications: the paste of its seeds are applied for relief from painful joints; the oil is applied in case of rheumatism, gout, scabies and other skin diseases; camphor-mixed oil is used for application on ringworm and the soap from oil has strong bactericidal and fungicidal properties (Sundur et al., 2014). The fruits of *Sonneratia caseolaris* are used in the preparation of poultice for sprains and swellings (Hajra & Sanjappa, 1996). Researchers have increasingly recognized the pharmacological potential of these mangrove plants.

Several bioactive chemical compounds have been identified to date from mangroves species, exhibiting various bioactivities including antimicrobial, anti-inflammatory, antioxidant, anticancer and antiviral properties. Such characteristics make them promising candidates for the development of novel pharmaceuticals and therapeutic agents. The phytochemicals qualitatively identified from mangrove species are listed in Table 2.1.

**Table 2.1:** Phytochemicals identified qualitatively from mangrove species [adapted from Mitra et al., 2021]

| <b>Mangrove Species</b>      | <b>Chemical Compounds</b>                                                                                                                                                    | <b>References</b>                                                                                |
|------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| <i>Avicennia africana</i>    | Naphthoquinones                                                                                                                                                              | Ito et al. (2000)                                                                                |
| <i>Avicennia alba</i>        | Lipids, hydrocarbons, naphthoquinones, flavonoids, phenolic groups, alkaloids, triterpenoids                                                                                 | Ito et al. (2000), Banadaranayake (2002)                                                         |
| <i>Avicennia marina</i>      | Alcohols, amino acids, carbohydrates, fatty acids, hydrocarbons, carboxylic acids, steroids, tannins, triterpenes, vitamins, flavonoids, phenolics, alkaloids, triterpenoids | Manilal et al. (2009, 2016), Ali & Bashir (1998), Padmakumar et al. (1993), Bandaranayake (2002) |
| <i>Avicennia officinalis</i> | Alkaloids, carbohydrates, flavonoids, glycosides, lipids, polyphenols, proteins, saponins, tannins                                                                           | Saxena (1975), Bhimba et al. (2010), Bandaranayake (2002)                                        |
| <i>Bruguiera cylindrica</i>  | Alkaloids, tannins, fatty acid, flavonoids, phenolic compounds, triterpenoids                                                                                                | Padmakumar & Ayyakkannu (1994,1997), Bandaranayake (2002)                                        |
| <i>Bruguiera gymnorhiza</i>  | Anthocyanins, carbohydrates, carotenoids, catechins, fatty acids, lipids, flavans, phenolic compounds, proteins, steroids, carboxylic acids, triterpenes                     | Rollet (1981), Hogg & Gillan (1984), Bandaranayake (2002)                                        |
| <i>Bruguiera sexangular</i>  | Anthocyanins, carbohydrates, fatty acids, hydrocarbons, lipids, phenolic compounds, proteins, steroids, alkaloids, tannins                                                   | Rollet (1981), Hogg & Gillan (1984), Bandaranayake (2002)                                        |
| <i>Ceriops decandra</i>      | Flavonoids, polyphenols, proteins, steroids, tannins, triterpenes, alkaloids                                                                                                 | Premanathan et al. (1996), Rollet (1981)                                                         |
| <i>Ceriops tagal</i>         | Fatty acids, hydrocarbons, steroids, alkaloids, polyphenols, proteins, tannins                                                                                               | Bandaranayake (2002), Rollet (1981)                                                              |
| <i>Excoecaria agallocha</i>  | Alkaloids, polyphenols, proteins, saponins, steroids, sugars, tannins, triterpenes, fatty acid                                                                               | Bandaranayake (2002), Rollet (1981), Padmakumar & Ayyakkannu (1994,1997)                         |
| <i>Heritiera fomes</i>       | Polyphenols, proteins, tannins                                                                                                                                               | Basak et al. (1996), Bandaranayake (2002)                                                        |
| <i>Lumnitzera racemosa</i>   | Cyclitols, sugars, tannins                                                                                                                                                   | Premnathan et al. (1992), Rollet (1981)                                                          |
| <i>Nypa fruticans</i>        | Acetic acid, ethanol, sugars                                                                                                                                                 | Rollet (1981)                                                                                    |
| <i>Rhizophora apiculata</i>  | Phenolic compounds, steroids, triterpenes, flavonoids, alkaloids, triterpenoids, carotenoids, tannins                                                                        | Subramonia & Kathiresan (1988), Bandaranayake (2002)                                             |
| <i>Rhizophora gymnorhiza</i> | Anthocyanins, anthocyanidins, steroids, tannins, triterpenes                                                                                                                 | Majumdar & Patra (1976)                                                                          |
| <i>Rhizophora mucronata</i>  | Alkaloids, flavonoids, lipids, inositols, triterpenes, phenolic compounds, tannins                                                                                           | Rollet (1981), Basak et al. (1996), Bandaranayake (2002)                                         |

As evident from Table 2.1, the majority of the identified bioactive molecules are secondary metabolites rather than peptides/proteins. A few studies have explored the bioactivities of

mangroves in Goa using crude extracts, yet there is a notable absence of research focusing on fractionation of active components or their functional characterization.

#### **2.1.4 Gaps in existing research**

- There are currently no available studies on the purification and identification of antimicrobial cationic peptides derived from mangrove species found along the West Coast of India, or from elsewhere.
- Comprehensive study of antimicrobial potential of mangrove plants found in Goa is lacking.

Since there is thus currently a glaring lack of information on antimicrobial peptides from mangroves, it was hoped that our research could significantly contribute to narrowing this knowledge gap, by screening potential mangrove species in Goa for production of bioactive cationic peptides and characterization of molecules of promise. The initial step in comprehensively understanding the potential of antimicrobial peptides derived from different mangroves and their plant parts would be crucial. By delving into this area, our study aims to shed light on the significance of mangroves and establish a groundwork for future investigations into the exploration and utilization of mangroves for the discovery of antimicrobial peptides.

#### **2.1.5 Cationic peptides from mangrove plants?**

Mangrove plants, thriving in the challenging intertidal zones, have evolved unique mechanisms to combat environmental stressors, including microbial pathogens (Kathiresan et al., 2005; Saddhe et al., 2017). They exhibit a wide range of bioactivities, making them significant resources for medicinal and pharmacological applications. Plants such as *Avicennia marina*, *Acanthus ilicifolius*, *Xylocarpus granatum*, *Excoecaria agallocha*, *Kandelia candel* and *Rhizophora mucronata* are known to show various activities such as antitumor, antifungal, antimicrobial, antiviral, anti-inflammatory, and insecticidal activity (Wu et al., 2008; Dahibhate et al., 2019; Bibi et al., 2019; Mitra et al., 2021). The unique chemical compounds found in mangrove plants have shown promising potential in the development of new drugs for various health conditions.

Mangrove plants have long been acknowledged for their abundant reserve of bioactive substances encompassing alkaloids, saponins, phenols and tannins, which showcase diverse biological activities. Alongside these traditional bioactive compounds, plants also produce a varied array of bioactive peptides crucial for their defence mechanisms (Patra & Mohanta, 2014).

Bioactive peptides exhibit a range of biological effects, including antimicrobial and antioxidant properties. Research into bioactive proteins and peptides, aimed at searching for compounds resistant to pathogens and possessing antimicrobial properties, has been increasing. These proteins and peptides display targeted toxicity towards pathogens, presenting promising prospects for novel antimicrobial agents.

Mangrove plants are a rich source of bioactive secondary metabolites but as compared to terrestrial plants and animal sources, they are unexplored in relation to cationic peptide production (Wu et al., 2023). Whilst a number of other compounds from mangrove plants have been identified with antimicrobial activity (Mitra et al., 2021), to the best of our knowledge none of these plants have been examined for peptide antimicrobials and therefore, exploring them for antimicrobial cationic peptides appeared to be a promising study.

Secondary metabolites are extracted according to their biochemical characteristics. The antimicrobial peptides (AMPs) in plants are small molecules with molecular weights ranging from 2 to 10 kDa, displaying amphiphilic properties, and typically carrying a positive charge at neutral and physiological pH. They exhibit significant diversity in both primary and secondary structures. Notably, many plant-derived AMPs are characterized by compact spatial structures, facilitated by intramolecular disulfide bonds, which confer stability against temperature, enzymes, and chemical agents. Plant AMPs are categorized into various families based on amino acid sequence similarity, cysteine motifs, disulfide bond locations, and secondary structure elements. These families include defensins, thionins,  $\alpha$ -hairpinins, hevein-like peptides, knottins, snakins, lipid-transfer proteins, and cyclotides. Additionally, there are peptides with unconventional characteristics, such as unique cysteine motifs, absence of disulfide bonds, cyclic peptides lacking cysteine knots, and peptides rich in glycine, histidine, or alanine (Tam et al. 2015). There are various methods by which AMPs are isolated from plant sources. General approaches to AMP isolation are as depicted in Fig. 2.1.

Obtaining high-quality protein samples is crucial for optimal outcomes in proteomic analysis. However, challenges arise due to co-extraction and the presence of non-protein components especially pronounced in plant tissues, where proteases, oxidative enzymes, etc. constitute a relatively small fraction compared to various other constituents including cell walls, vacuoles, pigments, lipids, starches, polysaccharides, organic acids, polyphenols, and secondary metabolites (Fei et al., 2021). Mangrove plants encounter specific hurdles in protein extraction, primarily due to elevated levels of interfering compounds such as tannins (Yan & Tam, 2011).

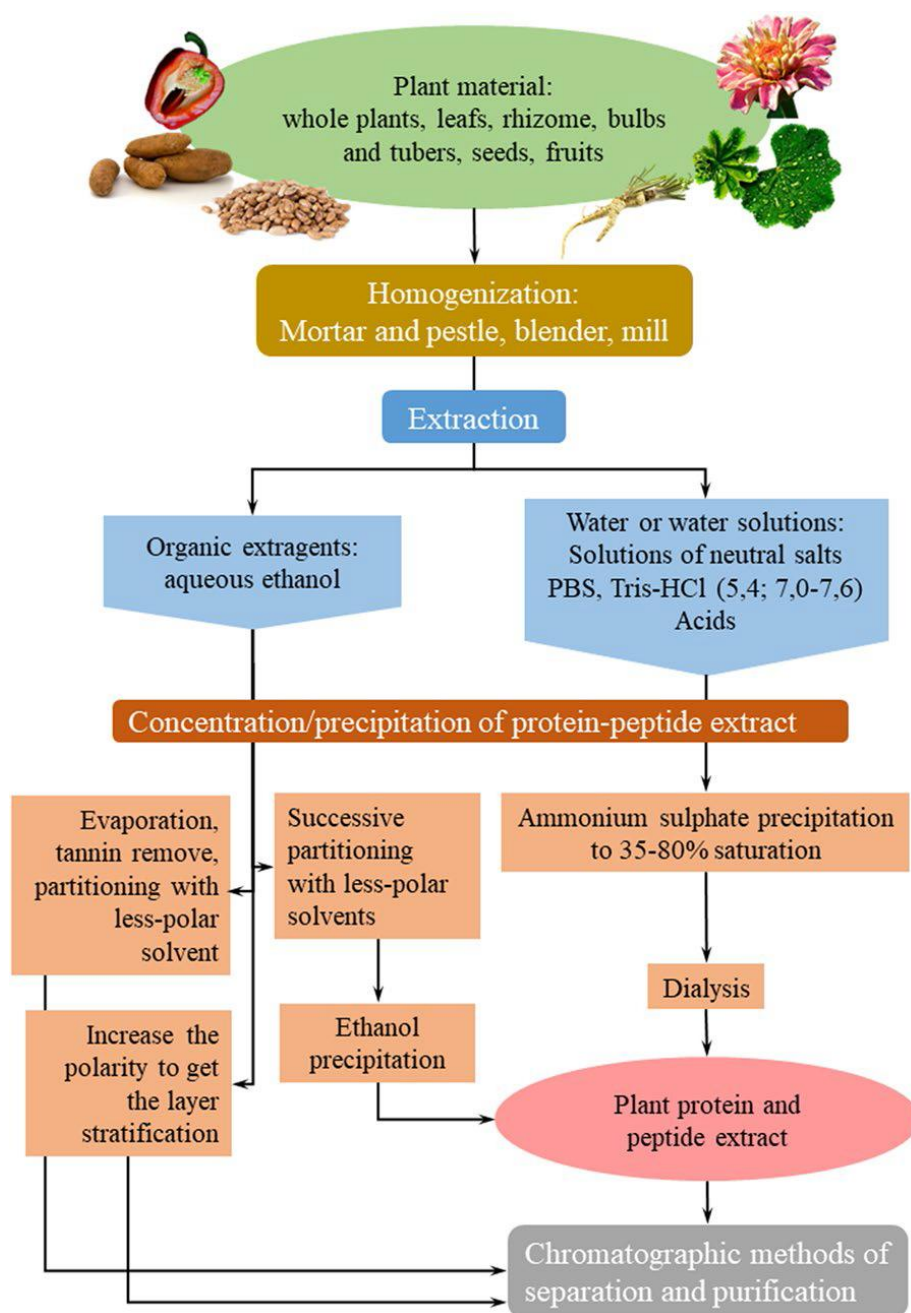


**Fig. 2.1:** Schematic diagram of AMP isolation procedures from different plant sources [adapted from Barashkova & Rogozhin, 2020].

The choice of protein extraction methods for plant samples would significantly influence subsequent experimental results. Several biochemical properties of antimicrobial peptides are taken into consideration to facilitate their isolation. Presently, two categories of solvents are employed for extracting AMPs from plant sources. The first is represented by water and water-based solutions including salt, acids, and buffer solutions; the second includes organic-based solutions, including water solutions of ethanol (Fig 2.1). The most common approaches to AMP extraction are outlined in Table 2.2.

**Table 2.2:** The most common approaches to AMP extraction [adapted from Barashkova & Rogozhin, 2020]

| Extractant                                              | AMP                        | Plant species and part                 | Extraction method                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Reference              |
|---------------------------------------------------------|----------------------------|----------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------|
| <b>Water and water-based solutions</b>                  | New cysteine-rich peptides | <i>Potentilla anserina</i> (roots)     | 1. Dried roots were extracted at room temperature for 1 h and centrifuged at 10,000 rpm for 20 min; 2. Concentration by adding ammonium sulfate to 80% relative saturation; 3. Desalting was performed using C18 reversed-phase flash column at a stepwise gradient of ethanol (40, 60, 80%)                                                                                                                                                                                                                          | Odintsova et al., 2008 |
|                                                         | 8-Cys hevein-like peptides | <i>Moringa oleifera</i> (fresh leaves) | 1. Plant material was extracted with an equal volume of water while blending (6 min). The mixture was centrifuged at 8000 rpm for 10 min; 2. The supernatant was filtered and loaded on C18 flash-column, elution was performed using increase of ethanol concentration (20, 70%)                                                                                                                                                                                                                                     | Chan & Ng, 2013        |
| <b>Acid solution, 50 mM H<sub>2</sub>SO<sub>4</sub></b> | Defensin                   | <i>Nicotiana glauca</i> (flowers)      | 1. Flowers were ground with liquid nitrogen by mortar and pestle, then extracted with sulfuric acid (3 mL/g wet weight) for 1 h. Insoluble material was filtered and centrifuged (25,000g, 15 min, 4 °C); 2. pH of supernatant was adjusted to 7.8 by adding NaOH, and stirred for 1 h, then centrifuged (25,000g, 15 min, 4 °C); 3. Concentration by adding solid ammonium sulfate to 80% relative saturation (stirring for 4–16 h at 4 °C); 4. The precipitate was dissolved in gel-filtration buffer, heated to 90 | Egorov et al., 2005    |

|                                                                                                                                 |                        |                                              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                            |
|---------------------------------------------------------------------------------------------------------------------------------|------------------------|----------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------|
|                                                                                                                                 |                        |                                              | °C and separated using Sephadex G-50 gel-filtration column                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                            |
| <b>Acid mixture: 1% v/v trifluoroacetic acid (TFA), 1 M HCl, 5% v/v formic acid, 1% w/v NaCl</b>                                | Gly/His-rich peptides  | <i>Capsella bursa-pastoris</i> (roots)       | 1. Roots were homogenized while blending with extraction mixture (1:4, w/v). Homogenate was filtered through a paper filter and centrifuged at 20,000g 30 min; 2. The supernatant was concentrated using the reversed-phase C18 Sep-Pack cartridge. Peptides were removed from the column by washing with 80% acetonitrile with 0.1% TFA.                                                                                                                                                                                                                                    | García-Olmedo et al., 1998 |
| <b>Buffer solution: 0.1 M Tris-HCl (pH 7.2)</b>                                                                                 | Defensin-like peptides | <i>Phaseolus limensis</i> (seeds)            | 1. Seeds were washed and soaked in water for 12 h. Then homogenized in a blender with buffer solution. The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C; 2. The supernatant was fractionated by two-step ammonium sulfate precipitation. In the first step, the solution was saturated to 20%; the resulting supernatant was saturated to 85%; 3. After centrifugation at 12,000 rpm for 20 min, the precipitate was collected, dissolved in 100 mL of 0.01 M Tris-HCl buffer (pH 7.2), dialyzed against the same buffer and subjected to further separation. | Evaristus et al., 2018     |
| <b>Buffer solution: 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 15 mM NaH<sub>2</sub>PO<sub>4</sub>, 100 mM KCl, 1. % EDTA, pH 5.4</b> | Thionin-like peptides  | <i>Capsicum anuum</i> (Fruits without seeds) | 1. <i>C. anuum</i> fruits were extracted with a buffer solution in a 1:5 ratio (w/v) for 2 h; 2. The extract was saturated with ammonium sulfate. The precipitate formed at 0-70% relative saturation was redissolved in distilled water and heated at 80 °C for 15 min and centrifuged; 3. The resulting suspension was extensively dialyzed against distilled water, freeze-dried and subjected to further fractionation by chromatographic method.                                                                                                                        | Kul'Ko et al., 2016        |
| <b>Organic solutions MeOH/CH<sub>2</sub>Cl/0.05% TFA in water (4:4:1)</b>                                                       | PawS-derived peptides  | <i>Zinnia haageana</i> (seeds)               | 1. Seeds (50 mg) were ground to a fine powder with mortar and pestle under liquid nitrogen with a pinch of 0.1 mm glass beads; 2. The extraction mixture (0.9 mL) was added to the seed powder, and the mixture was vortexed and centrifuged (3 min at 16,000g). If the phases were not separated at this point, 0.1 mL of chloroform or 0.1 mL of 0.05% TFA in water was added alternately, followed by short                                                                                                                                                               | Claeson et al., 1998       |

|                                                      |            |                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                     |
|------------------------------------------------------|------------|---------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
|                                                      |            |                                             | centrifugation after each addition, until phase separation was achieved; 3. After phase separation, the upper polar layer was collected, and dried under vacuum and re-dissolved in 0.5 mL, 5% (v/v) formic acid for the further peptide identification.                                                                                                                                                                                                                                                           |                     |
| <b>EtOH or MeOH 20% or 50% in water respectively</b> | Cyclotides | <i>Viola odorata</i> (aerial parts)         | 1. Dried plant material was finely ground; 2. Plant material was extracted with an extraction mixture in a 1:20 (w/v) ratio for 6 h.                                                                                                                                                                                                                                                                                                                                                                               | Barber et al., 1988 |
| <b>MeOH in water (1:1)</b>                           | Thionins   | <i>Viscum album</i> (Green and white parts) | 1. Plant material was crushed, the extraction mixture was added (1:5, w/v), the solution was filtered and its volume was reduced;<br>2. The aqueous phase was successively partitioned with cyclohexane, dichloromethane, and ethyl acetate;<br>3. Ethanol was added to the concentrated aqueous phase to achieve 85% (v/v) concentration; the precipitate was separated by centrifuging (2000g; 10 min);<br>4. The supernatant was concentrated, and ethanol was added to 85% (v/v). The precipitates were pooled | Limas et al., 1990  |

In the present study we have adopted the acid extraction protocol. Antimicrobial peptides are readily and often selectively soluble in acidic solvents that denature many larger proteins (Cole & Ganz, 2000). Apart from a small group of anionic antimicrobial peptides, most known antimicrobial peptides are cationic at neutral pH, which affords separation from the anionic molecules by ion-exchange matrices with positive-charge selectivity. By definition, antimicrobial peptides are less than 10 kDa in size and are separated from larger proteins by molecular filtration or size exclusion chromatography. In addition, most peptides confer an amphipathic “sidedness” in solution by exhibiting a conformation that regionalizes hydrophobic side chains. This feature also enables separation using organic (reverse-phase) solvents.

This Chapter focuses on the extraction of cationic peptides from selected mangrove plants and attempts to analyze the effects of seasonal and geo-climatic variations on cationic peptide production.

### **2.1.6 Description of the study area**

The Mandovi river originates from the Western Ghats, flows through Belagavi, Uttara Kannada in Karnataka and Cumbharjua, Divar and Chorão in Goa, and eventually merges into the Arabian sea. The Divar island (15°30'35"N and 73°52'28"E) area is separated from the mainland by the Mandovi estuary. This tropical shallow estuary, with an average depth of 5m, is flanked by mangrove forests and is influenced by semidiurnal tides. During the monsoon season (June–September) it receives a large inflow of freshwater from various sources (Sundar & Shetye, 2005). It is highly productive and forms a good nursery ground for oysters, fiddler crabs, fishes, etc. A variety of birds particularly pintail ducks, cormorants, the common sandpiper, etc. have made these mangrove swamps their abode. Dense populations of mangrove plants and their associate plants occur on the banks of the Mandovi estuary. These consist mainly of species such as *Acanthus ilicifolius*, *Pongamia pinnata*, *Cyperus* spp., *Bruguiera gymnorhiza*, *Avicennia officinalis*, *Caesalpinia* sp., *Sonneratia caseolaris* and *Rhizophora mucronata*. They are of immense ecological and economic value and support the livelihood of many islanders.

## **2.2 Materials and methods**

Analytical-grade chemicals were used in all the experiments and Milli-Q water or double-distilled water were used for all preparations. Unless specified otherwise, experiments were conducted in triplicate, and each dataset presented reflects the findings from a minimum of three independent experiments.

### **2.2.1. Mangrove plants under study**

The mangrove plants chosen for this study are described below:

**(A) *Sonneratia alba***



***Taxonomic position***

|          |                     |
|----------|---------------------|
| Kingdom  | : Plantae           |
| Division | : Magnoliophyta     |
| Class    | : Magnoliopsida     |
| Order    | : Magnoliales       |
| Family   | : Sonneratiaceae    |
| Genus    | : <i>Sonneratia</i> |
| Species  | : <i>alba</i>       |

**Fig. 2.2:** *Sonneratia alba* and its taxonomic classification

The mangrove plant *S. alba* has a root system of pneumatophores visible as popping up all around in the surrounding soil. They have a superior ovary and the fruit is a leathery berry seated on a calyx. They are found in East Africa, through the Indian subcontinent and southeast Asia, and have a number of medicinal uses. *S. alba* contains essential secondary metabolites which have an ability to act against Gram negative bacteria (Asad et al., 2013). Its bark extract showed significant anti-oxidant, cytotoxic and anti-microbial activities against certain organisms (Morada et al., 2016). *S. alba* fruits also have medicinal properties, the ripe fruits being used to expel intestinal parasites while the half-ripe ones are used to treat cough (Saad et al., 2012). *S. alba* had demonstrated antibacterial activity against human bacterial pathogens *Staphylococcus aureus*, *Streptococcus* sp., *Salmonella typhi*, *Proteus vulgaris* and *Proteus mirabilis* (Sahoo et al., 2012)

**(B) *Excoecaria agallocha***



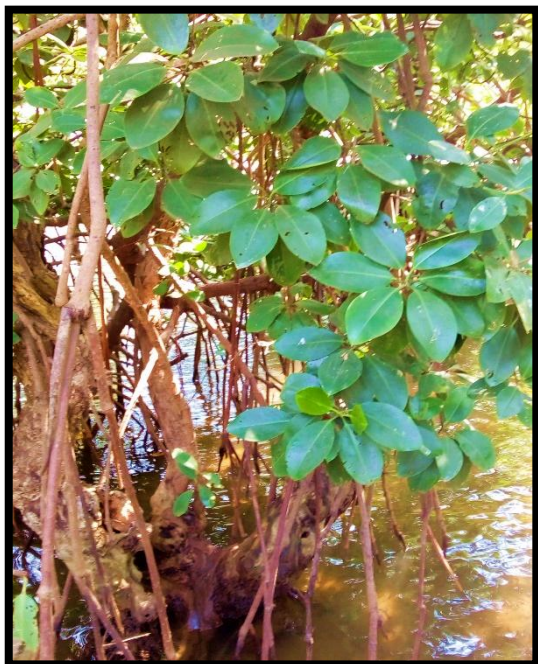
***Taxonomic position***

|          |                     |
|----------|---------------------|
| Kingdom  | : Plantae           |
| Division | : Tracheophyta      |
| Class    | : Magnoliopsida     |
| Order    | : Magnoliales       |
| Family   | : Euphorbiaceae     |
| Genus    | : <i>Excoecaria</i> |
| Species  | : <i>agallocha</i>  |

**Fig. 2.3:** *Excoecaria agallocha* and its taxonomic classification

Commonly known as “milky mangrove” species, *Excoecaria agallocha* is found along the coastal areas of Konkan and Tamil Nadu and in the tidal forest and swamps (Vadlapudi et al., 2009). It is called “blind eye mangrove”. The tree grows to 4-20 m in height. Its bark is grey, rough and covered with pores. Leaves of the mature plants have an exudate of salt on their lower epidermis (Peter et al. 1999). Clinical trials carried out on this plant have shown potential anticancer, anti-bacterial and anti-viral properties (Mastellar, 1997). The infusion of these leaves possesses anti-oxidant and anti-tumor promoting properties (Konishi et al., 2000). The bark oil had been effective against leprosy as well as paralysis. This plant has been used to treat sores and stings from marine creatures and ulcers. The leaf extracts had anti-oxidant, anti-cancer as well as anti-hyperglycaemic properties (Chan et al., 2018), while the stem ethanolic extracts were seen to have cytotoxic effects on human lung cancer cell lines (Patil et al., 2012). *E. agallocha* displayed promising anti-bacterial potential against fish pathogens (Shamsuddin et al., 2013).

**(C) *Rhizophora mucronata***



***Taxonomic position***

|          |   |                   |
|----------|---|-------------------|
| Kingdom  | : | Plantae           |
| Division | : | Magnoliophyta     |
| Class    | : | Magnoliopsida     |
| Order    | : | Malpighiales      |
| Family   | : | Rhizophoraceae    |
| Genus    | : | <i>Rhizophora</i> |
| Species  | : | <i>mucronata</i>  |

**Fig. 2.4:** *Rhizophora mucronata* and its taxonomic classification

*Rhizophora mucronata* is an evergreen mangrove that grows from East Africa through Madagascar, islands of Indian Ocean and the South Pacific Island. Commonly called “Asiatic mangrove”, this tree with its stilt roots grows to a height of 30 m, with a trunk 50-70 m wide. The leaves are glossy green with black spots over them. The bark of this tree is of significance for tannin production, in the leather industry, dye lines, fishing nets and ropes. This plant is used in a number of medicinal purposes such as for haematuria, diarrhoea, diabetes and dysentery. It is also used against hepatitis, elephantiasis and ulcers. Its bioactive toxic phytochemical is used against mosquito populations (Meenakshi et. al, 2014). Aqueous extract of this plant possesses antiviral activity against WSSV (White Spot Syndrome Virus) of *P. monodon* (Chakraborty et al., 2014).

**(D) *Acanthus ilicifolius***



**Taxonomic position**

|          |   |                    |
|----------|---|--------------------|
| Kingdom  | : | Plantae            |
| Division | : | Spermatophyta      |
| Class    | : | Dicotyledonae      |
| Order    | : | Dicotyledonae      |
| Family   | : | Acanthaceae        |
| Genus    | : | <i>Acanthus</i>    |
| Species  | : | <i>ilicifolius</i> |

**Fig. 2.5:** *Acanthus ilicifolius* and its taxonomic classification

*Acanthus ilicifolius*, often referred to as holly-leaved acanthus, sea holly, or holy mangrove, is a type of shrub or herb within the Acanthaceae plant family. Originating from Australia, Australasia, and Southeast Asia, it is employed for medicinal purposes in the treatment of asthma and rheumatism. The plant typically grows as a shrub, reaching heights of up to 2 m. It possesses shallow tap roots and may sporadically produce stilt roots. The fruits of the plant are kidney-shaped in appearance. Folklore claims for this plant are as aphrodisiac, blood purifier, diuretic, for treatment of asthma, diabetes, dyspepsia, hepatitis, leprosy, neuralgia, paralysis, ringworm, rheumatism, skin diseases, snakebite, stomach pains, leucorrhoea, leukaemia (Mastaller et al., 1997; Bandaranayake, 2002). The 2-benzoxazolinone (BOA) and benzoxazinoid compounds present in *A. ilicifolius* have been identified as anti-inflammatory agents (Wahidullah & Bhattacharjee, 2001; Gulcan et al., 2003).

### **2.2.2 Collection and identification of plant material**

Plant parts were collected from the Divar mangrove area of Goa, India from *Sonneratia alba* (N15°30' E73°52), *Excoecaria agallocha* (N15°30' E73°52), *Rhizophora mucronata* (N15°30' E73°52) and *Acanthus ilicifolius* (N15°30' E73°51') (Fig 2.6). The fresh leaf, stem and root samples were washed thoroughly with tap water to remove dirt and surface-sterilised with 70%

ethanol, followed by washing with distilled water. The samples were shade-dried at room temperature. The fresh samples were identified by Prof. M. K. Janarthanam, Department of Botany, Goa University, Goa and thereafter, a herbarium was deposited in the Department of Botany, with sample nos. PGN01, PGN02, PGN03 and PGN04 for *Sonneratia alba*, *Excoecaria agallocha*, *Rhizophora mucronata* and *Acanthus ilicifolius*, respectively.

For seasonal studies, leaf, stem and root samples of the above four plants were collected during the pre-monsoon months (March to May), active southwest monsoon period (July to September) and in the post-monsoon season (October to February).

**Table 2.3** : List of experimental mangrove plants.

| S. No | Mangrove plant              | Plant part | Abbreviation | Location   |
|-------|-----------------------------|------------|--------------|------------|
| 1.    | <i>Sonneratia alba</i>      | Leaf       | SA Leaf      | Divar, Goa |
|       |                             | Stem       | SA Stem      |            |
|       |                             | Root       | SA Root      |            |
| 2.    | <i>Excoecaria agallocha</i> | Leaf       | EA Leaf      | Divar, Goa |
|       |                             | Stem       | EA Stem      |            |
|       |                             | Root       | EA Root      |            |
| 3.    | <i>Rhizophora mucronata</i> | Leaf       | RM Leaf      | Divar, Goa |
|       |                             | Stem       | RM Stem      |            |
|       |                             | Root       | RM Root      |            |
| 4.    | <i>Acanthus ilicifolius</i> | Leaf       | AI Leaf      | Divar, Goa |
|       |                             | Stem       | AI Stem      |            |
|       |                             | Root       | AI Root      |            |

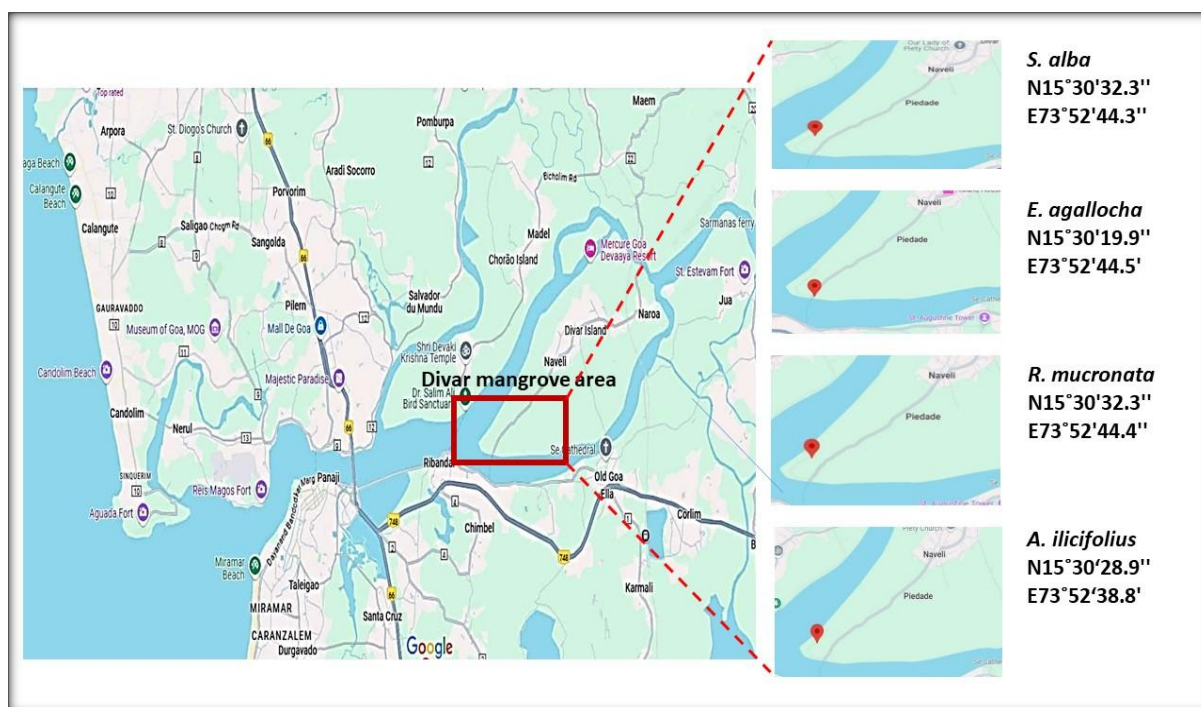


**Fig. 2.6:** Map showing locations of mangrove plants under study

### 2.2.3 Crude extract preparation (acetic acid extraction)

Leaf, stem and root tissue (10 g) of *S. alba*, *R. mucronata*, *E. agallocha* and *A. ilicifolius* were homogenized in 10% acetic acid in a blender and then vortex-mixed for 5 min, in order to selectively denature and precipitate larger proteins, leaving cationic peptides in solution. The 10% homogenate was left overnight at 4°C on a shaker, under mild agitation. The crude extract was filtered through a muslin cloth, centrifuged at 10,000 rpm for 30 min at 4°C and the pellet discarded. The supernatant ('acid extract') was stored at 4°C (Cole & Ganz, 2000).

### 2.2.4 Ammonium sulphate precipitation

Homogenates of leaf, stem or root extracts of *S. alba*, *R. mucronata*, *E. agallocha* and *A. ilicifolius* were subjected to 90% ammonium sulphate saturation, maintaining a temperature of 4°C. The proteinaceous pellet obtained upon centrifugation at 10,000 rpm for 30 min was suspended in 10 mM sodium acetate buffer (pH 5.6) or else stored as such at -20°C until further use.

### 2.2.5 Maintaining pathogen cultures

The microbial strains used in this study as listed were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh.

**Table 2.4:** Microbial strains used for the study

| S. No                 | Microbial strain                | MTCC Number | Characteristic |
|-----------------------|---------------------------------|-------------|----------------|
| Gram-negative strains |                                 |             |                |
| 1.                    | <i>Salmonella typhimurium</i>   | MTCC 3231   | Human pathogen |
| 2.                    | <i>Escherichia coli</i>         | MTCC 443    | Human pathogen |
| 3.                    | <i>Pseudomonas aeruginosa</i>   | MTCC 741    | Human pathogen |
| 4.                    | <i>Pseudomonas fluorescense</i> | MTCC 7200   | Fish pathogen  |
| 5.                    | <i>Aeromonas hydrophila</i>     | MTCC 1739   | Human pathogen |
| 6.                    | <i>Aeromonas sobria</i>         | MTCC 3613   | Fish pathogen  |
| Gram-positive strain  |                                 |             |                |
| 7.                    | <i>Staphylococcus aureus</i>    | MTCC 3160   | Human pathogen |
| Fungal strain         |                                 |             |                |
| 8.                    | <i>Candida albicans</i>         | MTCC 227    | Human pathogen |

All the bacterial pathogens were maintained on Nutrient Agar at 37 °C for 24 h and the fungal pathogen on Potato Dextrose Agar incubated at 37 °C for 48 h.

### 2.2.6 Antimicrobial Assay

Antimicrobial activity of the plant extracts was assessed by the well diffusion method (Shanmugaraju, 2012). The test pathogen was spread-plated onto nutrient agar (HiMedia) plates. Wells were punched into the agar using an 8 mm cork borer. Partially purified extract (100 µL) was added into the wells and the zone of inhibition noted after 24 h.

### 2.2.7 Standardisation of homogenate percentage

Homogenates at 5%, 10% and 15%, of *S. alba*, *R. mucronata*, *E. agallocha* and *A. ilicifolius* plant parts were prepared by grinding leaf, stem or root samples in 10% acetic acid. These extracts were then partially purified by ammonium sulphate precipitation, as outlined in Section 2.2.4. The proteinaceous pellet in 10 mM sodium acetate buffer, pH 5.6 was tested for activity against *S. typhimurium*, *A. hydrophila* and *C. albicans*, by the agar well diffusion method (as above).

### **2.2.8 Solvent percentage standardisation**

For the initial screening studies, 10% homogenate of root extract of *S. alba* was prepared in 0.3%, 1% and 10% acetic acid and then subjected to 90% ammonium sulphate precipitation. The pellet suspended in 10 mM sodium acetate buffer, pH 5.6 was used for antimicrobial tests against *S. typhimurium* and *C. albicans*, by the agar well diffusion method.

### **2.2.9 Estimation of total proteins by Bradford's method**

The protein content present in different protein pellets obtained after ammonium sulphate precipitation from various plant samples were determined by Bradford's method (Bradford, 1976), using bovine serum albumin (BSA) as standard.

### **2.2.10 Measurement of physico-chemical parameters at sampling sites**

Water samples were collected from four predetermined locations in the Divar mangrove area as shown in the map (Fig. 2.6). Sterile glass bottles were used for surface water collection, ensuring a volume of 1litre per sample. Salient parameters of the water samples, such as temperature, salinity, conductivity, Total Dissolved Solids (TDS) and Dissolved Oxygen (DO), were determined on the field using a multiparameter instrument (CyberScan PC 650, Eutech Instruments, Thermo Fisher Scientific, India) and pH was measured on a digital pH meter (Eutech pH meter-700, Thermo Fisher Scientific, India).

### **2.2.11 Absorption spectra**

The acid extracts of leaf, stem, and root samples from *S. alba*, *R. mucronata*, *E. agallocha*, and *A. ilicifolius* were diluted at a ratio of 1:3 and scanned using a UV-vis spectrophotometer within the range of 200-400 nm.

### **2.2.12 Phytochemical Analysis of the Extracts:**

A qualitative phytochemical screening was conducted to identify the primary classes of compounds (including tannins, flavonoids, alkaloids, phenols, and terpenoids) present in the extracts, following standard protocols (Evans, 2009; Chaudhary et al., 2010; Kancherla et al., 2019).

#### *Test for Alkaloids:*

Mayer's Test: 1 mL of the extract was combined with a few drops of Mayer's reagent. The

appearance of a cream-colored precipitate indicated the presence of alkaloids.

#### *Test for Tannins:*

Ferric Chloride Test: The extracts were treated with a few drops of FeCl<sub>3</sub> solution. The formation of a blackish precipitate indicated the presence of tannins.

#### *Test for Terpenoids:*

Sulfuric Acid Test: To 1 mL of the extract, 5 mL of chloroform was added. Then, 1 mL of concentrated H<sub>2</sub>SO<sub>4</sub> was carefully added along the walls of the tube and mixed. The formation of a reddish color in the lower layer indicated the presence of terpenoids.

#### *Test for Flavonoids:*

To 1 mL of the extract, a few drops of concentrated H<sub>2</sub>SO<sub>4</sub> were added, followed by 2 mL of ammonia solution. The appearance of an orangish precipitate indicated the presence of flavonoids.

### **2.2.13 SDS-PAGE**

The molecular weight structure was gauged by SDS-PAGE, using a 15% gel (Laemmli, 1970). After electrophoresis, the gel was stained with Coomassie Brilliant Blue G-250 as per Candiano et al. (2004). Low molecular weight protein markers (Sigma-Aldrich) ranging from 3.5 to 43 kDa were used as standard.

## **2.3 Results and discussion**

Mangrove plants thrive in challenging intertidal zones, providing vital ecological benefits such as coastal protection and habitat for diverse wildlife. Moreover, mangroves have developed an impressive array of chemical defences to protect themselves from various threats. These defences include secondary metabolites such as tannins, flavonoids and alkaloids, which have antimicrobial and insecticidal properties. These chemicals not only deter herbivores and pathogens but also aid in adaptation to their habitat (Fei et al., 2021). The current research investigates the production of antimicrobial peptides from these plants, tapping into their rich reservoir of bioactive compounds. The extraction of antimicrobial peptides from plants can be achieved through various methods, with the selection of extraction solvent playing a significant role in determining the success of the process. In this study, the extraction of AMPs from

leaves, stem and roots of *S. alba*, *E. agallocha*, *R. mucronata* and *A. ilicifolius* has been carried out for the first time.

Hydrophobic residues are a common occurrence in the AMP structure. The majority of AMPs are positively charged at physiological pH by virtue of an abundance of basic amino acid residues such as arginine and lysine, although some anionic AMPs have also been reported (López-García. et al., 2012). Such diversity in structure and chemical characteristics hence requires different extraction solvents with varying polarities, pH and degree of hydrophobicity, in order to extract different types of AMPs. Since the extraction of AMPs from these plants have not been recorded before, we began by investigating different percentages of homogenate and of solvent (acetic acid) to be used.

### 2.3.1 Percentage homogenate standardisation

The partially purified leaf, stem and root extracts of different percentages (5%, 10% and 15%) of *S. alba*, *E. agallocha*, *R. mucronata* and *A. ilicifolius* homogenates were evaluated for antimicrobial activity against *S. typhimurium*, *A. hydrophila* and *C. albicans*. The antimicrobial tests of the different extracts obtained demonstrated encouraging activity levels (Fig. 2.7).

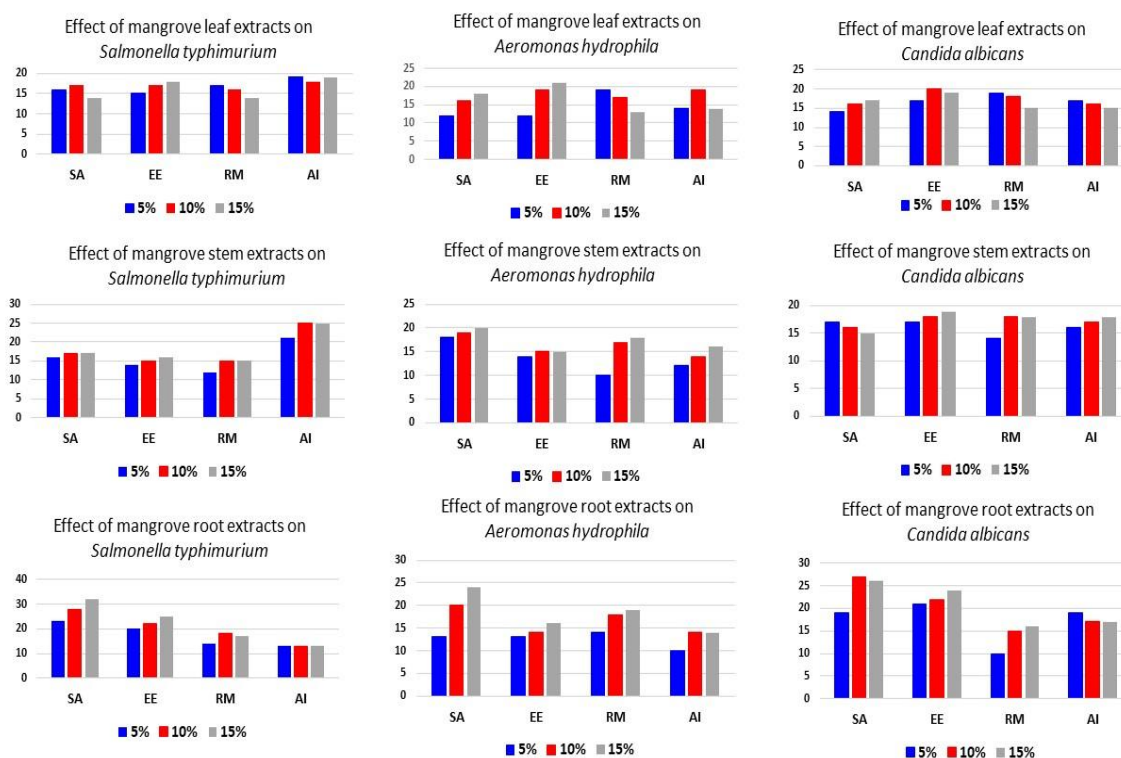


**Fig. 2.7:** Antimicrobial activity of partially purified percentage homogenates (5%, 10% and 15%) of *S. alba* (SA), *E. agallocha* (EA), *R. mucronata* and *A. ilicifolius* on *S. typhimurium*, *A. hydrophila* and *C. albicans*.

The antimicrobial effectiveness of the extracts from *S. alba*, *E. agallocha*, *R. mucronata*, and *A. ilicifolius* showed either a decline or no significant improvement beyond a 10% homogenate concentration when tested against *S. typhimurium*, *A. hydrophila*, and *C. albicans*. However, the exception was observed with the root extract of *S. alba*, which exhibited slightly elevated activity against *S. typhimurium* and *A. hydrophila* when a 15% extract concentration was used. Since the *S. alba* root extract showed higher activity as compared to the other extracts on all the 3 pathogens tested it was further used for solvent strength studies. Higher concentrations of mangrove plant homogenates may reduce antimicrobial activity due to interference by non-antimicrobial compounds, which can dilute or mask active antimicrobial agents (Mulia et al., 2023). Reduced solubility and bioavailability may lead to the precipitation of active compounds, further lowering their effectiveness (Kumar et al., 2011). Additionally, higher extract viscosity can hinder the diffusion of antimicrobial compounds, reducing their observed activity (Sahoo et al., 2012). Therefore, optimizing extract concentration is crucial for maximizing antimicrobial efficacy.

### 2.3.2 Solvent concentration studies

Homogenizing tissue with acetic acid of varying concentrations would affect extraction efficiency and bioactive compound properties. Higher concentrations could enhance yields but might alter structure and function, making optimization essential. *Sonneratia alba* root extracts were therefore prepared in different percentages (0.3%, 5%, 10%) of acetic acid. The extracts were partially purified by ammonium sulphate precipitation and their antimicrobial activity tested on *S. typhimurium* and *C. albicans* (Table 2.5).

**Table 2.5:** Antimicrobial activity of partially purified crude extracts of *S. alba* root, prepared in different concentrations of acetic acid

| Zone of inhibition (mm)  |                       |                          |                       |                          |                       |
|--------------------------|-----------------------|--------------------------|-----------------------|--------------------------|-----------------------|
| 0.3% acetic acid         |                       | 1% acetic acid           |                       | 10% acetic acid          |                       |
| S.<br><i>typhimurium</i> | C.<br><i>albicans</i> | S.<br><i>typhimurium</i> | C.<br><i>albicans</i> | S.<br><i>typhimurium</i> | C.<br><i>albicans</i> |
| 0                        | 0                     | 9                        | 9                     | 14                       | 15                    |

The results indicated that 10% acetic acid could best extract cationic peptides from these sources, and provided minimal interference in the plate assays, as observed from the control values of the zone of inhibition (which was invariably zero; data not shown, “control” referring

to 10 mM sodium acetate buffer only). Our results align with those of Cole and Ganz (2000), suggesting that 5–10% acetic acid is quite an optimal range for cationic peptide isolation.

### 2.3.3 Estimation of total proteins in ammonium sulphate suspensions by Bradford's method

Most previous studies indicated that plant antimicrobial peptides were primarily extracted from seeds (Golla et al., 2016). Seeds would tend to have a higher total protein content since they serve as the primary reservoir of proteins in plants, crucial for germination processes. Since obtaining a consistent supply of seeds and that too from tall trees was fraught with feasibility issues, it was decided to restrict our studies to root, stem and leaf samples from the selected mangrove plants.

The crude extracts were prepared and partially purified by ammonium sulphate precipitation at 90% saturation. The protein concentration was estimated in these partially purified mangrove plant extracts by the Bradford's method. The values ranged from 0.82 to 5.60 mg/g wet weight in the four plant species under study. While the highest amount was detected in *R. mucronata* root and stem extracts, *S. alba* extracts recorded the lowest (Table 2.6). For *E. agallocha*, comparatively higher values were obtained in the root extracts, yet these values were lower than those from the *A. ilicifolius* plant parts.

**Table 2.6:** Protein content in partially purified mangrove plant extracts

| Sample  | Total protein (mg/g wet wt.) |
|---------|------------------------------|
| SA Leaf | 1.02±0.07                    |
| SA Stem | 0.82±0.08                    |
| SA Root | 0.91±0.02                    |
| EA Leaf | 1.09±0.04                    |
| EA stem | 1.14±0.03                    |
| EA Root | 2.42±0.02                    |
| RM Leaf | 3.84±0.03                    |
| RM Stem | 5.13±0.15                    |
| RM Root | 5.60±0.04                    |
| AI Leaf | 3.44±0.15                    |
| AI Stem | 2.83±0.08                    |
| AI Root | 2.97±0.17                    |

### 2.3.4 Seasonal variation in peptidic content of mangrove plant parts

Mangrove plants play a crucial role in coastal ecosystems, providing unique habitats and

contributing to biodiversity. In addition to their ecological significance, mangroves have been recognized for their potential medicinal properties, particularly in the field of antimicrobial activity. The local geo-climate, seasonal changes, external factors such as light, temperature and humidity may affect the composition of secondary metabolites. The synthesis of secondary metabolites in response to environmental factors is nothing but an adoptable strategy enabling tolerance to abiotic stress (Kovvada et.al., 2019). Understanding the seasonal and geographical variations in the antimicrobial (antimicrobial peptide) production in mangrove plants would be essential for effectively harnessing their therapeutic potential, a factor which has been less explored and badly overlooked.

This Section explores the seasonal and geographical impact on antimicrobial peptide production in mangrove plants thriving in the unique environmental conditions of the Divar estuary.

Goa, being in the tropical zone and near the Arabian sea, has a hot and humid climate for most of the year. The month of May is the hottest, seeing day temperatures of over 35°C, coupled with high humidity. The monsoon rains arrive by early June. Most of Goa's annual rainfall is received through these which last till late September, with the annual average rainfall commonly less than 300 cm, and a dry season that may exceed nine months. Goa has short winters of low intensity, between mid-December and February.

All of the four plants under study (*S. alba*, *E. agallocha*, *R. mucronata* and *A. ilicifolius*) were marked and the sites visited during 2016-2019. Observations were made every month at each site, for all 12 months covering three seasons, pre-monsoon (March – June), monsoon (July – October), and post-monsoon (November –February). Physicochemical parameters of the water samples, such as temperature, salinity, conductivity, Total Dissolved Solids (TDS) and Dissolved Oxygen (DO) content were determined.

The acid extracts for selective extraction of cationic antimicrobial peptides were prepared for all the 12 selected mangrove plant parts, as described in section 2.2.3. The crude extracts were partially purified through 90% ammonium sulphate precipitation, the total protein obtained was redissolved in 10 mM sodium acetate buffer and estimated using Bradford's method (Fig. 2.8).

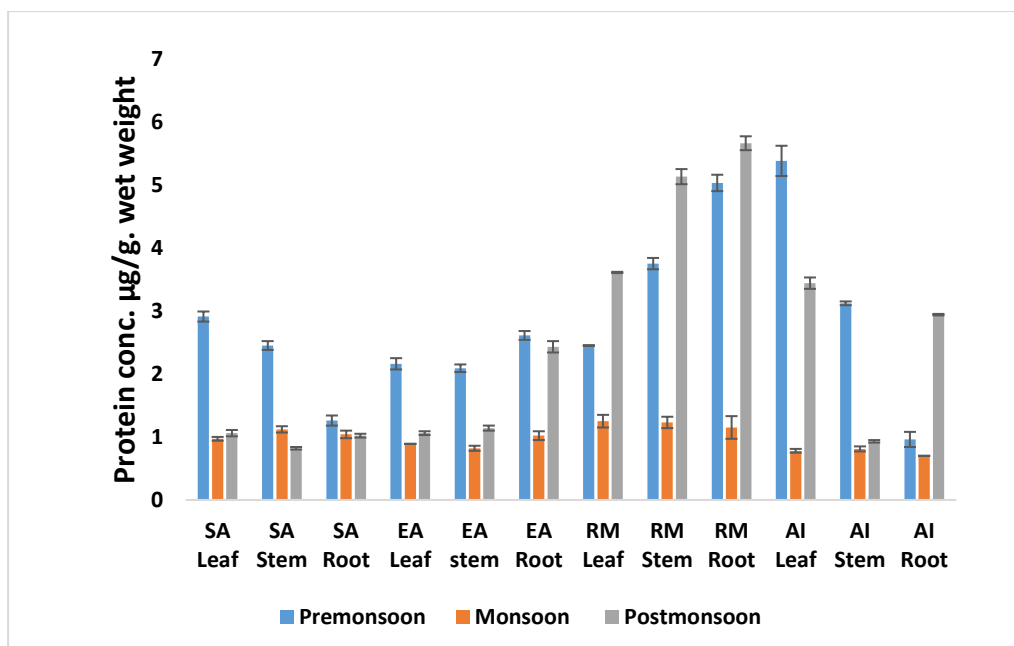


**Fig 2.8** Seasonal variation in peptidic content of mangrove plant parts

The influence of seasonal physiochemical variations was found to be significant in the production of antimicrobial peptides. The peptidic content ranged from 0.70 to 5.66 mg/g wet weight in all the seasons. In the premonsoon season, highest peptidic content was recorded in *A. ilicifolius* leaf (5.38 mg/g wet) and lowest in *A. ilicifolius* root extract (0.9638 mg/g wet). In the monsoon season *R. mucronata* leaf (1.25 mg/g wet) had the highest and *A. ilicifolius* root (0.70 mg/g wet) had the lowest peptidic content, whereas in the premonsoon season *R. mucronata* root (5.66 mg/g wet) had the highest and *S. alba* stem had the lowest (0.82 mg/g wet) values. In general, the peptide content was found to be higher in pre- and post- monsoon seasons, the values significantly dropping during the monsoon season. During the premonsoon and postmonsoon seasons, environmental stressors such as high temperatures and salinity might trigger the synthesis of antimicrobial peptides (AMPs). Ghosh and Roychoudhury (2024) highlight the role of plant peptides in responding to such stresses, emphasizing their involvement in signaling pathways that lead to AMP production and enhance plant defense mechanisms. Based on the above findings, it was evident that seasonal fluctuations significantly affected antimicrobial peptide production in mangrove plants.

### 2.3.5 Absorption spectra

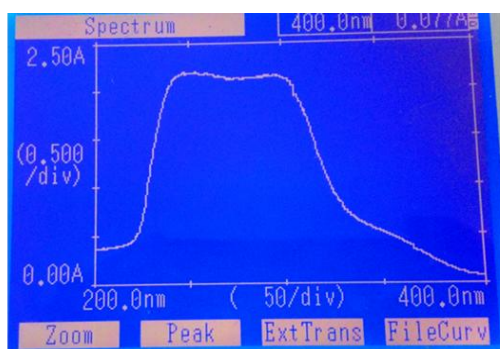
Given that mangroves are abundant in secondary metabolites, particularly tannins, initial

screening of extractable secondary metabolites was conducted by analysing the absorption spectra of the crude extracts. The general absorption characteristics of the major secondary metabolites that could be present are delineated in Table 2.7 The absorption peaks roughly characterized phytochemicals by their  $\pi$ -bonds, lone pairs of electrons,  $\sigma$ -bonds, aromatic rings, and chromophores.

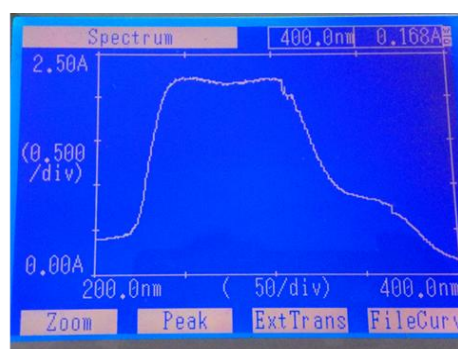
**Table 2.7:** Wavelength ranges representing specific secondary metabolites [adapted from Mabasa et. al., 2021].

| <b>Absorption Maxima (Wavelength Ranges)</b> | <b>Phytochemical Compounds (Metabolites)</b> | <b>References</b>                                                        |
|----------------------------------------------|----------------------------------------------|--------------------------------------------------------------------------|
| 234–676 nm                                   | Flavonoids, alkaloids, phenolic compounds    | [Patle et al., 2020; Karpagasundari & Kulothungan, 2014]                 |
| 230–285 nm (band I)                          | Flavonoids and their derivatives             | [Dhivya & Kalaichelvi, 2017]                                             |
| 230–290 nm (band I)                          | Flavonoids                                   | [Saxena & Saxena, 2012; Johnson & Fathima, 2018; Renuka et al., 2016]    |
| 300–350 nm (band II)                         | Flavonoids and their derivatives             | [Saxena & Saxena, 2012, Dhivya & Kalaichelvi, 2017, Renuka et al., 2016] |
| 350–500 nm                                   | Tannins                                      | [Patle et al., 2020]                                                     |
| 400–450 nm                                   | Carotenoids                                  | [Patle et al., 2020]                                                     |
| 400–550 nm                                   | Terpenoids                                   | [Saxena & Saxena, 2012; Johnson & Fathima, 2018; Renuka et al., 2016]    |
| 600–700 nm                                   | Chlorophyll                                  | [Saxena & Saxena, 2012; Johnson & Fathima, 2018; Renuka et al., 2016]    |

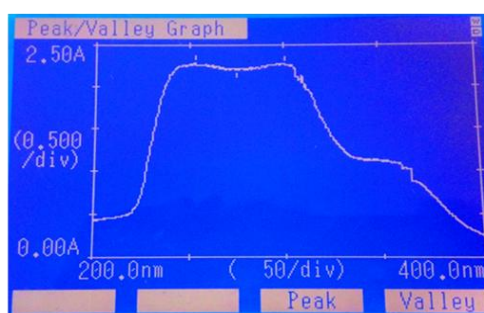
Detailed studies on phytochemicals in *S. alba* (Rahmania & Rozirwan, 2018), *E. agallocha*, *R. mucronata* and *A. ilicifolius* (Mitra et al., 2021) have been documented. The preliminary profiling for the presence of different secondary metabolites in our 12 crude extracts was evaluated through their absorption spectra. The UV-Vis spectra of the acid extracts of leaf, stem and root extracts of *S. alba*, *E. agallocha*, *R. mucronata* and *A. ilicifolius* were recorded in the range of 200 nm to 400 nm. Despite the acid extraction protocol followed, all the samples continued to suggest the presence of a variety of phytochemicals (Figs. 2.9 – 2.12).



SA Leaf extract

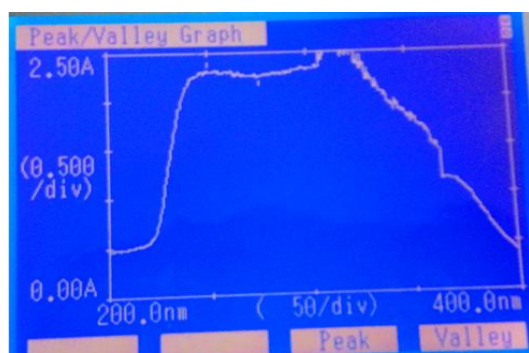


SA Stem extract

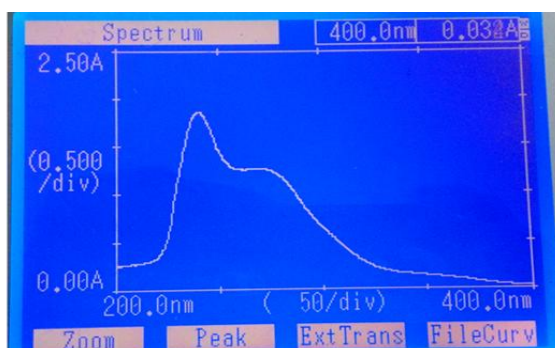


SA Root extract

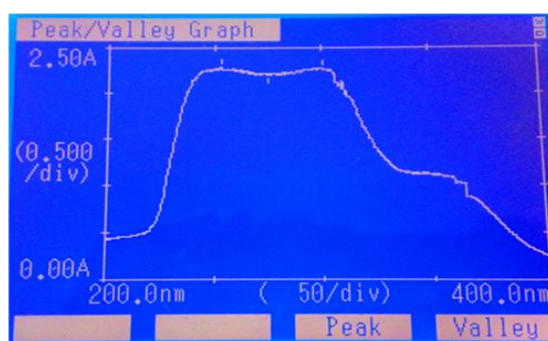
**Fig. 2.9:** UV-Vis spectra of the acid extracts of *S. alba*



EA Leaf extract

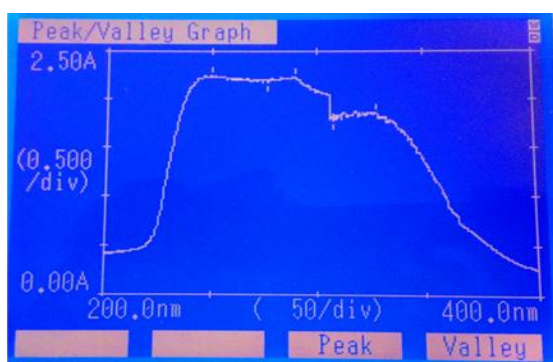


EA Stem extract

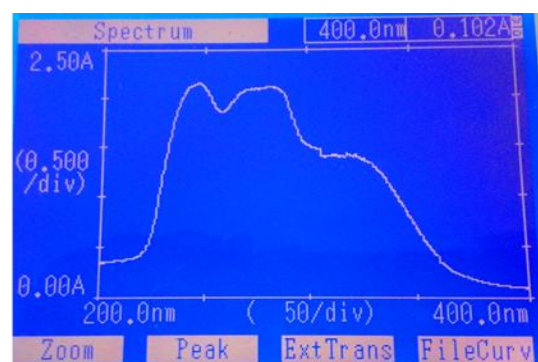


EA Root extract

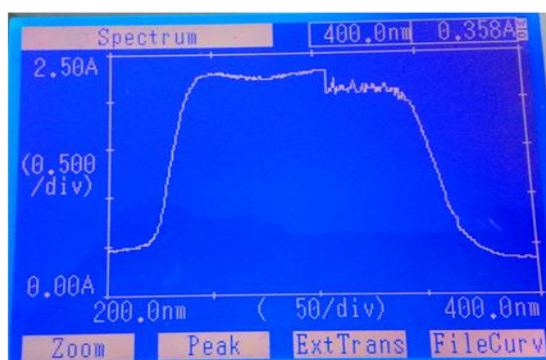
**Fig. 2.10:** UV-Vis spectra of the acid extracts of *E. agallocha*



RM Leaf extract

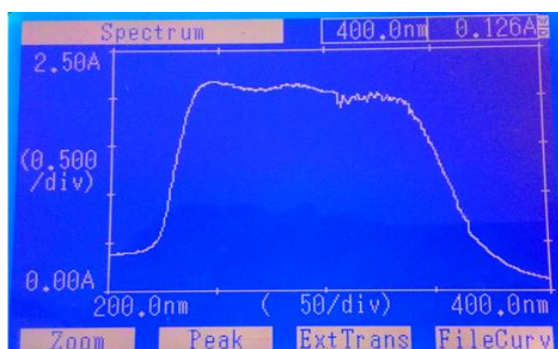


RM Stem extract

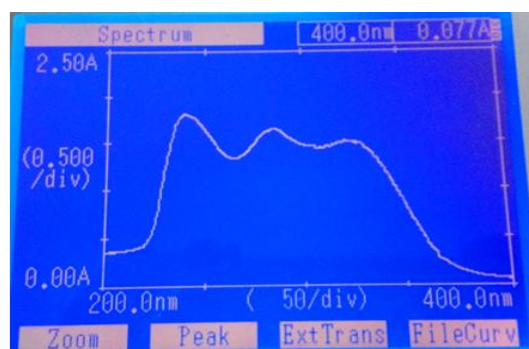


RM Root extract

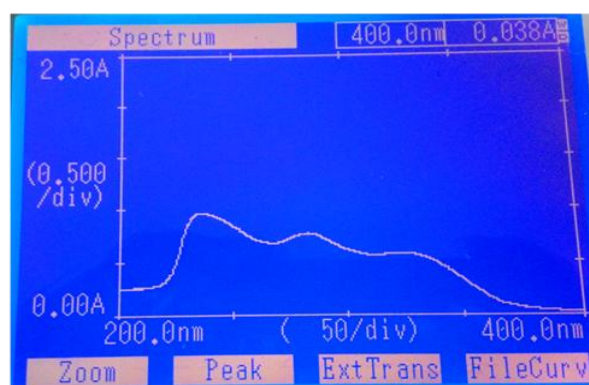
Fig. 2.11: UV-Vis spectra of the acid extracts of *R. mucronata*



AI Leaf extract



AI Stem extract



AI Root extract

Fig. 2.12: UV-Vis spectra of the acid extracts of *Acanthus ilicifolius*

### 2.3.6 Phytochemical analyses of the extracts

The results on phytochemical screening of the crude acid extracts from leaf, stem and root samples of all the four mangrove plants indicated the strong presence of alkaloids only in the stem and root extracts of *R. mucronata*. All the four mangrove plants showed the ubiquitous presence of flavonoids, except *Acanthus* stem. Terpenoids could also not be detected in *Acanthus* leaf and stem extracts. Tannins were found to be present in all the extracts of *Sonneratia*, leaf and stem extracts of *Excoecaria* and the stem extract of *Acanthus* (Table 2.8). Such heavy presence of these secondary metabolites would be likely to complicate the isolation procedures of the cationic peptides which are the focus of the present study.

Partial purification through ammonium sulphate precipitation was therefore attempted, in order to preferentially separate out the proteinaceous content from these mangrove plant extracts. This step successfully served to eliminate the alkaloid and terpenoid molecules, wherever they were present (Table 2.8). Flavonoids could also be removed from all leaf extracts except that of *Rhizophora* leaf. Stem and root extracts of all the plants, except *Acanthus* stem, continued to show the clear presence of flavonoids even after the partial purification step. Tannins, too remained associated in all cases wherever they were initially detected.

**Table 2.8:** Phytochemical evaluation of crude and partially purified extracts

| Extracts | Crude Extracts |            |            |         | Partially Purified Samples |            |            |         |
|----------|----------------|------------|------------|---------|----------------------------|------------|------------|---------|
|          | Alkaloids      | Flavanoids | Terpenoids | Tannins | Alkaloids                  | Flavanoids | Terpenoids | Tannins |
| SA Leaf  | -              | +          | +          | +       | -                          | -          | -          | +       |
| SA Stem  | -              | +          | +          | +       | -                          | +          | -          | +       |
| SA Root  | -              | +          | +          | +       | -                          | +          | -          | +       |
| EA Leaf  | -              | +          | +          | +       | -                          | -          | -          | +       |
| EA stem  | -              | +          | +          | +       | -                          | +          | -          | +       |
| EA Root  | -              | +          | +          | -       | -                          | +          | -          | -       |
| RM Leaf  | -              | +          | +          | -       | -                          | +          | -          | -       |
| RM Stem  | +              | +          | +          | -       | -                          | +          | -          | -       |
| RM Root  | +              | +          | +          | -       | -                          | +          | -          | -       |
| AI Leaf  | -              | +          | -          | -       | -                          | -          | -          | -       |
| AI Stem  | -              | -          | -          | +       | -                          | -          | -          | +       |
| AI Root  | -              | +          | +          | -       | -                          | +          | -          | -       |

Conspicuous peaks detected at around 250, 300, and 360 nm in *S. alba* leaf, stem, and root

extracts would suggest the presence of flavonoids and tannins (Mabasa et al., 2021), as indicated by phytochemical assessment (Table 2.8). Additionally, some amount of terpenoids was also observed, but these were removed after precipitation with ammonium sulphate.

Broad peaks observed at approximately 240, 300, and 380 nm in the leaf and stem extracts of *Excoecaria*, and at 240 and 280 nm in the root extract, suggest the presence of flavonoids in all extracts and tannins specifically in the leaf and stem extracts (Mabasa et al., 2021). Interestingly, these compounds persisted even after ammonium sulphate precipitation.

Peaks observed at approximately 240, 280, and 320 nm in the leaf and stem extracts of *Rhizophora*, and those at around 240 and 350 nm detected in the root extract, indicated the presence of alkaloids and flavonoids (Mabasa et al., 2021). According to Parlinska-Wojtan, et al. (2016), peaks at 280 and 320 nm correspond to terpenoids (Table 2.8). Here again, flavonoids remained present in all the extracts of *R. mucronata* even after ammonium sulfate precipitation.

The peaks observed in all the tested extracts of *A. ilicifolius* would suggest the presence of flavonoids (Mabasa et al., 2021). However, significant presence of flavonoids was detected only in leaf and root extracts (Table 2.8). Additionally, absorbance at 280 and 320 nm suggested the presence of terpenoids (Parlinska-Wojtan et al., 2016), although detected solely in the root extract. Furthermore, tannin presence was significant in the stem extract of *Acanthus*.

Since flavonoids and tannins were detected in many of the ammonium sulphate fractions, a more effective purification strategy had to be devised, as would be elaborated in the Chapters to follow.

### **2.3.7 SDS-PAGE analysis of the extracts**

The partially purified extracts (90% ammonium sulphate precipitates) of leaf, stem and root samples of the four mangrove plants (*viz.*, *S. alba*, *E. agallocha*, *R. mucronata* and *A. ilicifolius*) were subjected to SDS-PAGE analysis on a 15% separating gel, in the search for low molecular weight proteins / peptides (Fig. 2.12). *Sonneratia* showed bands > 43 kDa for all leaf, stem and root samples. *Excoecaria* leaf extract showed one band at approximately < 3.5 kDa. Stem extract showed a band at > 43 kDa. Stem and root extracts of *Rhizophora*

*mucronata* showed bands < 25 kDa. *Acanthus* stem and leaf extracts showed three bands, one > 43 kDa and the other two in the range of 14 – 43 kDa.

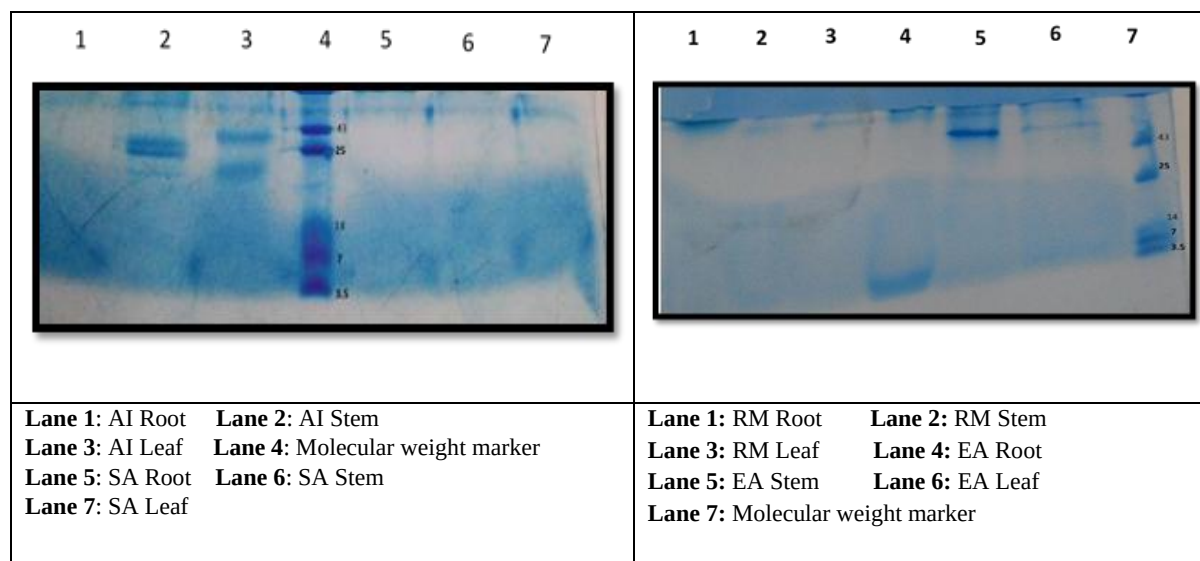


**Fig. 2.13:** SDS-PAGE analysis of the partially purified mangrove plant extracts

Mangrove plants, crucial components of estuarine ecosystems on intertidal shores, are challenging for protein extraction due to high levels of interfering compounds like tannins (Yan & Tam., 2021), as already evidenced in Section 2.3.6 above. Obtaining high-quality proteins is essential for proteomic studies of mangroves. The quality of protein extraction significantly impacts proteomic analysis results. The protein extracts from the mangrove plant exhibited smearing when analysed on 15% SDS-PAGE gels (Fig 2.13), attributable in part to the presence of additional interfering compounds such as secondary metabolites such as tannins, as was also reported by Fei et al. (2021).

In conclusion, the results presented in this Chapter showed that the examined mangrove species could be a source of antimicrobial peptides. Besides, the human pathogenic strains tested were significantly inhibited by the extracts. Homogenates at 10% and solvent concentration at 10% were found to be best effective against the tested bacterial and fungal pathogens. *Rhizophora* extracts showed highest peptidic content among all other extracts. In general, the peptide content was found to be higher in pre- and post- monsoon seasons, the values significantly dropping during the monsoon season. Secondary metabolites such as alkaloids, flavonoids, terpenoids, and tannins were identified, and although they were eliminated to some extent through partial purification, traces of flavonoids and tannins persisted in certain extracts. The

present study is the first record of systematic investigations of AMPs in mangrove plants and could project mangroves plants as a valuable medicinal source for antimicrobial product discovery. More detailed studies are presented in the next few Chapters.

# **CHAPTER 3:**

## **Activated Charcoal-based Decolourisation of Acid Extracts of Mangrove Plant Parts**

### 3.1 Introduction

Antimicrobial peptides (AMPs) are considered a new alternative to the conventional antimicrobials being used in therapy, as they destroy pathogenicity without inducing any antimicrobial resistance. In current AMP research, plant sources have been rather less explored as a source of AMPs, isolation from plant sources involves a major challenge in terms of the high level of interfering substances (such as phenolics, lipids, organic acids, carbohydrates, terpenes and pigments) and would involve sophisticated techniques to remove such contamination (Moreira et al., 2011). Some of these compounds even bind to proteins and prevent their absorption to the matrix of the ion exchanger, thus posing problems during the purification process. Polyphenolic compounds such as tannins, flavonoids and terpenoids, as well as pigments also interfere with the SDS gel resolution (Gupta et al., 2015). Plant extracts contain much lower levels of AMPs than of other bioactive molecules (Tang et al., 2018). The presence of polyphenolic compounds (tannins) has been detected even after partial purification of AMPs by ammonium sulphate precipitation (Moreira et al, 2011).

Mangroves are abundant sources of secondary metabolites, which encompass various classes of bioactive compounds. These compounds are plentiful in mangrove species and have been identified in mangrove seeds, leaves, and stem bark (Dahibhate et al., 2019; Mondal et al., 2017). Most secondary metabolites such as phenolics, terpenoids, and limonoids found in mangrove species exhibit pharmacological properties. These categories of chemicals, commonly containing ring structures with hydroxyl, amine, or sulfur groups, are naturally present in many plants (Wu et al., 2008).

*Excoecaria agallocha* (family Euphorbiaceae) has been known to produce various secondary metabolites with antimicrobial activity (Kaliyamurthi & Selvaraj, 2016), bringing up the need for efficient methods for removal of such compounds from our samples. Our study focusses on obtaining an extract of the plant material that was highly enriched in bioactive AMPs such that the activity detected therein could be ascribed to peptides, with a reasonable degree of certainty.

Removal of polyphenolic compounds from herbal extracts is often achieved by treating them with a bleaching agent or decolourising agents such as activated carbon, gelatin or bentonite, casein, ion-exchange resins and polyvinylpolypyrrolidone (PVPP) (Aikat et al., 2011). Previous studies on AMP (cyclotid) purification from plants (*Viola arvensis*, family Violaceae)

had developed fractionation protocols using different solvents for the removal of chlorophyll, polyphenol and the low molecular weight compounds abundant in plants (Göransson et al., 2004). Polyvinylpyrrolidone-based removal of phenolic compounds (de-tanninization) to obtain AMPs from *Magnolia officinalis*, *Rubus chingii*, *Scutellaria baicalensis* and *Terminalia chebula* extracts was carried out by Miyasaki et al. (2013).

On account of its highly porous nature, activated charcoal has outstanding adsorptive ability and can be favourably used in different processing conditions (Dina et al., 2012). Several applications, such as in the removal of phenolic and coloured compounds in foodstuff and fruit juices as well as in wastewater treatment have been extensively documented (Aikat et al., 2011; Ahmaruzzaman, 2008; Chee et al., 2013). Adsorption onto activated carbon is a well-established, economical and efficient method for the separation of the polyphenols from complex aqueous solutions (Ahmaruzzaman, 2008; Soto et al., 2008).

In the present investigation, activated charcoal-based first-level purification of mangrove plant extracts was attempted to develop a cost-effective screening method which could facilitate preliminary analysis of a large number of samples for their antimicrobial peptide production capability. The acetic acid extraction protocol used would in itself selectively precipitate many other proteins and favour more specific isolation of antimicrobial cationic peptides (Cole & Ganz, 2000).

Optimization of the decolourisation conditions can be done using the classical one-factor-at-a-time (OFAT) approach which would support easy data interpretation but would fail to evaluate the synergistic effects of the different parameters under study. Response Surface Methodology (RSM) was hence employed to optimize the parameters required for best decolourisation of the plant extracts using activated charcoal. This Chapter documents efficient methods for separation of AMPs and removal of polyphenolic compounds by activated charcoal at the initial stages itself, so as not to affect further purification strategies. Although there are many reports on decolourisation of herbal extracts by activated charcoal, there have been no documented studies on using this process for purification of antimicrobial peptides/proteins.

## 3.2 Materials and methods

Analytical-grade chemicals were used for all the experiments. Each experiment was conducted in triplicate, and the data points plotted represent the mean  $\pm$  standard deviation (S.D.) of values obtained from three or more independent experiments.

### 3.2.1 The OFAT approach

The "one-factor-at-a-time" (OFAT) approach involves varying and analyzing one experimental parameter at a time while keeping other factors constant. It is particularly helpful in identifying the key factors that affect a given process (Singh et al., 2011).

#### 3.2.1.1 Activated charcoal concentration optimization

The adsorption of coloured compounds in the crude extracts was carried out in 1.5 mL microcentrifuge tubes at different concentrations of activated charcoal. Crude acid extract (1 mL) of initial OD ( $c^0$ ) at 380 nm was used for all experiments. The extract was treated with 10-40 mg of activated charcoal (HiMedia, India) for 10 min at room temperature (30°C), centrifuged at 7,000 rpm for 5 min and the supernatant analyzed spectrophotometrically. The percentage decrease in absorbance in treated samples ( $c^*$ ) was monitored at 380 nm. A graph of remaining colour intensity (%) vs concentration of activated charcoal was plotted (Aikat et al., 2001).

Remaining colour intensity (%) =  $(c^*/c^0) \times 100$ ;

$c^0$  being the initial 'solute' concentration in liquid before adsorption and  
 $c^*$  the equilibrium 'solute' concentration in liquid after adsorption',  
( 'solute' here, pertaining to 'colour' ).

#### 3.2.1.2 Treatment time variation studies

Using the optimum ratio of mg charcoal per mL crude extract, the treatment time was varied from 5 - 40 min; the colour intensity remaining was determined by the absorption at 380 nm.

#### 3.2.1.3 Temperature variation studies

The effect of different temperatures on the adsorption process on activated charcoal was evaluated spectrophotometrically at 380 nm, under the optimized conditions of activated charcoal concentration and incubation time.

### 3.2.2 Adsorption spectra

The acid extracts of leaf, stem and root samples of *S. alba*, *R. mucronata*, *E. agallocha* and *A. ilicifolius* were scanned in the wavelength range of 200-800 nm using a UV-vis spectrophotometer.

### 3.2.3 Total phenolic content determination

Total phenolic content (TPC) was determined using Folin-Ciocalteu's reagent (Singleton et al., 1965). Briefly, 100  $\mu$ L of sample was mixed with 200  $\mu$ L of 10% (v/v) Folin-Ciocalteu's reagent for 2 min at room temperature, following which 800  $\mu$ L of 0.7 M sodium carbonate was added, the reaction mixture vortexed and incubated in the dark for 2 h at room temperature ( $25 \pm 1$  °C). The absorbance was measured at  $\lambda = 765$  nm. Gallic acid (GA) was used as standard for preparation of the calibration curve, the results being expressed as total gallic acid equivalents at equilibrium (GAEE, g/L). All determinations were carried out in triplicate.

### 3.2.4 Phytochemical analyses of the extracts

The phytochemical analyses of the extracts for presence of alkaloids, flavonoids, tannins and terpenoids were carried out as described in Chapter 2 (section 2.2.11).

### 3.2.5 Response Surface Methodology (RSM)

The RSM is a statistical and mathematical experimental design which uses regression analysis for modelling and optimization of a response influenced by several independent input parameters, by understanding their interactions and their effects (Box et al., 1951; Myers et al., 2016). The various techniques used in RSM are Central Composite Design (CCD), Box Behnken statistical experimental design (BBD) and two-level Full Factorial Design (FFD). The statistical experimental design for adsorption studies has been applied for phenol adsorption, metal removal and removal of dyes onto activated charcoal using FFD and CCD (Tabassi et al., 2017; Rao et al., 2018; Machrouhi et al., 2019). In the present study, CCD was used to find the optimum conditions for adsorption of polyphenols onto activated charcoal. The 'Design Expert 11.0' (Minneapolis, USA) software package was used to analyze the experimental design by CCD.

### 3.2.5.1 Experimental design

Activated charcoal-based adsorption of phytochemicals was carried out by considering the effect of some important parameters such as charcoal concentration, contact time and temperature. Data obtained from the OFAT approach were used further in the RSM experiments to study interactions among the three parameters and to reduce the number of experimental trials required to evaluate the effect of each parameter in the OFAT approach.

### 3.2.6 Adsorption studies

*Adsorption models* : Adsorption isotherms serve as mathematical frameworks elucidating the allocation of adsorbate entities within a liquid and adsorbent system. These models depend on a range of assumptions, mainly pertaining to the homogeneity or heterogeneity of adsorbents, the nature of coverage, and the likelihood of interactions among adsorbate species. Typically, adsorption data are explained through specific isotherms such as the Langmuir and the Freundlich models. In the context of this study, these isotherms evaluate a connection between the concentration of polyphenolic compounds adsorbed per unit mass of activated charcoal and the acetic acid extract. The data obtained from OFAT method was fitted in Langmuir and Freundlich isotherm models.

#### 3.2.6.1 The Langmuir isotherm

The Langmuir equation, proposed by Irving Langmuir in 1916, is a fundamental equation in surface chemistry and adsorption science. It describes the relationship between the amount of adsorbate molecules (such as gases or solutes) on a solid surface and the concentration of the adsorbate in the surrounding solution or gas phase at equilibrium. Some key points about this isotherm (Langmuir, 1918) include the following:

- Assumes that adsorption occurs in a single layer on the surface on a homogenous adsorbent, forming a complete monolayer coverage. This implies that each adsorption site can only accommodate one adsorbate molecule.
- Adsorbent exterior has definite number of adsorption sites.
- No interaction exists between the molecules adsorbed on the exterior of the adsorbent.
- Adsorption and desorption processes are reversible. Once the surface is saturated with adsorbate molecules, further adsorption is hindered, and adsorbate molecules may also desorb from the surface back into the bulk phase.

The equation for the adsorption isotherm is

$$a = ABc^*/(1 + Ac^*) \dots\dots\dots (1)$$

where  $a$  is the amount of solute adsorbed by unit weight of adsorbent ( $= v (c_0 - c^*)$ , from Equation (1)),  $c^*$  is the equilibrium concentration of solute in the free solution, and  $A$  and  $B$  are constants depending on the properties of the adsorbent and the substance being adsorbed. A plot of  $1/c^*$  vs  $1/a$  would be a straight line.

### 3.2.6.2 The Freundlich isotherm

The Freundlich isotherm is an empirical equation used to describe the adsorption of solutes on to solid surfaces. It was proposed by Herbert Freundlich in 1906 and is widely applied in various fields including chemistry, environmental science, and soil science. The basic assumptions of the model include (Freundlich, 1906; Allen et al., 2004) the following:

- This isotherm is suitable for heterogeneous surfaces where adsorption sites vary in their affinities for the solute.
- It allows for multilayer adsorption, meaning that adsorption can occur on multiple layers of adsorbate molecules on the surface.

The Freundlich equation for adsorption is:

$$C^* = k (v(c_0 - c^*))^n \dots\dots\dots (2)$$

where  $v(c_0 - c^*)$  is the apparent adsorption per unit mass of adsorbent and  $k$  and  $n$  are constants. The value of  $n$  is an indicator of the adsorption characteristics,  $n = 2$  to  $10$  representing good,  $1$  to  $2$ , moderately difficult, and  $n < 1$ , poor adsorption. A plot of  $\ln c^*$  vs  $\ln v(c_0 - c^*)$  would be a straight line.

### 3.2.7 Antimicrobial Assay

The antimicrobial activity of the crude acid extract (labelled as ‘control’) and the charcoal-treated (decolourised) extracts was evaluated by the well diffusion method (Shanmugaraju, 2012) as described in Section 2.2.5.

### 3.2.8 Desorption studies

Desorption studies were carried out by the method described by Tabassi et al. (2017), with minor modifications. The activated charcoal (35 mg) obtained after adsorption of 1mL of crude extract was washed twice with distilled water to remove the unadsorbed surface content. To

the washed activated charcoal, 1mL of NaOH was added at varying concentrations, incubated for 2 h, centrifuged for 10 min and the intensity of colour estimated by the absorbance at 380 nm.

### **3.3 Results and discussion**

Plant AMPs are widespread and have been implicated in diverse biotic and abiotic stress responses. Their purification from plant sources often involves sophisticated techniques, is time-consuming and laborious. Mangrove plants possess antimicrobials which have been isolated by different polar and non-polar solvents and belong to diverse chemical classes such as alkaloids, phenolics, steroids, terpenoids and tannins (Patra & Mohanta, 2014). To the best of our knowledge this the first report of any systematic study on selective isolation of AMPs using acid extraction, along with treatment with activated charcoal to develop a comparatively speedy and cost-effective screening method which could enable preliminary analysis of a large number of plant samples for their peptide production potential. As AMPs are known for their remarkable stability under extreme pH and high temperatures, as well as their abundance of positive charges, we have cashed in on the acid extraction protocol and further purification procedures involving activated charcoal. Acid extraction with 10% acetic acid was hence employed, such that most other proteins would be precipitated while selective isolation of cationic peptides would be favoured (Cole & Ganz, 2000). Various polyphenols were also extracted together with the peptides, which imparted colour to the extracts (Moreira et al., 2011). The strategy of adsorption of polyphenols from the acetic acid extracts onto activated charcoal was therefore adopted to effect decolourisation and removal of contaminants.

#### **3.3.1 Decolourisation of acid extracts by activated charcoal**

Decolourisation experiments were conducted as detailed in Section 3.2. After a treatment time ranging from 5-160 min, the supernatant was analysed for colour intensity and total protein content. Both crude and charcoal-treated samples were analysed thus.

The adsorption efficiency of coloured compounds on activated charcoal was evaluated by determining the percentage decrease of the absorbance at 380 nm upon treatment of the crude extracts. Extracts (1 mL) of *S. alba*, *E. agallocha*, *R. mucronate* and *A. illicifolius* were treated with 5-160 mg of activated charcoal for 10 min. A graph of % RCI (Remaining Colour Intensity) vs concentration of activated charcoal was plotted.

Quantities equivalent of an initial OD ( $c^0$ ) of crude extracts of *S. alba* (leaf  $c^0 = 2.142$ ), (stem  $c^0 = 2.204$ ), (root  $c^0 = 2.658$ ); *E.agallocha* (leaf  $c^0 = 1.442$ ), (stem  $c^0 = 0.743$ ), (root  $c^0 = 0.893$ ); *R. mucronata* (leaf  $c^0 = 2.142$ ), (stem  $c^0 = 2.204$ ), (root  $c^0 = 2.658$ ); *A. illicifolius* (leaf  $c^0 = 1.055$ ), (stem  $c^0 = 1.326$ ), (root  $c^0 = 0.827$ ) were treated with 20-60 mg of activated charcoal for 10 min. The variation of adsorption with charcoal amount (Fig 3.1, 3.2, 3.3 & 3.4) is a clear consequence of the available area for adsorption increasing with the amount of charcoal.

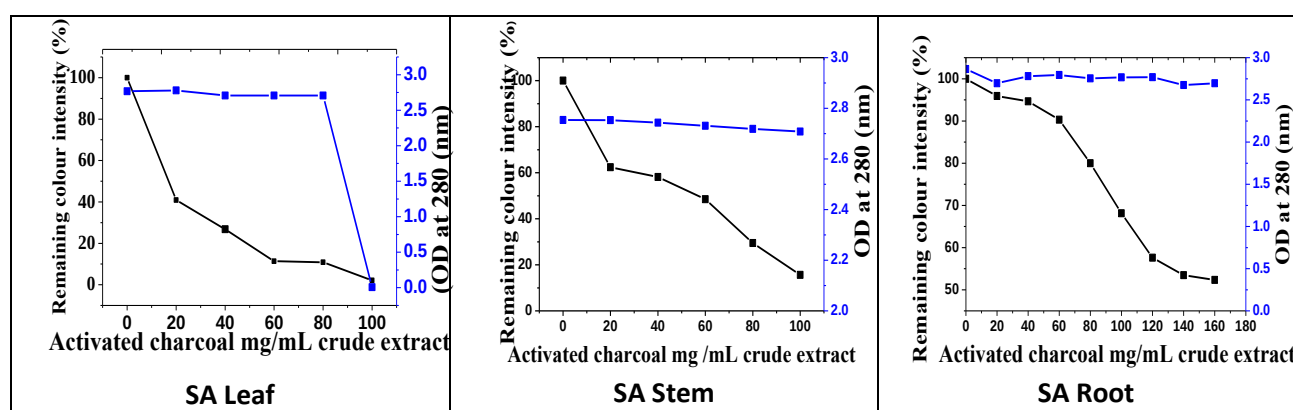


**Fig 3.1** Effect of activated charcoal on remaining colour intensity of leaf, stem and root extracts of *S. alba*.

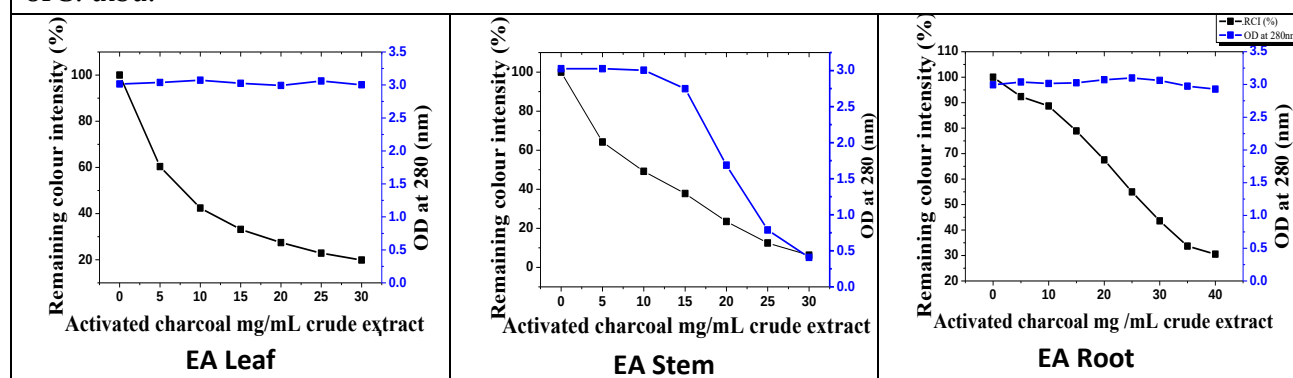


**Fig 3.2** Effect of amount of activated charcoal on remaining colour intensity of leaf, stem and root extracts of *E. agallocha*.

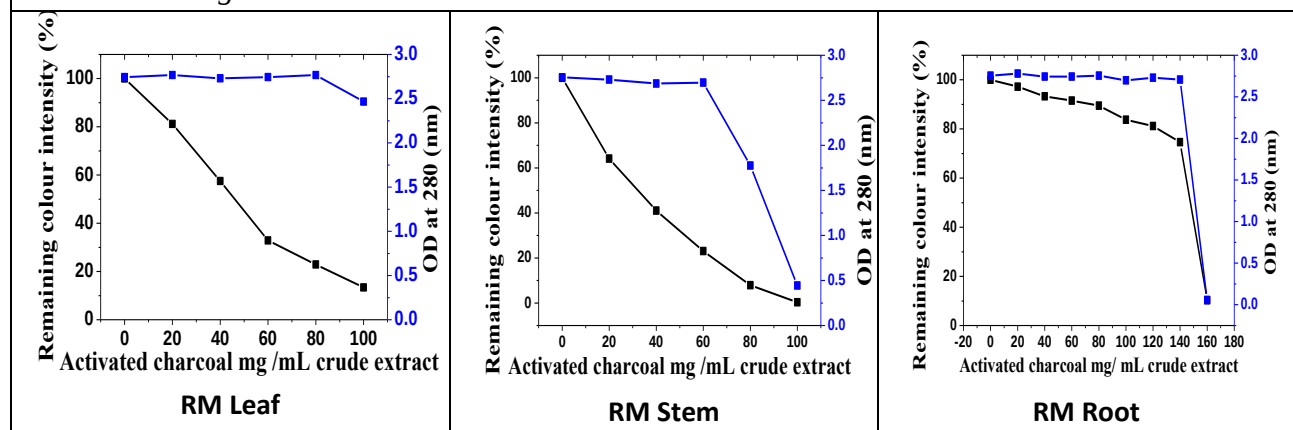


**Fig.3.3** Effect of amount of activated charcoal on remaining colour intensity of leaf, stem and root

extracts of *R. mucronata*.

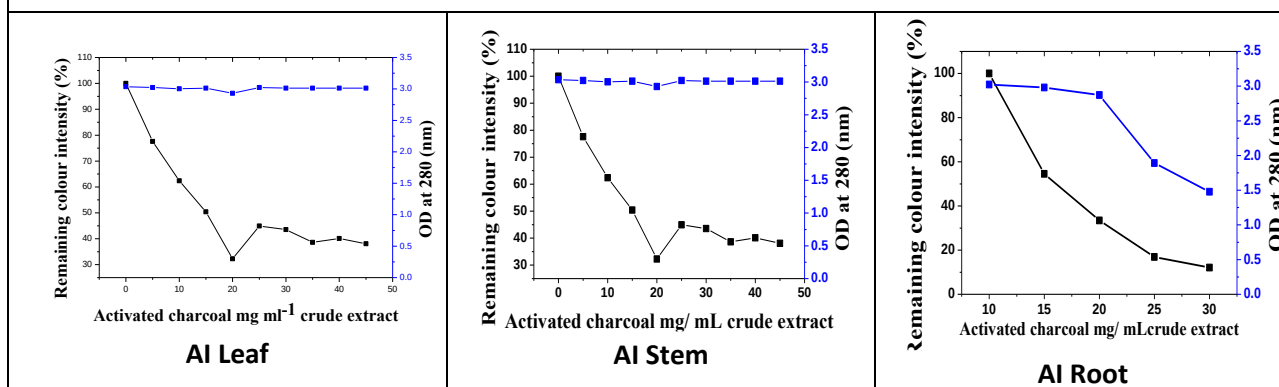


**Fig.3.4** Effect of amount of activated charcoal on remaining colour intensity of leaf, stem and root extracts of *A. ilicifolius*.

### 3.3.1.1 Plant extracts before and after charcoal treatment

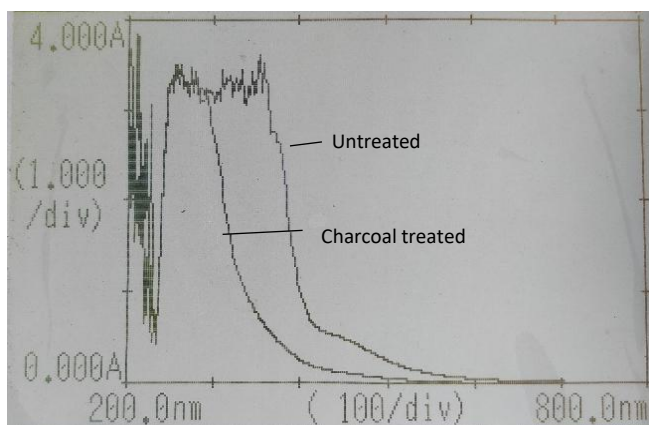
Subsequent to optimal decolourisation of the mangrove plant extracts as per conditions worked out above, the quantification of 10% remaining colour intensity (as per Gutcho, 1974) yielded results as laid out in Table 3.1.

**Table 3.1:** Decolourisation of the mangrove plant extracts to 90% extent

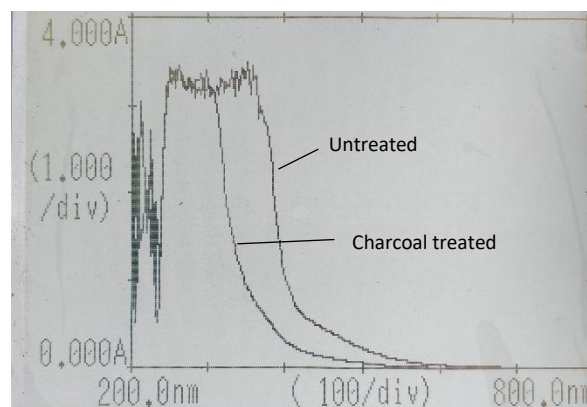
| Extract | Minimum charcoal concentration for 90% decolourisation (mg/mL) |
|---------|----------------------------------------------------------------|
| SA Leaf | 60-80                                                          |
| SA Stem | >100                                                           |
| SA Root | >> 160                                                         |
| EA Leaf | > 30                                                           |
| EA Stem | 25-30                                                          |
| EA Root | > 45                                                           |
| RM Leaf | > 100                                                          |
| RM Stem | 70-80                                                          |
| RM Root | 160                                                            |
| AI Leaf | >45                                                            |
| AI Stem | >45                                                            |
| AI Root | 30                                                             |

### 3.3.2 Adsorption spectra

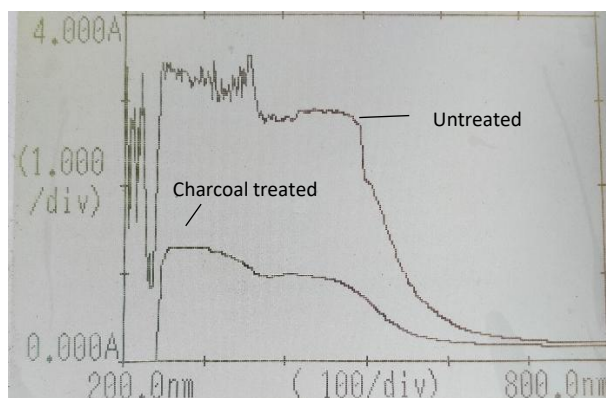
The absorption spectra of all the 12 mangrove plant samples were recorded before and after charcoal treatment (Fig.3.5- 3.8).



(a) *Sonnerartia alba* leaf

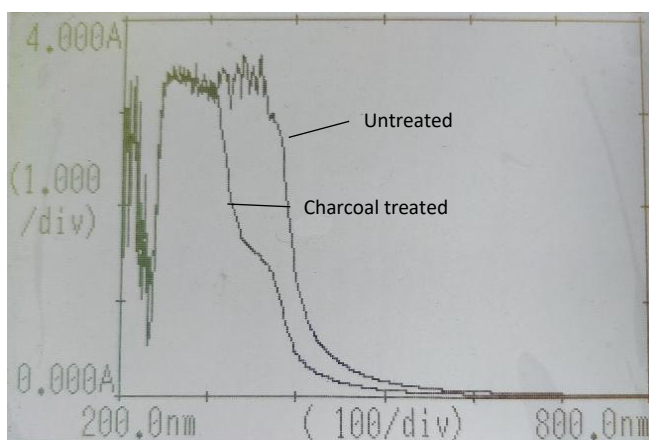


(b) *Sonnerartia alba* stem

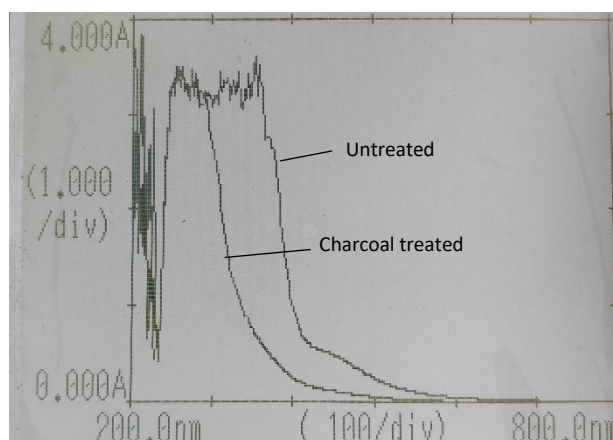


(c) *Sonnerartia alba* root

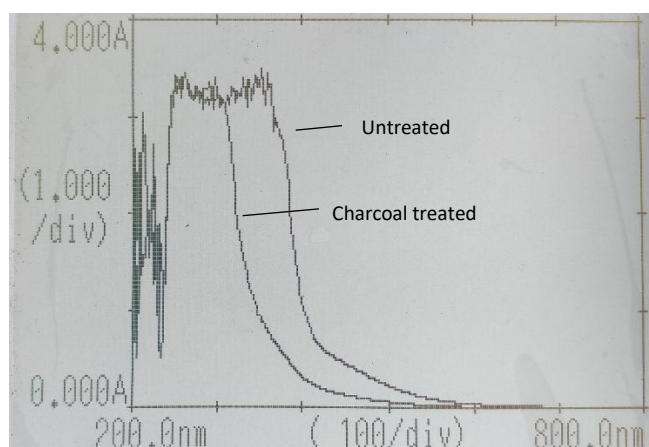
**Fig 3.5** Spectral analysis of *Sonnerartia alba* extracts



*Excoecaria agallocha* leaf

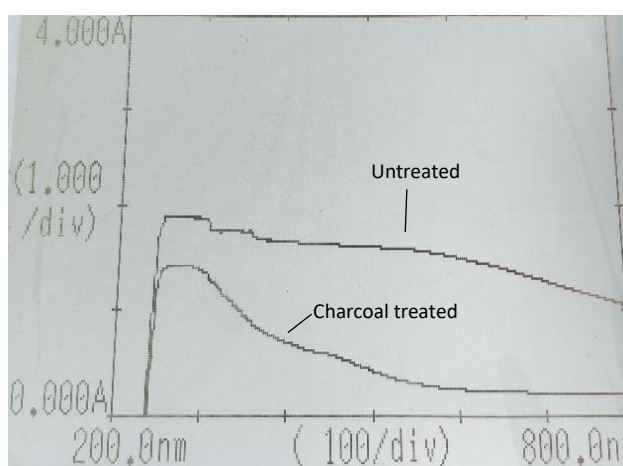


*Excoecaria agallocha* stem

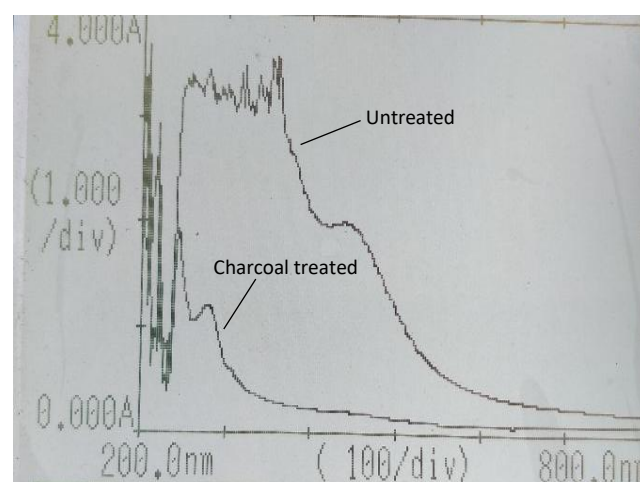


*Excoecaria agallocha* root

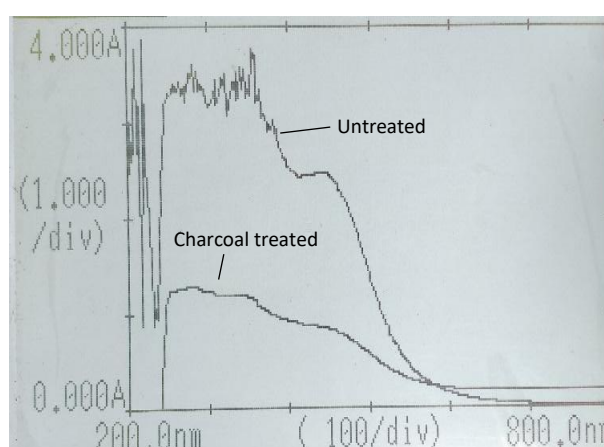
**Fig 3.6** Spectral analysis of *Excoecaria agallocha* extracts



(a) *Rhizophora mucronata* leaf

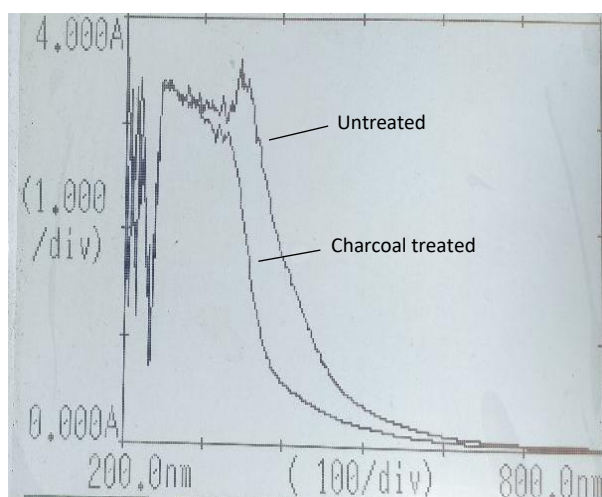


(b) *Rhizophora mucronata* stem

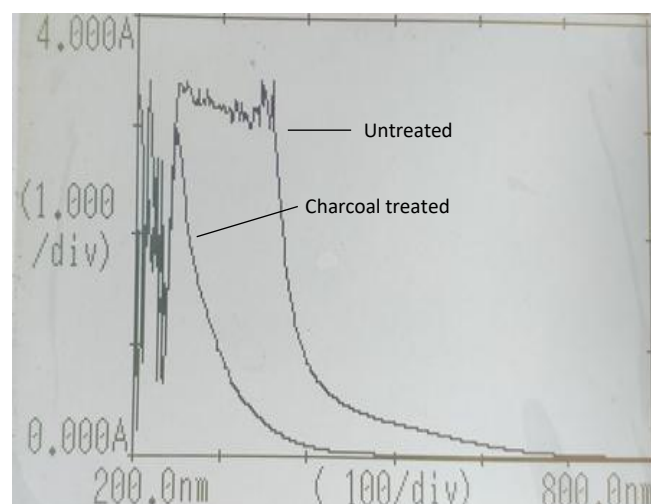


(c) *Rhizophora mucronata* root

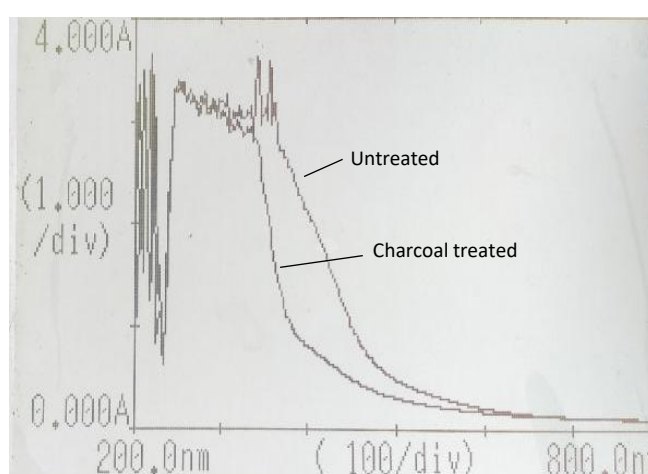
**Fig 3.7** Spectral analysis of *Rhizophora mucronata* extracts



*Acanthus illifolius* leaf



*Acanthus illifolius* stem



*Acanthus illifolius* root

**Fig 3.8** Spectral analysis of *Acanthus illifolius* extracts

The SDS-PAGE evaluation of the *Excoecaria* leaf extract had revealed a low molecular weight band of our interest, as mentioned in Section 2.3.7. This sample was hence selected for further investigations into the adsorption properties, as detailed below.

### 3.3.3 Effect of charcoal concentration, contact time and temperature on the decolourisation process: Optimization by the OFAT approach

Variations in adsorption of polyphenols (coloured molecules) on activated charcoal were studied in crude leaf extracts of *E. agallocha* (leaf  $c^o = 2.142$ ) by the one-factor-at-a-time (OFAT) method. Enhanced adsorption took place with increase in charcoal concentration, a significant extent being achieved by 10 mg/mL, beyond which a more gradual decolourisation was observed. Likewise, although drastic decolourisation of the extract was observed within

10 min, an ideal contact time of 20 min could ensure near total adsorption of coloured compounds. The most effective decolourisation occurred at 28°C, a slight decrease in decolourisation being observed when the temperature was either raised or lowered. In effect, optimized charcoal concentration of 30 mg/mL, contact time of 20 min and temperature of 28°C (Fig 3.9), were adopted for further experiments, as they could bring about ~ 90 % decolourisation. The changes to protein content were monitored by the absorbance at 280 nm.

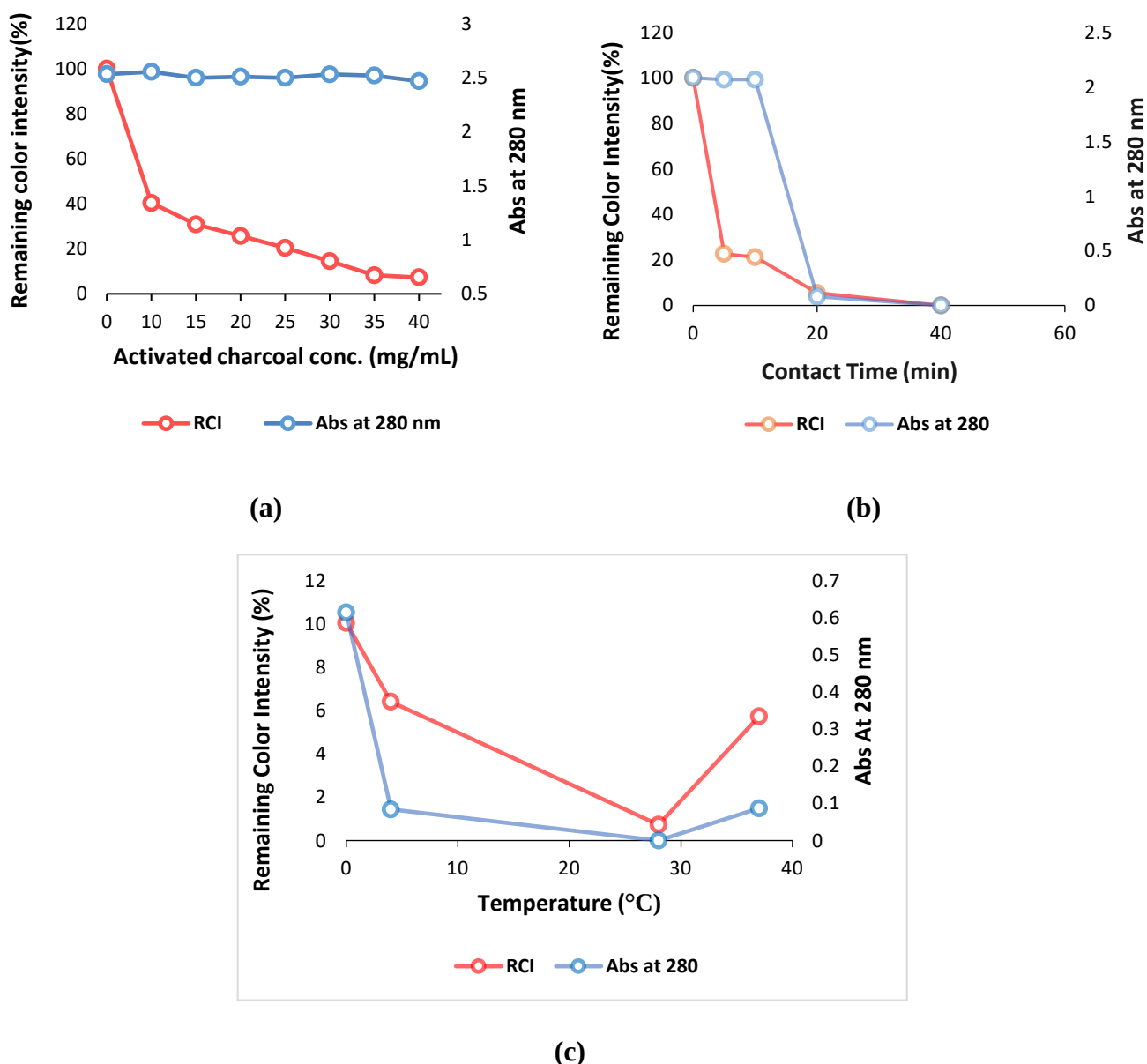


**Fig. 3.9:** Effect of (a) charcoal concentration (b) contact time and (c) temperature on the decolourisation process.

### 3.3.4 Total polyphenolic and protein content

Phenolic compounds are the main contributors to the intense dark colour of herbal extracts (Chee et al., 2013). Quantification of the polyphenol adsorption onto activated charcoal was carried out by the Folin-Ciocalteu reagent method (García-Pérez et al., 2019) and the results expressed in terms of gallic acid equivalent per litre (GAEe g L<sup>-1</sup>). Protein was estimated as detailed in Section 2.2.8.

It was observed that concentration of polyphenolic compounds decreased with increasing activated charcoal concentration (Table 3.2). When activated charcoal is added to crude extracts containing polyphenolic compounds, these molecules are attracted to and adsorbed on to the surface of the activated charcoal, in effect removing them from the solution (García-Pérez et al., 2019).

**Table 3.2:** Total polyphenolic and protein content of extracts after treatment with different concentrations of activated charcoal

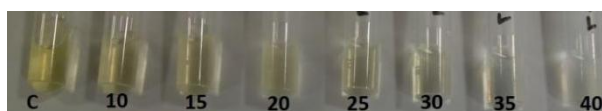
| <b>EA leaf extract treated with different concentrations of charcoal</b> | <b>GAEe of EA leaf extract (g L<sup>-1</sup>)</b> | <b>Protein concentration (µg/mL)</b> |
|--------------------------------------------------------------------------|---------------------------------------------------|--------------------------------------|
| <b>0</b>                                                                 | 0.289                                             | 21.47                                |
| <b>5</b>                                                                 | 0.282                                             | 17.91                                |
| <b>10</b>                                                                | 0.269                                             | 15.27                                |
| <b>15</b>                                                                | 0.235                                             | 11.71                                |
| <b>20</b>                                                                | 0.162                                             | 9.46                                 |
| <b>25</b>                                                                | 0.132                                             | 9.53                                 |
| <b>30</b>                                                                | 0.063                                             | 5.66                                 |

### 3.3.5 Phytochemical analyses of the extracts treated with different concentrations of activated charcoal

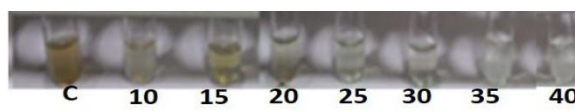
The qualitative phytochemical screening of *E. agallocha* extracts was performed following standard protocols, in order to identify the main classes of compounds (tannins, flavonoids, alkaloids, phenols and terpenoids) present in the extracts. During treatment with increasing activated charcoal concentrations, it was observed that charcoal at 35-40 mg/mL could effectively remove flavonoids, tannins and terpenoids (Table 3.3 and Fig. 3.10).

**Table 3.3:** Effect of charcoal concentration on phytochemical adsorption

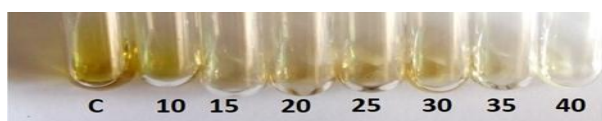
| Phytochemicals | Charcoal conc. (mg/mL) |    |    |    |    |    |    |    |
|----------------|------------------------|----|----|----|----|----|----|----|
|                | 0                      | 10 | 15 | 20 | 25 | 30 | 35 | 40 |
| Alkaloids      | -                      | -  | -  | -  | -  | -  | -  | -  |
| Flavonoids     | +                      | +  | +  | +  | +  | +  | -  | -  |
| Terpenoids     | +                      | -  | -  | -  | -  | -  | -  | -  |
| Tannins        | +                      | +  | +  | +  | +  | +  | +  | -  |



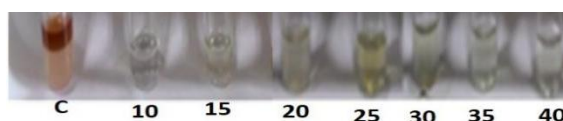
**Visual observation of decolourisation**



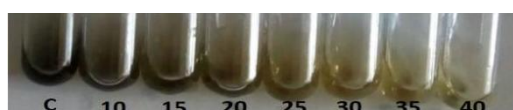
**Test for Alkaloids**



**Test for Flavonoids**



**Test for Terpenoids**



**Test for Tannins**

**Fig. 3.10:** Visual observation of decolourisation and phytochemical analysis of extracts treated with different concentrations of activated charcoal

### 3.3.6 Effect of charcoal concentration, contact time and temperature on the decolourisation process: Statistical optimization by RSM

The RSM approach was used to study the combined effect of three influencing variables on the adsorption of phytochemicals on activated charcoal. The adsorption data for *E. agallocha* leaf extract was modelled with the help of RSM using the Design Expert software. As detailed

earlier, a preliminary study was first carried out by the OFAT method to identify appropriate ranges for the parameters under study viz., charcoal concentration, contact time and temperature. The parameter values for the adsorption of polyphenols from acid extracts of *E. agallocha* leaf on to activated carbon were then optimized using RSM. Each parameter was considered at lower and upper limit values of charcoal concentration (5 and 35 mg/mL), contact time (5 and 20 min) and temperature (0 and 28 °C), respectively. As indicated by this design, 20 experiments (Table 3.4) were carried out in triplicate for estimating the experimental uncertainty variance. The response value in each trial was the average of triplicates measured as absorption at 380 nm for detection of polyphenols and protein concentration expressed in µg/mL. The process variable of (A) Charcoal conc. showed the greatest significant positive effect while the other variables of (B) Contact time and (C) Temperature had negligible influence on the decolourisation process. Likewise, the effect of process parameters on the protein concentration was monitored. Charcoal concentration had a significant effect on protein concentration, as compared to the other factors of contact time and temperature.

**Table 3.4:** Coded and actual values (in %) of variables of experimental design

| Variables                             | Code | Levels of variables |      |    |
|---------------------------------------|------|---------------------|------|----|
|                                       |      | +1                  | 0    | -1 |
| <b>Charcoal concentration (mg/mL)</b> | A    | 5                   | 20   | 35 |
| <b>Contact time (min)</b>             | B    | 5                   | 12.5 | 20 |
| <b>Temperature (°C)</b>               | C    | 0                   | 14   | 28 |

**Table 3.5:** Central composite design of the experiment

| Run | Factor 1<br>A. Charcoal<br>conc.<br>(mg/mL) | Factor 2<br>B. Contact<br>time<br>(min) | Factor 3<br>C.<br>Temperature<br>(°C) | Response1:<br>Decolourisation<br>(%) | Response 2:<br>Protein conc.<br>(µg/mL) |
|-----|---------------------------------------------|-----------------------------------------|---------------------------------------|--------------------------------------|-----------------------------------------|
| 1   | +1                                          | -1                                      | +1                                    | 94.7                                 | 6.60                                    |
| 2   | 0                                           | 0                                       | 0                                     | 81.2                                 | 6.49                                    |
| 3   | 0                                           | 0                                       | +1                                    | 83.8                                 | 6.10                                    |
| 4   | 0                                           | 0                                       | 0                                     | 81.2                                 | 6.49                                    |
| 5   | -1                                          | +1                                      | +1                                    | 53.0                                 | 18.17                                   |
| 6   | -1                                          | 0                                       | 0                                     | 45.0                                 | 19.18                                   |
| 7   | +1                                          | +1                                      | -1                                    | 96.9                                 | 7.00                                    |
| 8   | -1                                          | -1                                      | +1                                    | 53.0                                 | 16.15                                   |
| 9   | -1                                          | +1                                      | -1                                    | 44.8                                 | 15.77                                   |
| 10  | 0                                           | 0                                       | 0                                     | 81.2                                 | 6.49                                    |
| 11  | 0                                           | 0                                       | 0                                     | 81.2                                 | 6.49                                    |
| 12  | -1                                          | -1                                      | -1                                    | 43.1                                 | 20.06                                   |
| 13  | +1                                          | 0                                       | 0                                     | 96.4                                 | 7.44                                    |
| 14  | +1                                          | +1                                      | +1                                    | 96.0                                 | 9.65                                    |
| 15  | +1                                          | -1                                      | -1                                    | 96.1                                 | 9.05                                    |
| 16  | 0                                           | 0                                       | 0                                     | 81.2                                 | 6.49                                    |
| 17  | 0                                           | 0                                       | -1                                    | 84.5                                 | 8.64                                    |
| 18  | 0                                           | -1                                      | 0                                     | 85.2                                 | 6.68                                    |
| 19  | 0                                           | 0                                       | 0                                     | 81.2                                 | 6.49                                    |
| 20  | 0                                           | +1                                      | 0                                     | 83.3                                 | 6.10                                    |

**Table 3.6:** ANOVA for response surface quadratic model (for Response 1 : Decolourisation).

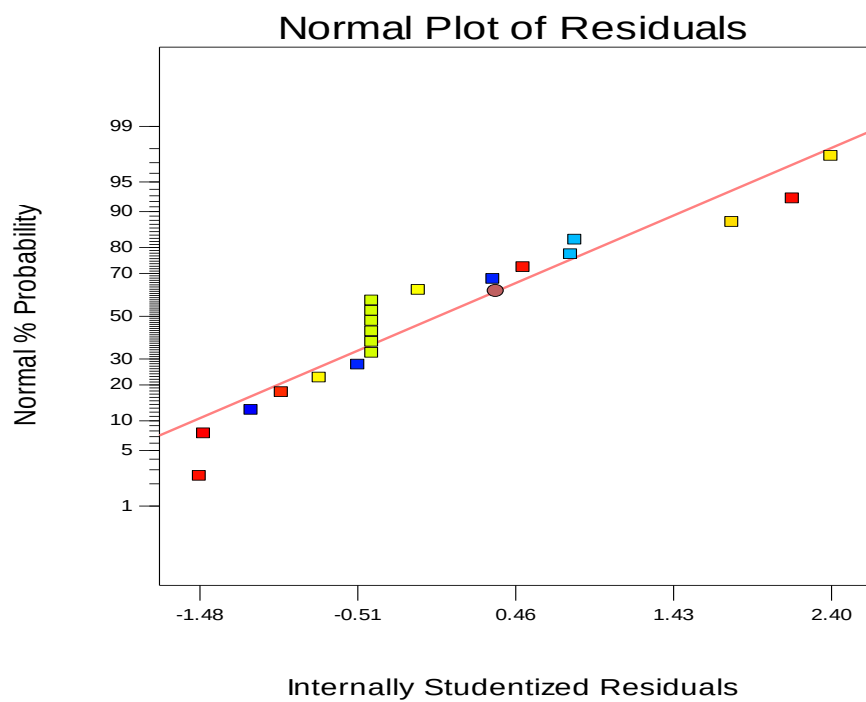
| <b>Source</b>      | <b>Sum of Squares</b> | <b>Degrees of freedom</b> | <b>Mean Square</b> | <b>F Value</b> | <b>p-value Prob &gt; F</b> |
|--------------------|-----------------------|---------------------------|--------------------|----------------|----------------------------|
| <b>Model</b>       | 6460.75               | 9                         | 717.86             | 260.28         | < 0.0001                   |
| <b>Residual</b>    | 27.58                 | 10                        | 2.76               |                |                            |
| <b>Lack of Fit</b> | 27.58                 | 5                         | 5.52               |                |                            |
| <b>Pure Error</b>  | 0                     | 5                         | 0                  |                |                            |
| <b>Cor Total</b>   | 6488.33               | 19                        |                    |                |                            |

C.V. % =2.15;  $R^2$  =0.99; Adj  $R^2$  = 0.99; Pred  $R^2$  = 0.97.

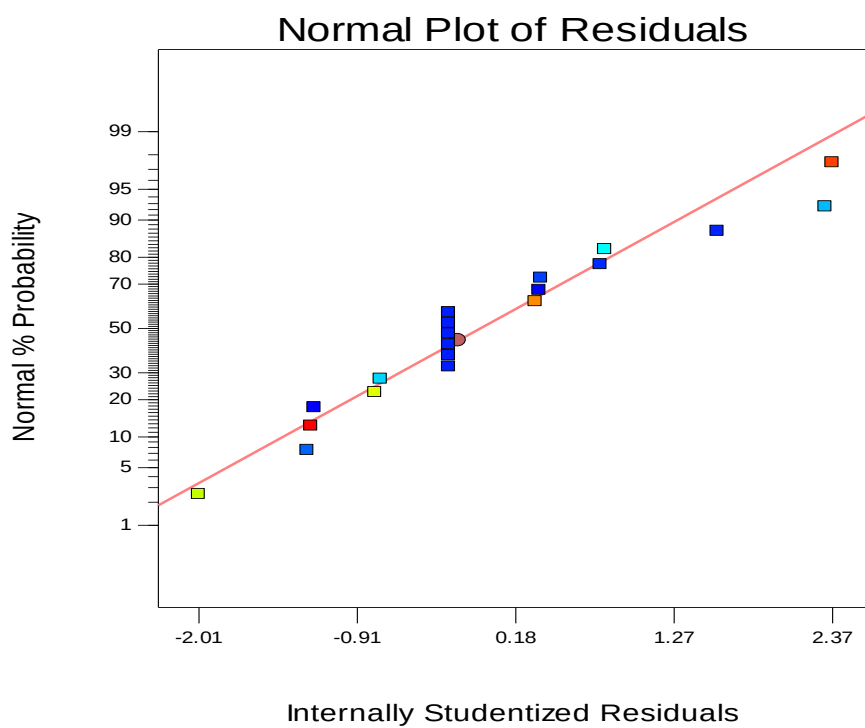
**Table 3.7:** ANOVA for response surface quadratic model (for Response 2 : Protein conc.)

| <b>Source</b>      | <b>Sum of Squares</b> | <b>Degrees of freedom</b> | <b>Mean Square</b> | <b>F Value</b> | <b>p-value Prob &gt; F</b> |
|--------------------|-----------------------|---------------------------|--------------------|----------------|----------------------------|
| <b>Model</b>       | 462.54                | 9                         | 51.39              | 103.87         | < 0.0001                   |
| <b>Residual</b>    | 4.95                  | 10                        | 0.49               |                |                            |
| <b>Lack of Fit</b> | 4.95                  | 5                         | 0.99               |                |                            |
| <b>Pure Error</b>  | 0                     | 5                         | 0                  |                |                            |
| <b>Cor Total</b>   | 467.49                | 19                        |                    |                |                            |

C.V. % =7.19;  $R^2$  =0.98; Adj  $R^2$  = 0.97; Pred  $R^2$  = 0.91.



(a)



(b)

**Fig. 3.11:** Normal plot of residual of for (a) decolourisation and (b) protein concentration

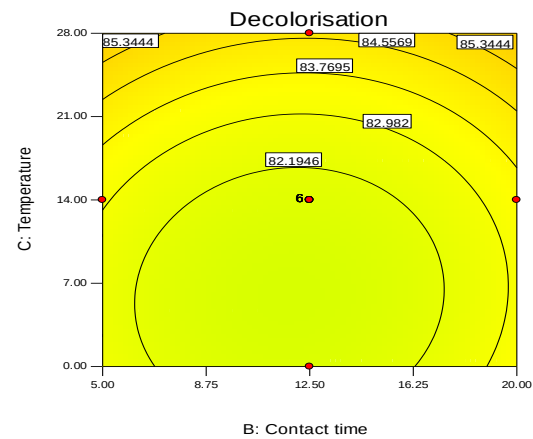
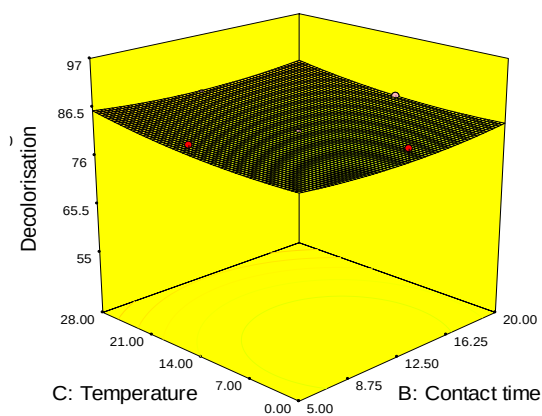
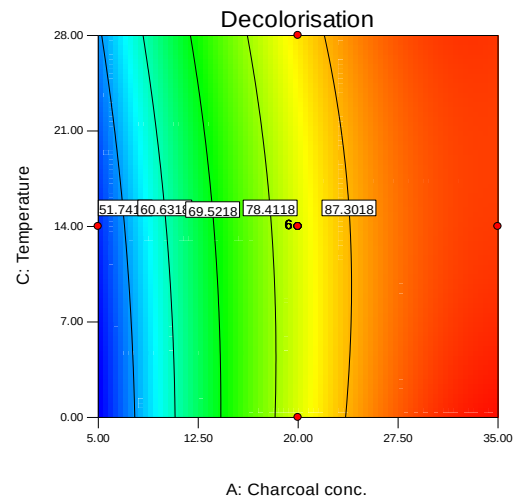
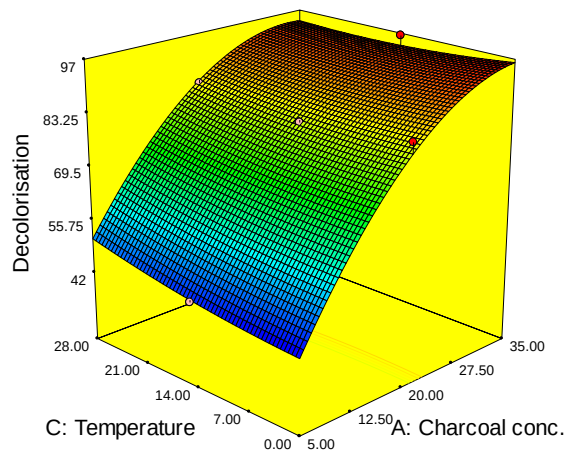
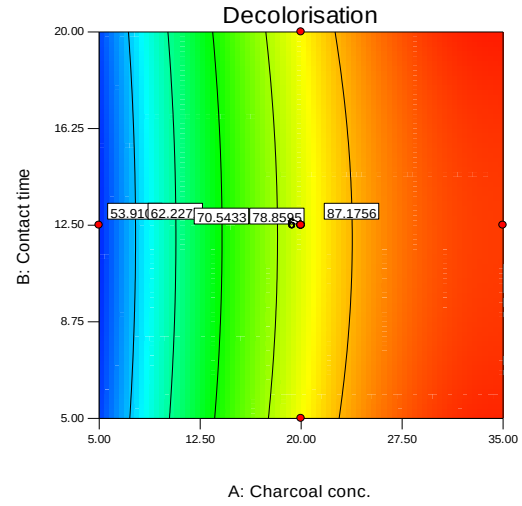
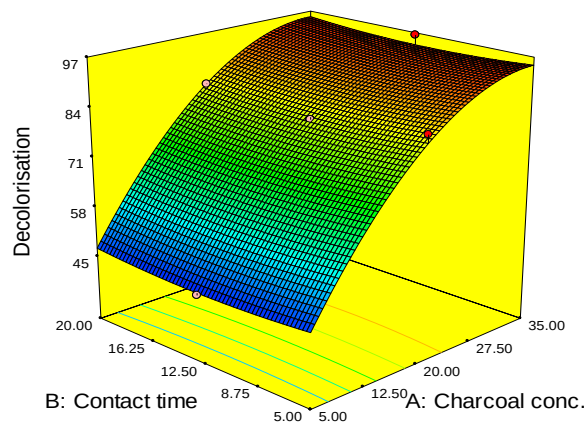


**Fig. 3.12:** Response surfaces and contour plots showing the mutual effect of (a) Contact time and charcoal conc. (b) Temperature and charcoal conc. (c) Temperature and contact time, on the decolourisation process.



Final Equation in Terms of Coded Factors:

$$\text{Decolourisation} = + 81.85 + 24.12 * A + 0.19 * B + 1.51 * C + 0.050 * A * B - 2.55 * A * C - 0.1 * B * C - 12.14 * A^2 + 1.41 * B^2 + 1.31 * C^2 \dots\dots\dots(3)$$

$$\text{Protein conc} = + 6.69 - 4.96 * A - 0.19 * B - 0.39 * C + 0.41 * A * B + 0.21 * A * C + 1.43 * B * C + 6.35 * A^2 + 0.57 * B^2 + 0.41 * C^2 \dots\dots\dots(4)$$

where A, B, and C represent the values of the independent variables charcoal concentration, contact time and temperature, respectively.

The second-order polynomial equations, expressed in coded values, were employed to fit the experimental data of the CCD for the prediction of both decolourisation (Equation 3) and protein conc. (Equation 4).

ANOVA indicated that the model was highly significant, with an F-value of 260.28 and  $p < 0.0001$  for decolourisation and F-value of 103.87 and  $p < 0.0001$  for protein concentration. The calculated  $R^2$  values were 0.9957 and 0.98 for decolourisation and protein concentration respectively, confirming the suitability of the model for response prediction. The adjusted coefficient ( $\text{adj } R^2$ ) was 0.9919 and predicted coefficient ( $R^2$ ) was 0.9704 for decolourisation and ( $\text{adj } R^2$ ) 0.97 and predicted coefficient ( $R^2$ ) was 0.91 for protein concentration, suggesting that the regression models using the second-order polynomial equations provided a very good fit to the experimental data. A higher  $R^2$  Adj indicates a larger proportion of the variability in the dependent variable, implying a more reliable and accurate predictive model. The adjusted precision assesses the signal-to-noise ratio, the values of 46.001 and 29.207 for decolourisation and protein concentration, respectively, pointing to satisfactory signal and indicating that the model was significant for the process. The optimal values obtained from RSM analysis were (A) = 25.81 mg/L, (B) = 20 min, (C) = 28°C. The model-predicted optimum decolourisation was 92%, with 45.77% protein recovery. The verification experiment was carried out using optimized conditions, which gave a decolourisation efficiency of 89.15 % and protein recovery of 44.41% , close to the predicted values.

### 3.3.7 Adsorption isotherms

The effect of charcoal concentration on the adsorption of polyphenols was examined across various concentrations. The adsorption data were then fitted to two isotherm models, namely the Langmuir and Freundlich equations, and the goodness of fit was evaluated.

The Langmuir isotherm is linked to the adsorption of a monolayer on a uniform adsorbent surface, where all adsorption sites possess the same affinity for the adsorbate. There is assumed to be no interaction among the adsorbate (polyphenols). The adsorption results obtained were fitted to the linear form of the Langmuir isotherm (Eq. 1).

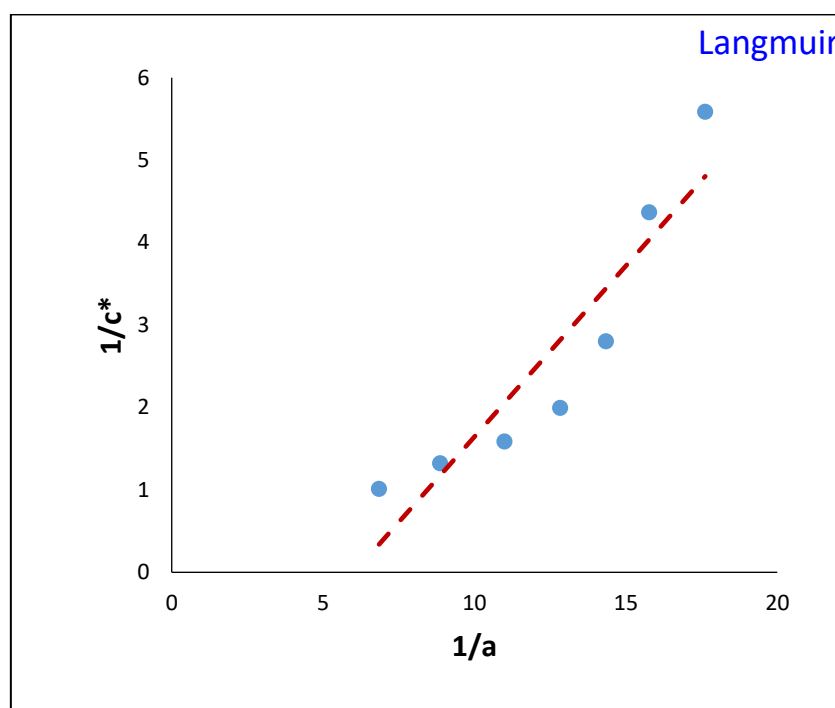


**Fig. 3.14:** Fitting of decolourisation data to the Langmuir model

A non-linear plot was obtained over the studied range of activated charcoal concentration (Fig.3.14) indicating that the model was not suitable for describing the adsorption of polyphenols on activated charcoal.

**Freundlich isotherm.** The Freundlich isotherm which considers multilayer adsorption on heterogeneous surfaces is described by Eq. (2).

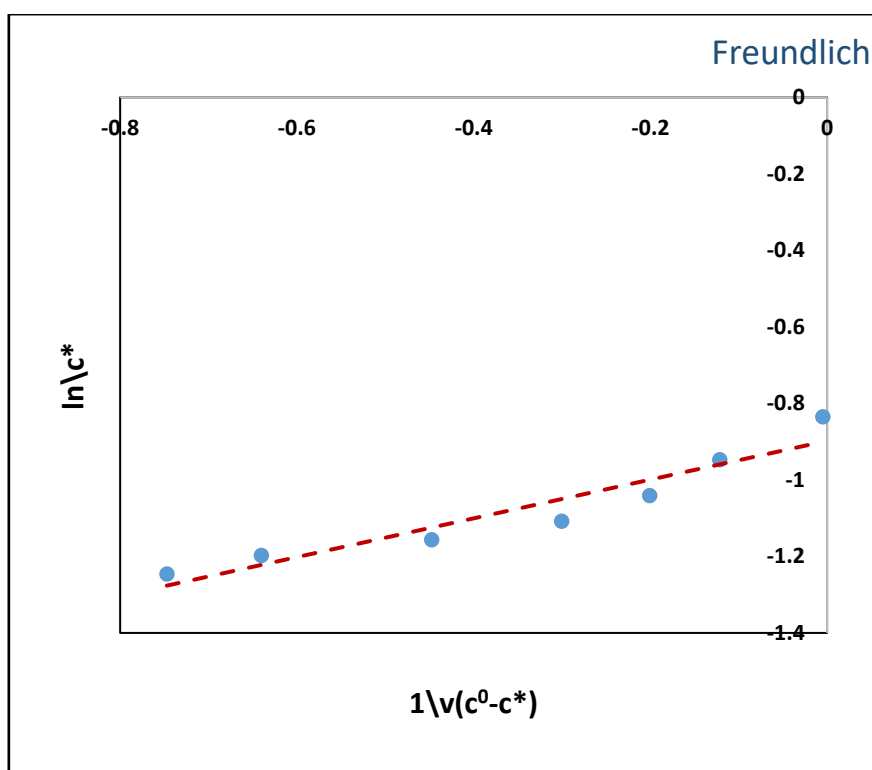


**Fig. 3.15:** Fitting of decolourisation data to the Freundlich model

The results (Fig. 3.15) suggest that the adsorption process of polyphenols on activated charcoal exhibited more of a Freundlich behaviour. García-Pérez (2019) reported similar findings while analyzing the adsorption of polyphenols from *Bryophyllum* extracts, observing that the results followed the Freundlich isotherm and did not conform to the Langmuir model.

### 3.3.8 Antimicrobial activity in decolourised extracts

*E. agallocha* extracts, both before and after charcoal treatment, were evaluated for their activity against *S. aureus*. The antimicrobial activity of the leaf preparations remained stable even after the treatment to achieve 90% decolourisation of the extracts (Fig. 3.16).

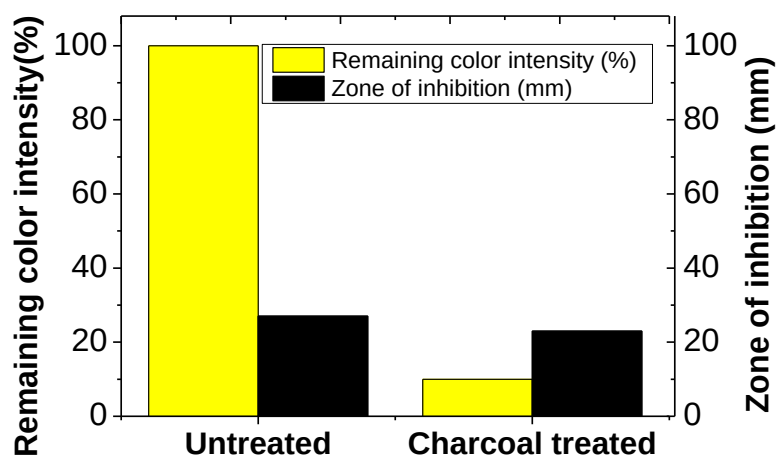


**Fig. 3.16:** Antimicrobial assay

### 3.3.9 Desorption studies

Recycling of the sorbent would be a very important step from an economy point of view. Desorption studies were hence carried out as detailed under Materials and methods. Near-total desorption was achieved with NaOH at a concentration 0.1M (Fig.3.17 & 3.18), indicating successful recycling of the sorbent for repeated use. In summary, adsorption on activated carbon could be successfully employed to remove polyphenols from plant extracts by employing effective, sustainable, economically viable and environmentally friendly procedures.

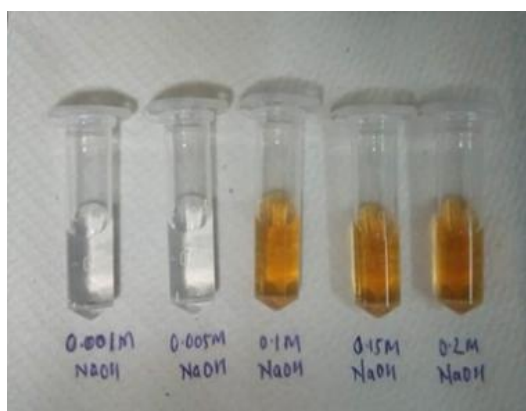


**Fig. 3.17:** Desorption studies

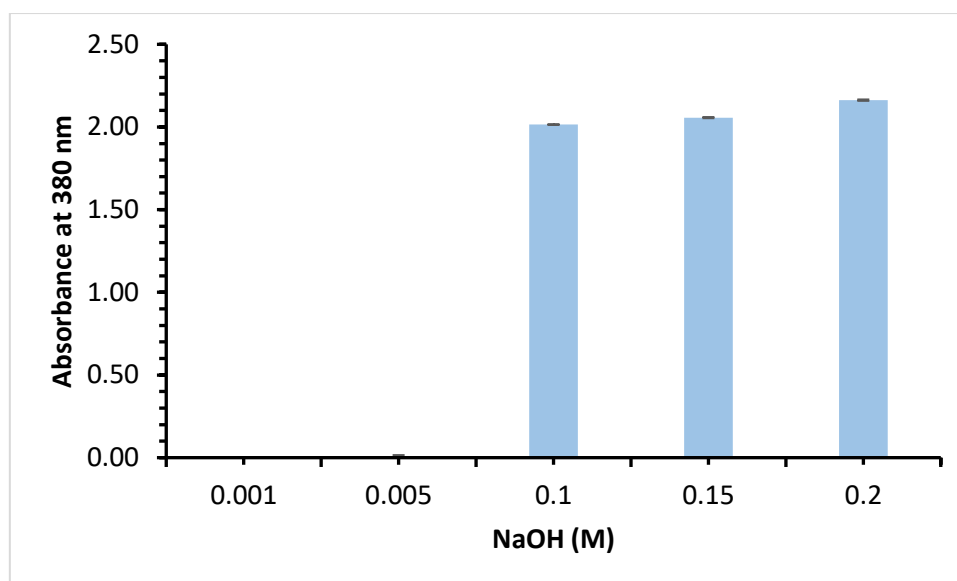


**Fig. 3.18:** Absorbance of desorbed polyphenols at 380 nm

In conclusion, the results presented in this Chapter highlight a novel approach proposed for the removal of polyphenolic compounds from mangrove plant extracts using sustainable adsorption methodology. Activated carbon was employed as an environmentally friendly and economical means to effectively eliminate polyphenols from the plant extracts. The adsorption of polyphenols was characterized, providing valuable insights into the adsorption behaviour of complex plant extracts. Equilibrium experiments revealed that the Freundlich isotherm model could successfully predict the experimental data for the *E. agallocha* extract. This finding suggests a promising starting point for future applications of activated charcoal in the recovery of bioactive peptides from plant extracts. Moreover, utilizing this approach might help overcome the typical disadvantages associated with traditional extraction and purification procedures.

The RSM analysis based on the CCD approach was applied to evaluate, predict, and optimize the decolourisation process and protein concentration. The individual and interaction effects of three important parameters, including charcoal concentration, contact time and temperature were sufficiently modeled and optimized. Charcoal concentration was found to be the main significant factor affecting decolourisation. The predicted decolourisation ( $R^2=0.9939$ ) and protein concentration ( $R^2=0.9939$ ) values were in good agreement with the experimental results. Based on RSM plots, the optimum conditions were (A) = 25.81 mg/L, (B) = 20 min,

(C) = 28°C. The decolourisation efficiency of 89.15 % and protein recovery of 44.41% were close to the predicted value of 92% decolourisation with 45.77% protein recovery. Overall, this study introduces a sustainable and effective method for polyphenol removal and the recovery of peptides from *E. agallocha* extracts.

The results presented in this Chapter have thus demonstrated how a simple generalised strategy using activated charcoal could be applied towards purification of cationic peptides from crude extracts and thereby dramatically increase the prospects of developing AMPs from the rich pool of bioactive molecules, especially from mangrove plant extracts.

# **Chapter 4:**

## **Bioactivities of Cationic Peptides from Four Mangrove Plants**

## 4.1 Introduction

Mangroves are unique ecosystems of the intertidal zones of tropical and subtropical regions. The living environment of mangrove flora is more complex compared to that of terrestrial and aquatic plants, exposing them to a wider array of microbial pathogens. Consequently, mangrove plants may possess highly efficient Host Defense Peptides (HDPs), although reported literature on them is minimal. As per a bioinformatic study, the mangrove plants *Kandelia obovata*, *Avicennia corniculatum*, and *Avicennia marina* were reported to possess antimicrobial peptides of the Snakin/GASA family (Shang et al., 2023).

The bioactivities of mangrove plant – derived cationic peptides have been a subject of growing interest of late. While they are known to produce a plethora of secondary metabolites with diverse bioactivities, the isolation of HDPs from these plants remains largely unexplored. Although some studies have hinted at the presence of bioactive peptides in mangrove plants, serious characterization of these molecules is wanting, the research, where attempted, still being at its infancy. Nonetheless, the recorded bioactivities of secondary metabolites from mangrove plants indicate their potential in various biological processes and their applications in medicine and other fields are being looked into.

Indian mangroves such as *Rhizophora mucronata*, *Sonneratia alba* and *Excoecaria agallocha* from Goa have been reported to possess antibacterial activity against human pathogens such as *Staphylococcus aureus*, *Streptococcus* sp., *Salmonella typhi*, *Proteus vulgaris* and *Proteus mirabilis* (Sahoo et. al., 2012). *Avicennia marina*, *A. officinalis*, *Bruguiera sexangula*, *Excoecaria agallocha*, *Lumnitzera racemosa*, and *Rhizophora apiculata* crude extracts were evaluated against antibiotic resistant pathogenic bacteria (Abeyasinghe et. al., 2010). *Suaeda maritima*, another mangrove from Bhitarkanika, Odisha was investigated for *in vitro* antioxidant and antimicrobial activities (Patra et. al., 2011). Furthermore, the mangrove plant extracts were found to possess significant antioxidative and potent antibacterial abilities against seven food-borne pathogens (Suganthi et. al., 2016).

*Sonneratia caseolaris* (Yompakdee et al., 2012), *Lumnitzera littorea* (Saad et al., 2011), and *Heritiera fomes* (Wangensteen et al., 2009), among other mangrove species from various regions worldwide, have been reported for their antimicrobial potential. *Avicennia marina*, for

instance, has been found to synthesize abietane diterpenoids with cytotoxic and antimicrobial activities (Han et al., 2008).

Phytochemical evaluations of mangroves have identified bioactive compounds in species such as *Bruguiera gymnorhiza* (bruguierols A–C) (Han et al., 2005), *Avicennia marina* (naphthoquinone derivatives) (Han et al., 2007), and *Derris indica* (flavonoids) (Koysomboon et al., 2006), among others. Below is a compilation of chemical compounds derived from mangrove species, along with their associated bioactivities (Table 4.1).

**Table 4.1:** Bioactivities of chemical compounds from mangroves species [adapted from Dahibhate et al., 2018].

| Biological Properties | Species                  | Bioactive Compounds                                                        | Reference                                                              |
|-----------------------|--------------------------|----------------------------------------------------------------------------|------------------------------------------------------------------------|
| <b>Anticancer</b>     | <i>A. corniculatum</i>   | Aegicoroside A                                                             | Titus et al., 2018                                                     |
|                       | <i>E. agallocha</i>      | Excoecarin L and Excoecarin D                                              | Greenwell et al., 2015                                                 |
|                       | <i>R. stylosa</i>        | Rhizostyloside                                                             | Homhual et al., 2006                                                   |
|                       | <i>S. paracaseolaris</i> | Paracaseolin A-E                                                           | Losad et al., 2017                                                     |
|                       | <i>B. cylindrica</i>     | Taraxeryl-trans-p-chlorocinnamate                                          | Ni et al., 2018                                                        |
|                       | <i>X. granatum</i>       | Thaixylogranins A-H,<br>Xylogranatumines A-G,<br>Xylomexicanins E-H        | Van et al., 2018, Gong et al., 2017, Roy et al., 2006                  |
|                       | <i>C. tagal</i>          | Tagalphenylpropinoidin A-B,<br>Tagalenes J-K, Tagalons A-D,<br>Tagalsine X | Liao et al., 2016, Zhou et al., 2014, Wu et al., 2014, Ni et al., 2017 |
|                       | <i>E. agallocha</i>      | Agallochanins A-K, Agallolides A-M                                         | Jiang et al., 2020, Jiang et al., 2019                                 |
|                       | <i>C. decandra</i>       | Decandrols A-I                                                             | Jiang et al., 2018                                                     |
| <b>Antibacterial</b>  | <i>C. tagal</i>          | Isopimar-8(14)-en-16-hydroxy-15-one                                        | Wu et al., 2016                                                        |
|                       | <i>E. agallocha</i>      | ent--hydroxy-atisane-3,4-lactone                                           | Nithyamol et al., 2018                                                 |
|                       | <i>A. corniculatum</i>   | Acornin 1 and Acornine 2                                                   | Wang et al., 2009                                                      |
| <b>Antifungal</b>     | <i>A. corniculatum</i>   | Acornin 1 and Acornine 2                                                   | Wang et al., 2009                                                      |
|                       | <i>X. granatum</i>       | 3-(1-hydroxyethyl)-4,4-dimethyl-4-butyrolactone                            | Ponnapalli et al., 2012                                                |

|                          |                          |                                                                                                       |                                          |
|--------------------------|--------------------------|-------------------------------------------------------------------------------------------------------|------------------------------------------|
| <b>Antiviral</b>         | <i>E. agallocha</i>      | Excoecariphenols A-D                                                                                  | Gupta et al., 2014                       |
|                          | <i>X. granatum</i>       | Sundarbanxylogranins B                                                                                | Shijie et al., 2009                      |
|                          | <i>S. paracaseolaris</i> | Paracaseolin D                                                                                        | Losada-Echeberria et al., 2017           |
| <b>Anti-inflammatory</b> | <i>X. moluccensis</i>    | Thaixylomolin B, 7-deacetylgedunin                                                                    | Sanna et al., 2015, Hopkins et al., 2010 |
|                          | <i>E. agallocha</i>      | Agallochaols K, Agallochaols O, Agallochaols Q                                                        | Pandey et al., 2009                      |
|                          | <i>K. candel</i>         | Kandelside, Blumenol C glucoside, Corchoionoside C                                                    | Chacha et al., 2010                      |
|                          | <i>R. annamalayana</i>   | Benzoxepins                                                                                           | Raola et al., 2017                       |
| <b>Antioxidant</b>       | <i>A. marina</i>         | Maricaffeolylide A, Maricyclohexene A                                                                 | Ravangpai et al., 2011                   |
|                          | <i>B. gymnorhiza</i>     | Brugymnoside A                                                                                        | Li et al., 2010                          |
|                          | <i>R. mucronata</i>      | Olean-18(19)-en-3b-yl-(3,6-dimethyl-3E,6Z-dienoate), (13a)-27-frido-olean-14(15)-en-(17a)-furan-3b-ol | Thao et al., 2015                        |
|                          | <i>X. granatum</i>       | Dihydrocaffeic acid--epicatechin                                                                      | Foti et al., 2007                        |

## 4.2 Materials and methods

All chemicals were sourced from HiMedia Laboratories, Pvt., Ltd., India, unless otherwise specified. All experiments were conducted in triplicate.

### 4.2.1 Test pathogens

Pure cultures of *Salmonella typhimurium* (MTCC 98), *Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 741), *Pseudomonas fluorescence* (MTCC 7200), *Candida albicans* (MTCC 227) *Aeromonas hydrophila* (MTCC 1739) and *Aeromonas sobria* (MTCC 3613) were used for the study.

### 4.2.2 Partial purification of the peptides by ammonium sulphate precipitation

Acid extracts of leaf stem and root of *S. alba*, *R. mucronata*, *E. agallocha* and *A. illicifolius*

were prepared and subjected to 90% ammonium sulphate precipitation at 4°C (as described in Chapter 2 Sections 2.2.5).

#### **4.2.3 Antimicrobial Assay**

The antimicrobial activity of the crude acid extract is evaluated by well diffusion method (as described in Chapter 2 Sections 2.2.5).

#### **4.2.4 Antioxidant potential (DPPH Assay)**

Absolute methanol (3.7mL) was taken in all test tubes except blank in which 3.8 ml methanol was added. 100 µl of Butylated hydroxytoluene (BHT) (1.6 mg/mL in methanol) was added to tubes marked as standard and 100 µl of respective samples to all other tubes marked as tests. Thereafter, 200 µL of DPPH solution (1 mg/mL in methanol) was added to all tubes, including the blank. The mixture was then incubated in the dark at room temperature for at least 30 minutes, after which the absorbance of the samples was measured at 517 nm (Brand-Williams et al., 1995).

Calculation

% Antioxidant activity = {(absorbance at blank) – (absorbance at test) / (absorbance at blank)} x 100.

#### **4.2.5 Antidiabetic Assays (Alpha Amylase Inhibition Assay)**

*In vitro* amylase inhibition was studied by the method of Bernfeld (Sangeetha and Vedaasree, 2012). In brief, 100 µl of acarbose (2 mg/ml) or 100 µl of the test extract was allowed to react with 200 µl of α-amylase enzyme and 100 µl of 2 mM phosphate buffer (pH-6.9). After a 20 min incubation, 100 µl of 1% starch solution was added. The same was performed for the controls, wherein 200 µl of the enzyme was replaced by buffer. After incubation for 5 min, 500 µl of dinitro-salicylic acid reagent was added to both control and test samples and kept in a boiling water bath for 5 min. The absorbance was recorded at 540 nm and the percentage inhibition of α-amylase enzyme calculated applying the formula:

Inhibition (%) = {(control–test)/control} x 100.

## **4.3 Results and discussion**

### **4.3.1. Antimicrobial activity**

While the antimicrobial activity study in Chapter 2 focused on determining the percentage homogenate and solvent percentage standardization for selected extracts, this Chapter presents a systematic investigation of the antimicrobial activity of all four mangrove plants and their various parts. Additionally, a comparison of the antimicrobial activity amongst these plants and their parts is also brought out.

As detailed in Table 4.2 the root extract of *Sonneratia alba* elicited highest activity against *Salmonella typhimurium*. All the extracts showed activity against *Aeromonas hydrophila* and *Candida albicans*.

**Table 4.2:** The zone of inhibition exhibited by partially purified acid extracts from leaf, stem and root samples of *Sonneratia alba*, *E. agallocha*, *R. mucronata* and *A. ilicifolius* tested against Gram positive (*S. aureus*), Gram negative bacteria (*S. typhimurium*, *E. coli*, *P. aeruginosa*, *P. fluorescens*, *A. hydrophila* and *A. sobria* ) and a fungus (*C. albicans* ).

| Plant samples               |               | <i>Salmonella typhimurium</i> (MTCC 98) | <i>Candida albicans</i> (MTCC 227) | <i>Staphylococcus aureus</i> (MTCC 3160) | <i>Escherachia coli</i> (MTCC 443) | <i>Pseudomonas aeruginosa</i> (MTCC 741) | <i>Pseudomonas fluorescens</i> (MTCC 7200) | <i>Aeromonas hydrophilla</i> (MTCC.), | <i>Aeromonas sobria</i> (MTCC 3613) |
|-----------------------------|---------------|-----------------------------------------|------------------------------------|------------------------------------------|------------------------------------|------------------------------------------|--------------------------------------------|---------------------------------------|-------------------------------------|
| <i>Sonneratia alba</i>      | Leaf          | 14.0 ± 0.57                             | 10.3 ± 0.33                        | 14.0 ± 0.00                              | 13.0 ± 0.00                        | 18.0 ± 0.00                              | 14.3 ± 0.33                                | 17.0 ± 0.00                           | 12.0 ± 0.00                         |
|                             | Stem          | 15.3 ± 0.33                             | 12.6. ±0.33                        | 15.3 ± 0.33                              | 14.0 ± 0.00                        | 20.8 ± 0.16                              | 15.3 ± 0.33                                | 17.5 ± 0.2                            | 14.0 ± 0.00                         |
|                             | Root          | 10.0 ± 0.00                             | 11.0 ± 0.00                        | 11.3 ± 0.33                              | 10.0 ± 0.00                        | 16.3 ± 0.33                              | 16.3 ± 0.33                                | 17.0 ± 1.00                           | 00.0 ± 0.00                         |
| <i>Excoecaria agallocha</i> | Leaf          | 17.0 ± 0.57                             | 10.3 ± 0.33                        | 14.0 ± 0.00                              | 17.6 ± 0.57                        | 17.0 ± 0.00                              | 20.6 ± 0.33                                | 14.0 ± 0.00                           | 12.0 ± 0.00                         |
|                             | Stem          | 15.3 ± 0.88                             | 10.3 ± 0.33                        | 17.3 ± 0.33                              | 15.6 ± 0.66                        | 18.3 ± 0.33                              | 19.3 ± 0.33                                | 15.0 ± 0.00                           | 13.1 ± 0.08                         |
|                             | Root          | 16.0 ± 0.00                             | 13.0 ± 0.00                        | 16.0 ± 0.00                              | 16.3 ± 0.33                        | 17.0 ± 1.52                              | 18.0 ± 0.00                                | 11.6 ± 0.33                           | 12.3 ± 0.33                         |
| <i>Rhizophora mucronata</i> | Leaf          | 14.0 ± 0.00                             | 19.0 ± 0.00                        | 12.0 ± 0.00                              | 15.3 ± 0.33                        | 21.0 ± 0.00                              | 17.3 ± 0.57                                | 16.3 ± 0.33                           | 00.0 ± 0.00                         |
|                             | Stem          | 14.0 ± 0.00                             | 17.0. ±0.00                        | 15.0 ± 0.00                              | 14.0 ± 0.00                        | 20.0 ± 0.00                              | 16.0 ± 0.00                                | 15.0 ± 0.00                           | 14.3 ± 0.33                         |
|                             | Root          | 13.3 ± 0.33                             | 15.0 ± 0.00                        | 14.0 ± 0.00                              | 14.0 ± 0.00                        | 17.0 ± 0.00                              | 14.6 ± 0.66                                | 15.0 ± 0.00                           | 00.0 ± 0.00                         |
| <i>Acanthus ilicifolius</i> | Leaf          | 14.3 ± 0.33                             | 12.0 ± 0.00                        | 14.0 ± 0.00                              | 13.0 ± 0.00                        | 20.3 ± 0.33                              | 17.3 ± 0.33                                | 15.0 ± 0.00                           | 18.0 ± 0.00                         |
|                             | Stem          | 14.0 ± 0.57                             | 13.6. ± 0.33                       | 17.3 ± 0.33                              | 14.6 ± 0.33                        | 19.6 ± 0.33                              | 16.6 ± 0.33                                | 13.0 ± 0.00                           | 12.6 ± 0.33                         |
|                             | Root          | 14.0 ± 0.00                             | 15.3 ± 0.33                        | 18.3 ± 0.33                              | 14.0 ± 0.00                        | 18.6 ± 0.66                              | 16.0 ± 0.00                                | 15.3 ± 0.33                           | 18.6 ± 0.33                         |
| Antibiotic (+ control)      | Ampicillin    | 27± 0.33                                | -                                  | 36± 0.28                                 | 33± 0.00                           | 32± 0.00                                 | -                                          | 25± 0.39                              | 29± 0.00                            |
|                             | Ciprofloxacin | 33± 0.00                                | -                                  | 37 ± 0.08                                | 35± 0.28                           | 40± 0.85                                 | 40± 0.33                                   | 36± 0.00                              | 40± 0.10                            |
|                             | Kanamycin     | 38± 0.28                                | -                                  | -                                        | 19± 0.00                           | 18± 0.00                                 | 23± 0.00                                   | 26± 0.33                              | 26± 0.33                            |
|                             | Streptomycin  | -                                       | -                                  | 29± 0.33                                 | 26± 0.57                           | 27± 0.33                                 | 25 ± 0.08                                  | 30± 0.28                              | 30± 0.33                            |
| Tetracycline (+ control)    |               | 33± 0.00                                | -                                  | 38± 0.00                                 | 36± 0.00                           | 23± 0.47                                 | 22± 0.28                                   | 25± 0.00                              | 41± 0.00                            |

\*Antibiotic concentrations (per 100 µl): Ampicillin (10 µg), Ciprofloxacin (5µg), Kanamycin (30 µg), Streptomycin (10µg), Tetracycline (30µg)

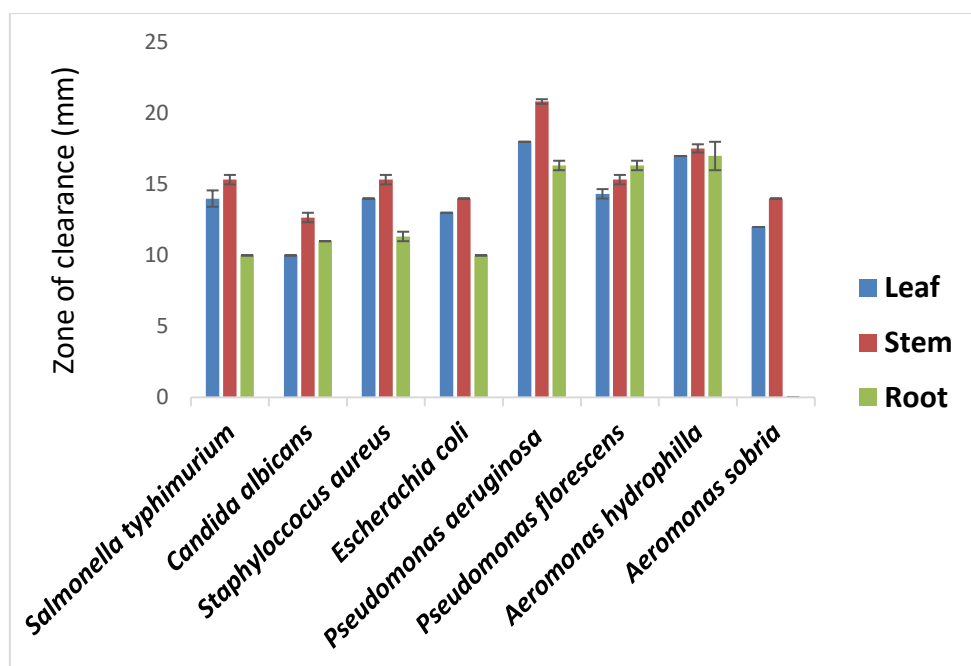


**Fig. 4.1:** Antimicrobial activity of *Sonneratia alba* extracts



**Fig. 4.2:** Antimicrobial susceptibility testing by the well diffusion method

- Leaf extract of *Sonneratia alba* showed highest antimicrobial activity against *P. aeruginosa* and lowest activity against *C. albicans*.
- Stem extracts of *Sonneratia alba* showed highest activity against *P. aeruginosa* and lowest activity against *C. albicans*.
- Root extract of *Sonneratia alba* showed highest activity against *A. hydrophila* and lowest activity against *S. typhimurium* and *E. coli*.
- Among leaf, stem and root extract of *Sonneratia alba*, stem extract showed highest activity against *P. aeruginosa*

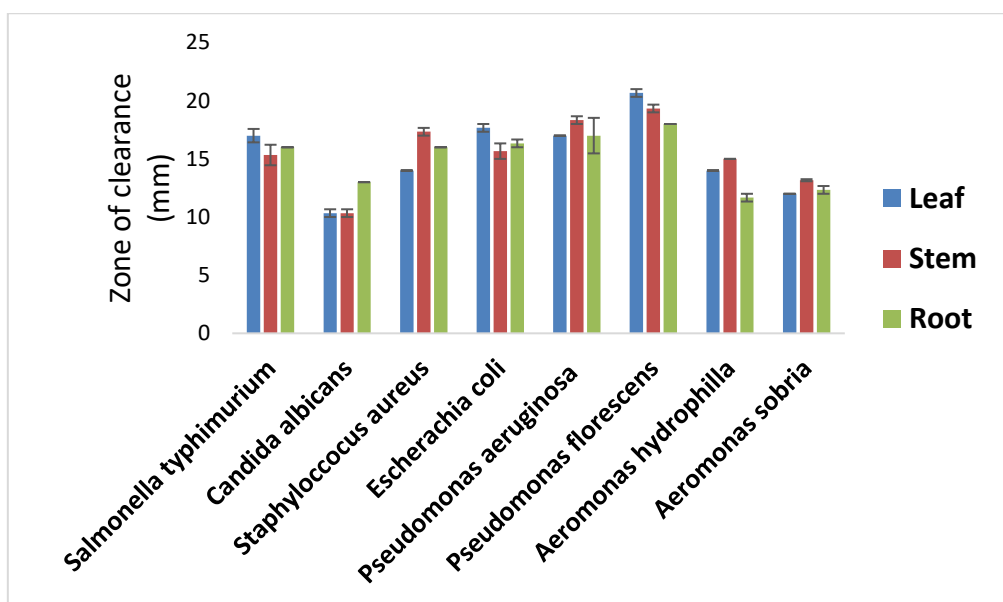


**Fig. 4.3:** Antimicrobial activity of *Excoecaria agallocha* extracts

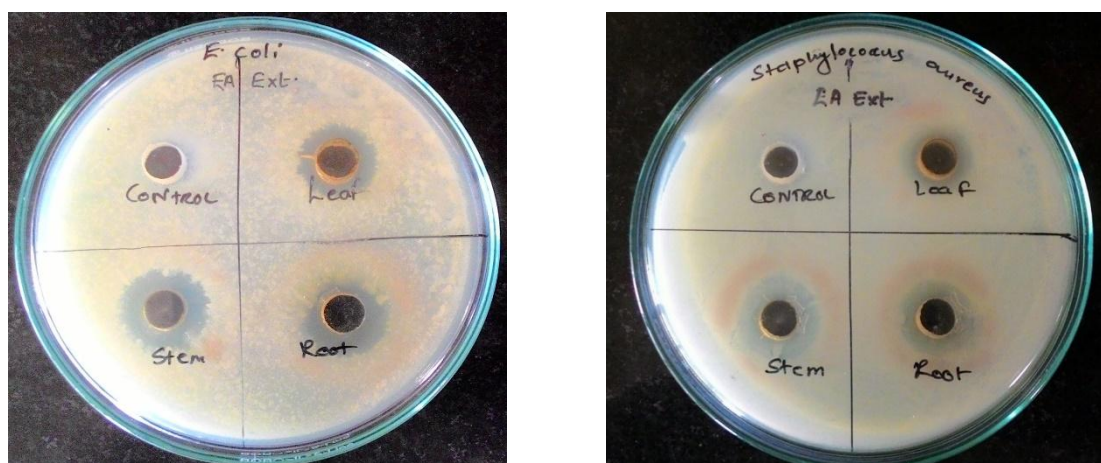


**Fig. 4.4:** Antimicrobial susceptibility testing using the well diffusion method

- Leaf extract of *Excoecaria agallocha* showed highest antimicrobial activity against *P. aeruginosa* and lowest activity against *C. albicans*.
- Stem extract of *Excoecaria agallocha* showed highest activity against *P. fluorescence* and lowest activity against *C. albicans*.
- Root extract of *Excoecaria agallocha* showed highest activity against *P. fluorescence* and lowest activity against *A. hydrophila*.
- Among leaf, stem and root extract of *Excoecaria agallocha*, leaf extract showed the highest activity against *P. fluorescence*.

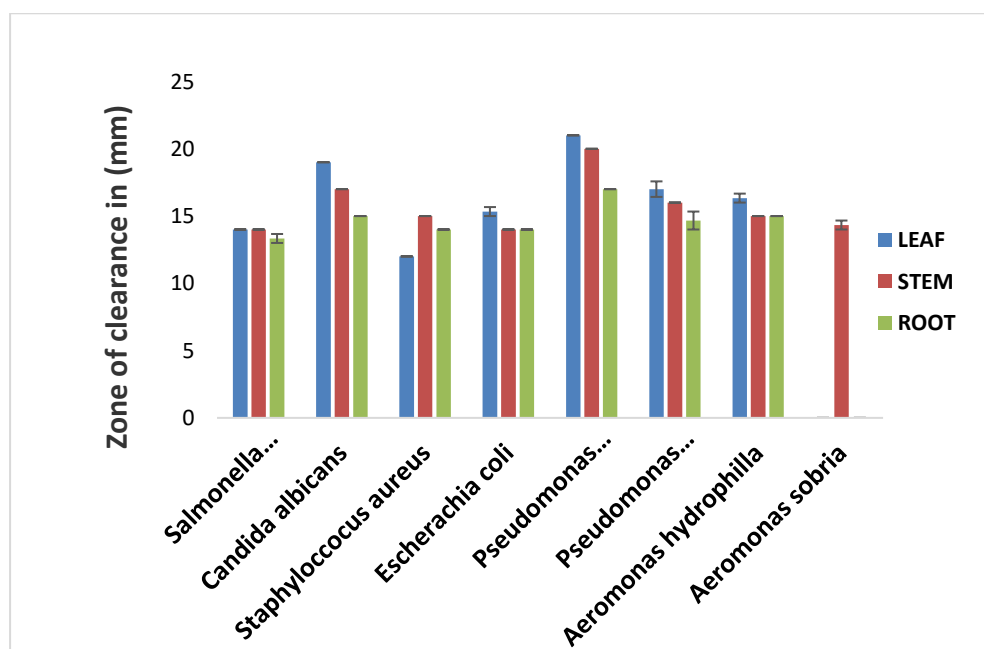


**Fig. 4.5:** Antimicrobial activity of *Rhizophora mucronata* extracts

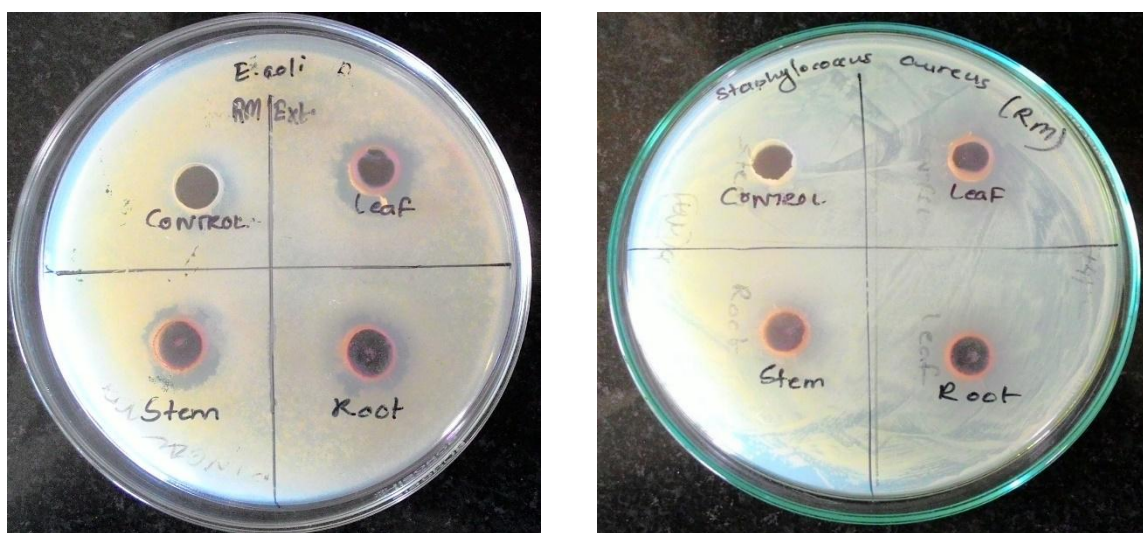


**Fig. 4.6:** Antimicrobial susceptibility testing using the well diffusion method

- Leaf extract of *Rhizophora mucronata* showed highest antimicrobial activity against *P. aeruginosa* and lowest activity against *A. sobria*.
- Stem extracts of *Rhizophora mucronata* showed highest activity against *P. aeruginosa* and lowest activity against *S. typhimurium* and *E. coli*.
- Root extract of *Rhizophora mucronata* showed highest activity against *P. aeruginosa* while no activity was observed against *A. sobria*.
- Among leaf, stem and root extracts of *Rhizophora mucronata*, leaf extract showed highest activity against *P. aeruginosa*.

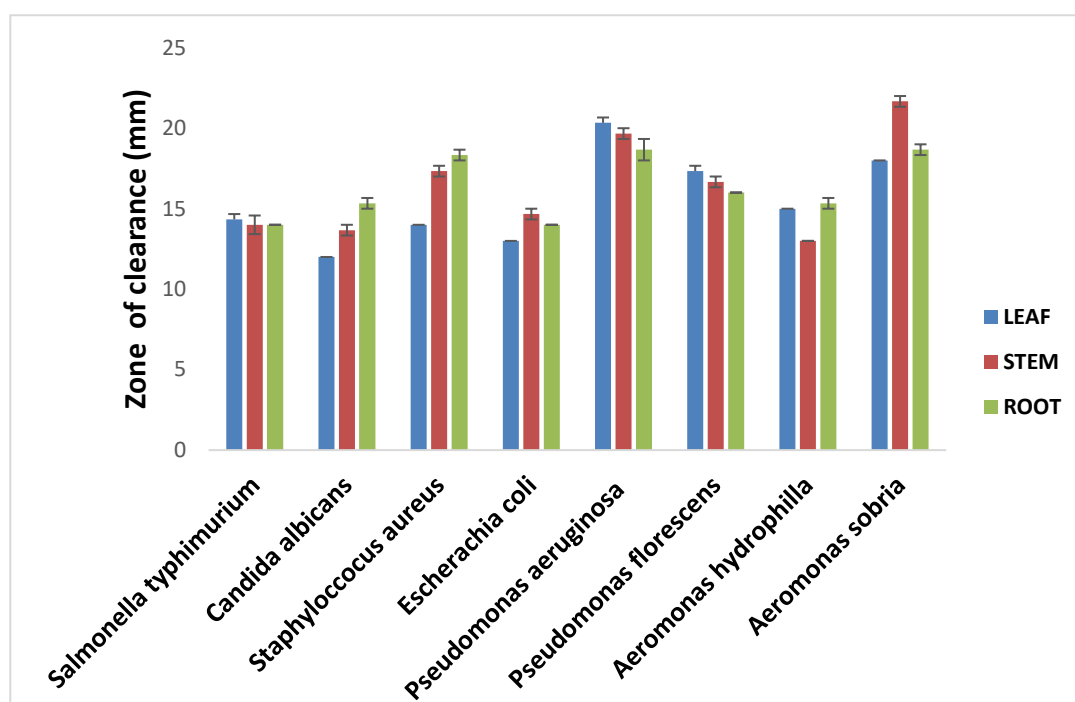


**Fig. 4.7:** Antimicrobial activity of *Acanthus ilicifolius* extracts



**Fig. 4.8:** Antimicrobial susceptibility testing using the well diffusion method

- Leaf extract of *Acanthus ilicifolius* showed highest antimicrobial activity against *P. aeruginosa* and lowest activity against *C. albicans*.
- Stem extracts of *Acanthus ilicifolius* showed highest activity against *A. sobria* and lowest activity against *A. aeruginosa*.
- Root extract of *Acanthus ilicifolius* showed highest activity against *P. aeruginosa* and lowest activity against *E.coli*.
- Among leaf, stem and root extracts of *Acanthus ilicifolius*, stem extract showed highest activity against *P. aeruginosa* .

### 4.3.2 Antioxidant potential (DPPH Assay)

Leaf, stem and root extracts from all the four mangrove plants tested showed radical scavenging activity to various extents (Fig. 4.9) The activity of the extracts surpassed that of the standard (BHT). Significantly, those extracts with highest peptide content also recorded best antioxidant potential.

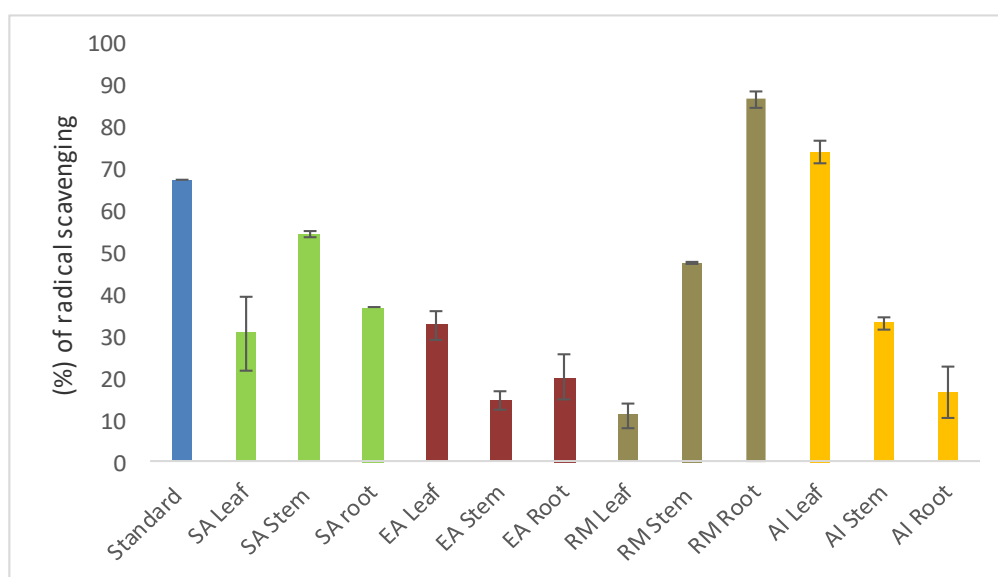


**Fig. 4.9:** Antioxidant activity of the mangrove plant extracts

### 4.3.3 Antidiabetic Assay

#### Alpha Amylase Inhibition Assay

Extracts from the leaves, stems, and roots of all four tested mangrove plants exhibited varying degrees of alpha amylase inhibition assay, as illustrated in Figure 4.10.

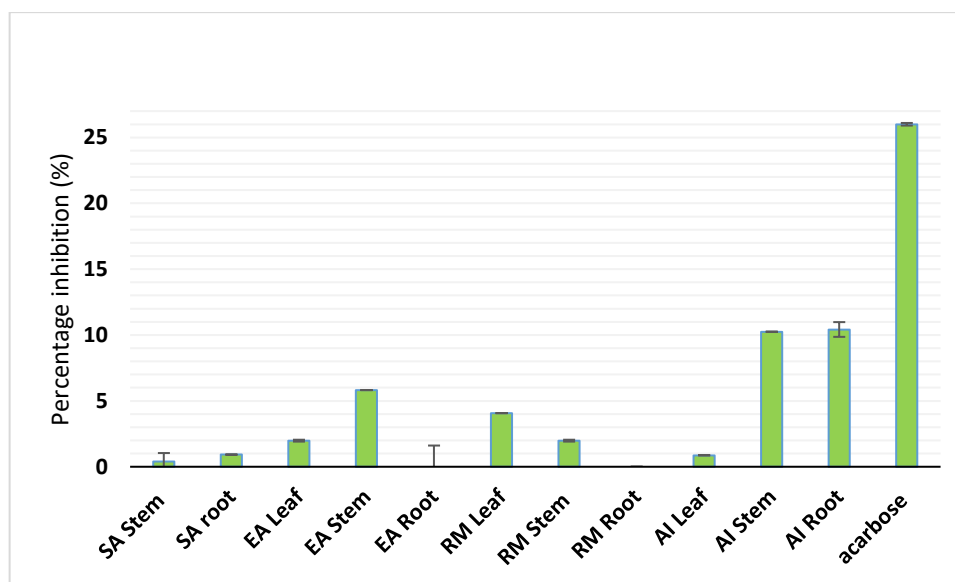


**Fig. 4.10:** Antidiabetic activity of the mangrove plant extracts

All the extracts exhibited minimal alpha-amylase inhibitory activity. The highest activity was observed in the *Acanthus ilicifolius* root sample, with 10.42% inhibition at a protein concentration of 193 $\mu$ g/mL. However, this activity was significantly lower compared to acarbose (40  $\mu$ g/mL), which showed 26% inhibition.

In conclusion, a variety of bioactivities, including antimicrobial, antioxidant and antidiabetic activities, were evaluated in the four mangrove plants. The extracts exhibited significant antimicrobial and antioxidant activities, the latter surpassing the standard (BHT) in some cases. Moreover, they demonstrated moderate antidiabetic activity, with *Acanthus ilicifolius* root and stem samples showing 10.42% and 10.25% inhibition, respectively.

# Chapter 5:

## Purification and Characterization of a Cationic Peptide from *Excoecaria agallocha*

## 5.1 Introduction

Plant extracts typically contain AMPs in lower quantities compared to other compounds (He et al., 2010). This makes it important to concentrate the extract in order to effectively isolate the AMPs. Various methods have been devised to concentrate supernatants containing AMPs, with ammonium sulphate precipitation and organic solvent extraction being two of the most widely used. After initial purification, the use of ion exchange chromatography using resins or columns that preferentially bind cationic peptides based on their charge and then eluting bound peptides using a gradient of increasing ionic strength or pH is a common approach. The Fabatin peptide, which was isolated from an extract of the fava bean flower, was separated using ion exchange chromatography with CM-Sepharose (Zhang & Lewis, 1997). Cationic peptides can be also separated based on their molecular size using size exclusion chromatography. Reversed-phase chromatography which separates peptides based on their hydrophobicity has sometimes been employed for additional purification if needed. Peptides are loaded on to a C18 column and eluted using a gradient of organic solvent concentration, as described in studies such as the isolation of Gly/His-rich peptides from *Capsella bursa-pastoris* roots (García-Olmedo et al., 1998) and the isolation of 8-Cys hevein-like peptides from fresh leaves of *Moringa oleifera* (Chan & Ng, 2013). For determination of molecular weights, purified peptide fractions are often analysed using mass spectrometry (MS). Techniques such as MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight) or LC-MS (Liquid Chromatography-Mass Spectrometry) can be employed to perform sequencing using techniques such as Edman degradation or tandem mass spectrometry (MS/MS) (Tang et al., 2018).

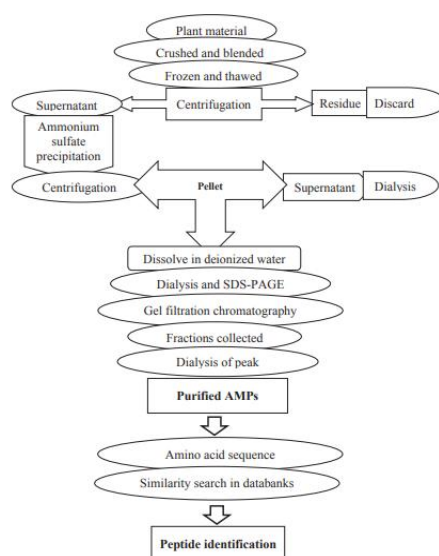


**Fig.5.1:** Diagram of different strategies used for isolation, purification and identification of antimicrobial peptides from plant sources [adapted from Tang et al., 2018].

## **5.2 Materials and methods**

### **5.2.1. Measurement of physico-chemical parameters at sampling sites**

Salient parameters of the water samples, such as temperature, salinity, conductivity, Total Dissolved Solids (TDS) and Dissolved Oxygen (DO), were determined on the field using a multiparameter instrument (CyberScan PC 650, Eutech Instruments, Thermo Fisher Scientific, India) and pH was measured on a digital pH meter (Eutech pH meter-700, Thermo Fisher Scientific, India).

### **5.2.2. Seasonal variation in protein content**

The protein content was determined using Bradford method as described in chapter 2, section 2.2.9

### **5.2.3 AU-PAGE**

Acid-urea polyacrylamide gel electrophoresis (AU-PAGE) resolves peptides and proteins based on their cationic charge density and is highly suited for the analysis of antimicrobial peptides. Small peptides with a high concentration of basic residues migrate more cathodally than larger or less positively charged peptides and proteins. The AU-PAGE was performed on a 15% resolving gel containing 6 M urea and 5% acetic acid (Panyim and Chalkley 1969). The gel was then stained with Coomassie Brilliant Blue G-250 and the banding compared with a known peptide standard (lysozyme) run simultaneously.

### **5.2.4 SDS-PAGE**

The molecular weight was analysed by SDS-PAGE, using a 15% gel as described in Chapter 2, section 2.2.13

### **5.2.5 Sephadex G-50 column chromatography**

The pellet obtained following ammonium sulfate precipitation of the leaf extract of *E. agallocha* (as detailed in Chapter 2, section 2.2.4) was subjected to size exclusion column chromatography on a Sephadex G-50 column (12 x 500 mm) pre-equilibrated with 10 mM sodium acetate buffer, pH 5.6. The protein pellet obtained upon ammonium sulphate precipitation to 90% saturation of the crude extract was suspended in the same buffer and

loaded on to the column. The samples were eluted with the buffer at a flow rate of 12 mL/h and the absorbance of the eluting fractions monitored at 220 and 280 nm. Aliquots from the eluted fractions were tested for antimicrobial activity following the agar well diffusion method (Shanmugaraju, 2012). Active fractions were pooled and concentrated by lyophilisation.

#### **5.2.6 MALDI-TOFF MS analysis**

Peptide molecular weight determination was carried out using mass spectrometry in positive ionization mode on a MALDI-TOFF/ TOF instrument. Purified and lyophilized peptide was reconstituted with 10  $\mu$ L of 0.1% trifluoroacetic acid (v/v). Aliquot of each peptide solution (1  $\mu$ L) was applied directly to a ground steel MALDI target plate and immediately after, an equal volume of a freshly-prepared 5 mg/mL solution of 4-hydroxy- $\alpha$ -cyano-cinnamic acid (Sigma) in 50% aqueous (v/v) acetonitrile containing 0.1% trifluoroacetic acid (v/v). Bruker flex Analysis software was used to perform the spectral processing and peak list generation of MS spectra. Deduced b- and y- ion series were overlaid onto their fragmentation spectra using this software.

### **5.3. Results and Discussion**

#### **5.3.1 Physicochemical parameters at the sampling site of *E. agallocha***

Our initial studies (Section 2.3.4) have highlighted that the production of antimicrobial peptides in *E. agallocha* could be influenced by seasonal variations in the physicochemical parameters of the sampling sites. In order to maximize the yield of peptides obtained from *E. agallocha*, a detailed study of the physicochemical parameters at the sampling sites and peptide production periods was hence conducted through monthly sampling for an entire year.

As observed in Fig. 5.2, water temperature at these sites varied from 30 to 25.5°C. The pH value, which represents the degree of ionisation of the medium, ranged from 6.7 to 7.5. Dissolved oxygen, being a limiting factor for growth and functioning of marine microorganisms, is a very useful parameter to determine water quality. Here, dissolved oxygen values ranged between 3.68 to 6.81 mg/L. Salinity variations were from 26.9 to 33.4 psu, being influenced by the input of freshwater from the Mandovi river. Total Dissolved Solids (TDS) comprise of inorganic salts (bicarbonates, chlorides and sulphates of calcium, potassium, magnesium and sodium) and some amount of organic matter. The values ranged from 26.9 to

33.4 ppt. Electrical conductivity depends upon quantity of dissolved salts in the water and the measured values ranged from 35.57 to 51.11 ms.

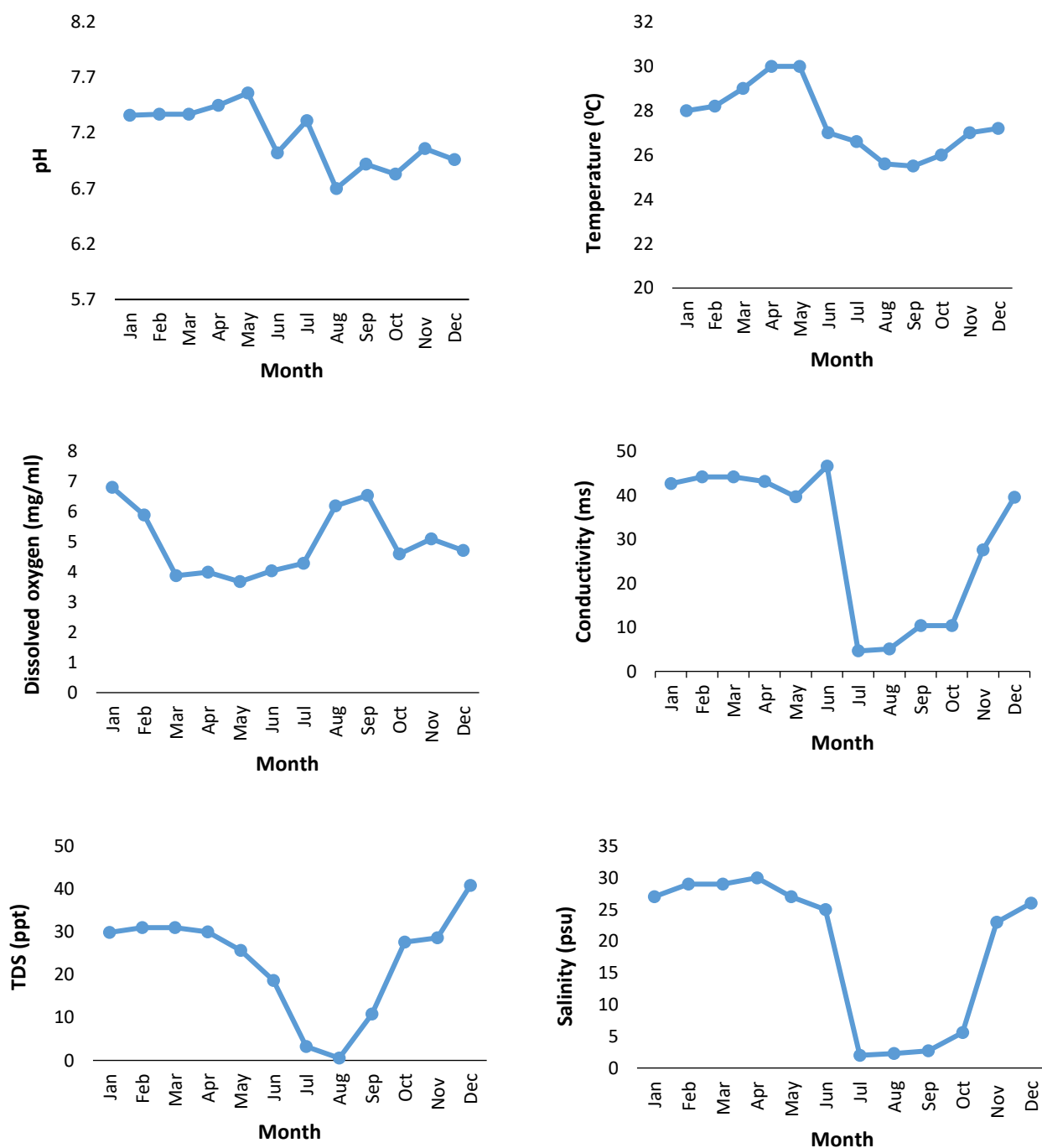


**Fig. 5.2:** Seasonal variations of physicochemical parameters recorded at the sampling site of *E. agallocha*

Overall, the pH, temperature, conductivity and salinity values were higher during the pre-monsoon season, decreasing during the active monsoon period and then further increasing during the post-monsoon season, while the DO was comparatively higher in the monsoon season.

### 5.3.2. Seasonal variation in protein content

Seasonal variations in proteinaceous content in leaves of *E. agallocha* was studied. The values were highest in the pre-monsoon season, dipping as the precipitation intensified (Fig. 5.3). Absorption at 220 nm (as an indicator of peptidic content) was found slightly higher during the pre-monsoon months.

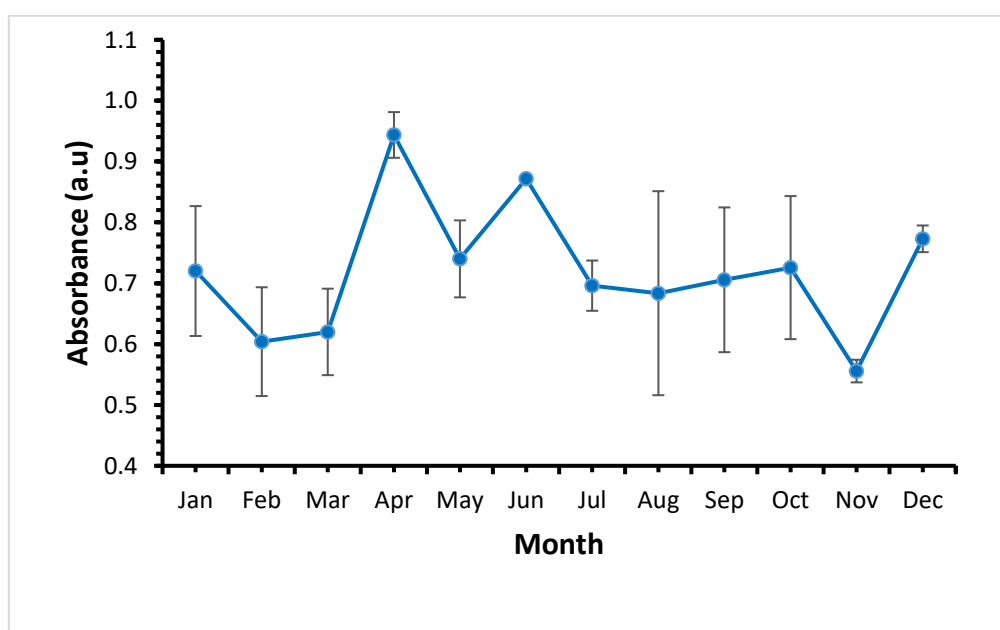


**Fig. 5.3:** Seasonal variation in protein content in the crude extract of *E. agallocha* leaf

The corresponding seasonal variations of protein content in the partially purified samples are depicted in Fig. 5.4. Except for the post-monsoon data, the variations were to a good extent in agreement with approximations derived through absorbance estimates at 220 nm.

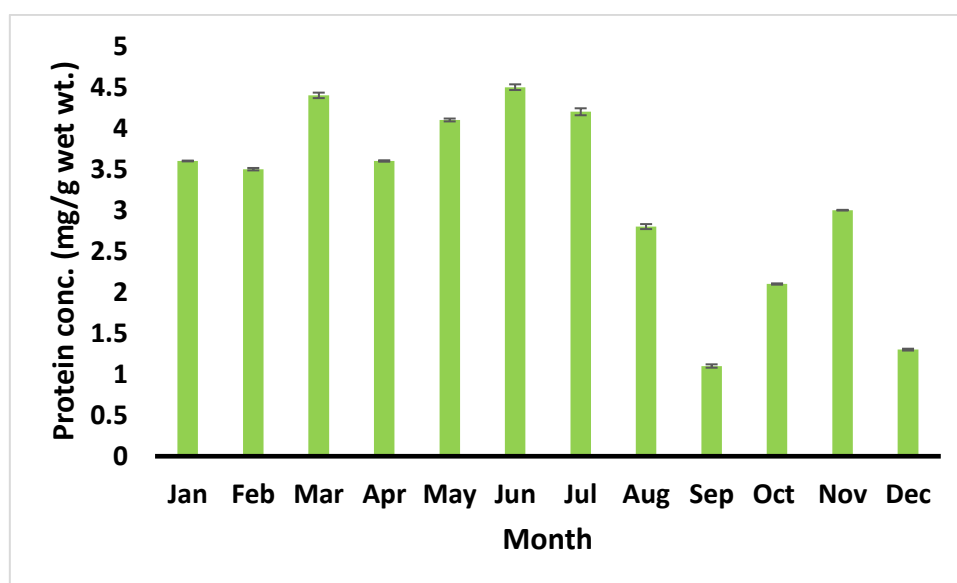


**Fig. 5.4:** Seasonal variation of proteinaceous content in partially purified extracts of *E. agallocha* leaf

### 5.3.3 Purification of the Cationic Peptide by Sephadex G-50 Column Chromatography

Out of all the plant extracts analysed using 15% SDS-PAGE, the *E. agallocha* leaf extract displayed a band which could signify a molecular weight of around 3 kDa or less (Fig. 2.16). This active sample was hence singled out for further purification through gel filtration chromatography.

As from Fig. 2.16, the *E. agallocha* leaf extract showed the presence of multiple peptides in a wide range of sizes, a preliminary standardization of the Sephadex G-50 column was done. This involved independently running pure preparations of standard small proteins/peptides, such as cytochrome C (molecular weight 12.3 kDa) and aprotinin (molecular weight 6.5 kDa) through the column (Fig 5.5 a &b).

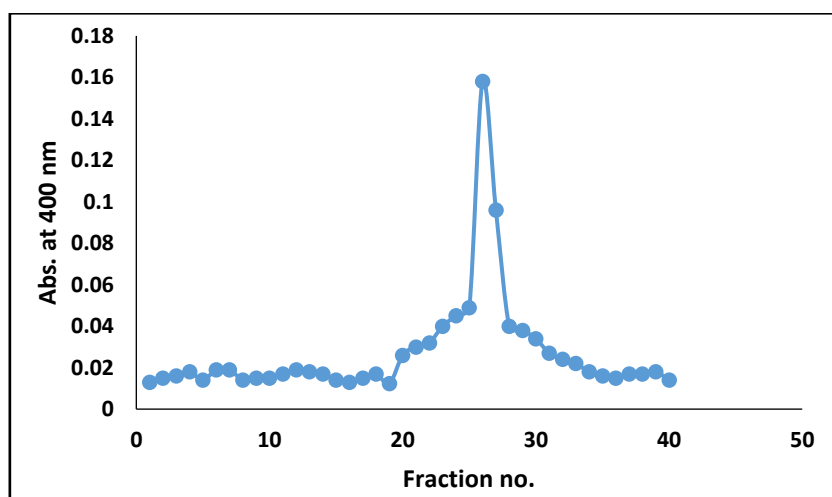


**Fig 5.5: a.** Elution profile of Cytochrome c

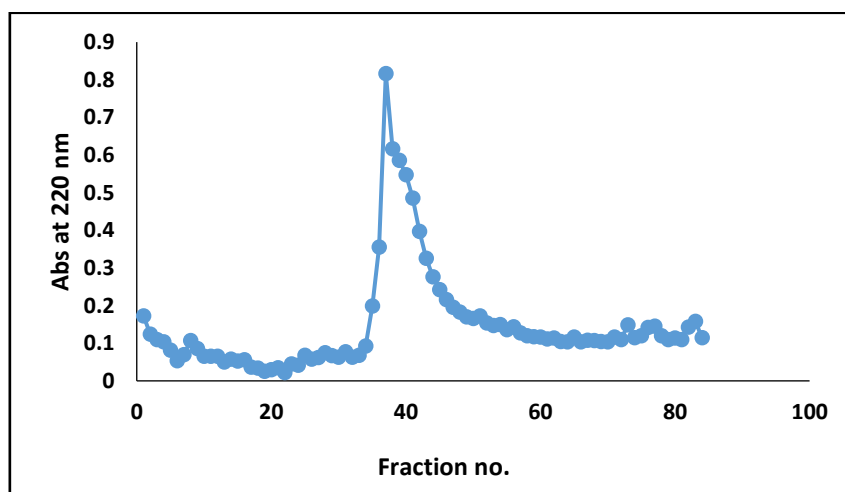


**Fig 5.5: b.** Elution profile of Aprotinin

The elution volumes for the standard markers used, *i.e.*, cytochrome *c* and aprotinin were 27 mL and 41 mL, respectively. Based on the SDS-PAGE profile of the crude *E. agallocha* leaf sample (Fig. 2.12), it could therefore be reasonably expected that our active peptide of interest would elute after aprotinin.

The partially purified sample of *E. agallocha* extract (90% ammonium sulphate precipitated pellet in suspension) was therefore subjected to gel filtration chromatography on the Sephadex G-50 column (Fig. 5.6). In view of the relatively low peptidic content of the loaded sample, absorbance at 220 nm rather than at 280 nm was chosen for plotting the elution profile, so as

to reduce the dependence on aromatic amino acid content in the already diluted eluting fractions.

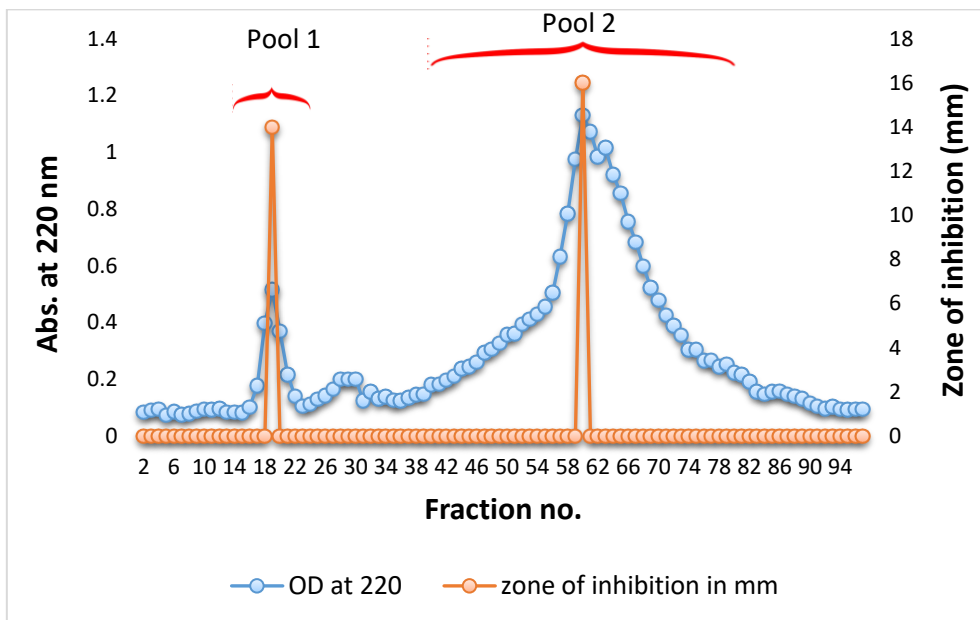


**Fig. 5.6:** Elution profile of 90% ammonium sulphate precipitated fraction of leaf extract of *Excoecaria agallocha*. Antimicrobial activity of pooled fractions was as tested against *S. aureus* by the agar well diffusion assay.

The single band observed on SDS-PAGE (Fig 2.12), would suggest a homogeneous population of proteins in the sample. However, upon further purification using gel filtration chromatography, two distinct peaks were obtained. Following this, fractions were also tested for antimicrobial activity.

**Table 5.1:** Antimicrobial activity in the eluted fractions

| Pooled Fractions              | Cumulative eluted volume (mL) | Zone of Inhibition (mm) |
|-------------------------------|-------------------------------|-------------------------|
| Pool 1<br>(fraction # 16-23 ) | 12                            | 14                      |
| Pool 2<br>(fraction # 45-80)  | 43                            | 16                      |

As shown in Table 3.1, the fractions from a single peak were combined and concentrated using a lyophilizer. The concentrated fractions of peak 1 were dissolved in 300  $\mu$ L of 10 mM sodium

acetate buffer, while peak 2 fractions were dissolved in 500  $\mu$ L of the buffer. They were then tested for antimicrobial activity against *S. aureus*, which had proven to be a sensitive test culture providing consistent data. The peak 2 concentrated pool, being of lower molecular weight, was selected for further studies on ACP activity.

#### 5.3.4 AU-PAGE analysis

The purified peptide was subjected to PAGE on an AU gel; a sample of pure lysozyme protein was run alongside as a positive control (Fig. 5.7).

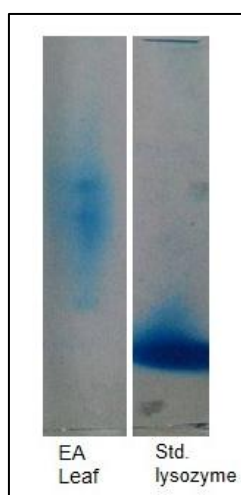


**Fig. 5.7** AU-PAGE profile of the purified *E. agallocha* leaf peptide

Cathodal migration of the purified *E. agallocha* leaf peptide served to confirm its cationic / basic nature.

### 5.3.5 SDS-PAGE profile of the purified peptide

The sample of concentrated fractions (peak 2) was electrophoresed on a 15% SDS-PAGE (Fig. 5.8).

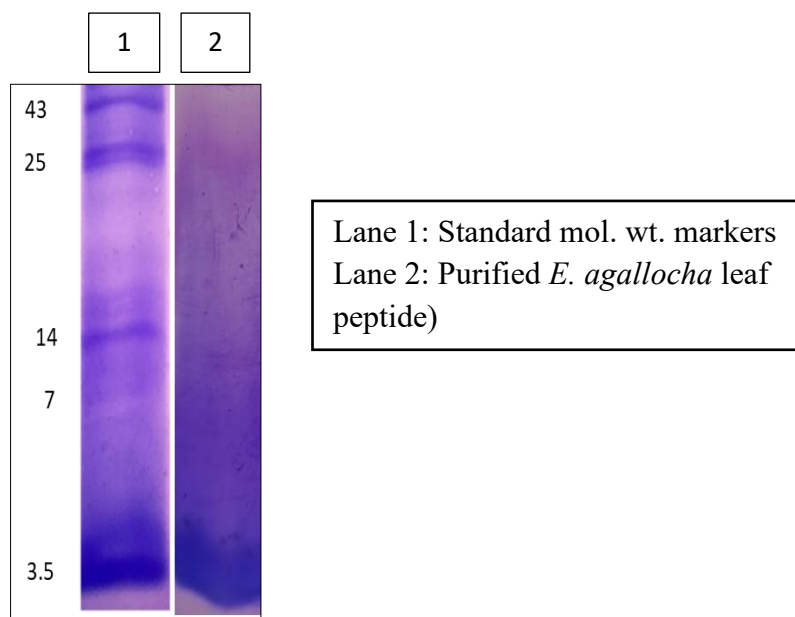


**Fig. 5.8** SDS-PAGE profile of the purified *E. agallocha* leaf peptide sample

The SDS-PAGE profile of the purified peptide too continues to be masked with a smear, despite repeated attempts, for probable reasons as discussed in Section 2.3.7. There was, however, evidence of a broad band that migrated even ahead of the lowest standard molecular weight marker of 3.5 kDa.

### 5.3.6 MALDI-TOF MS Analysis

The purified peptide corresponding to the low molecular weight active pool from gel filtration chromatography was subjected to MALDI-TOFF MS analysis (Fig. 5.9). Based on adjacent  $m/z$  values, the intact molecular mass of the *E. agallocha* leaf peptide was assessed as 1904.615 Da.

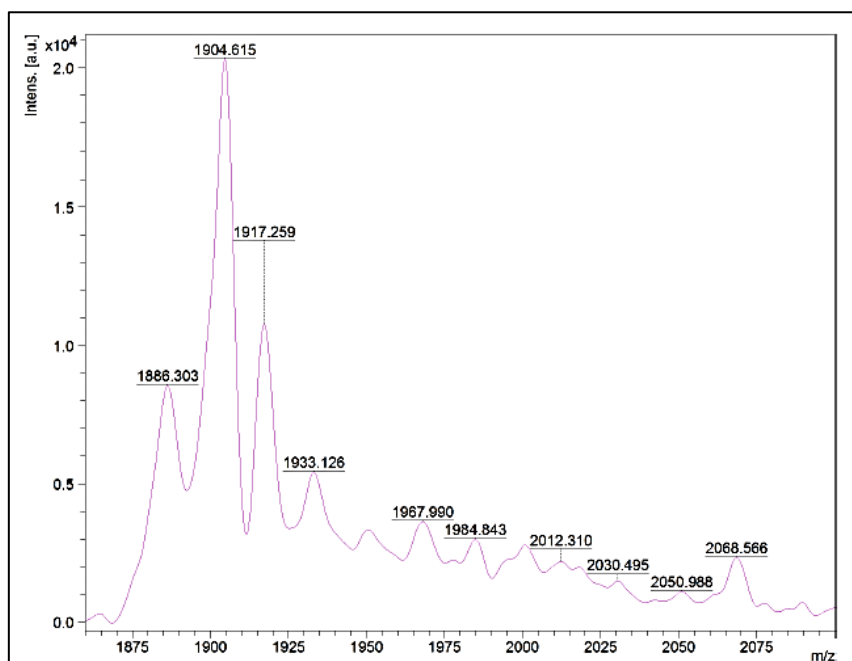


**Fig. 5.9** MALDI-TOF MS analysis of the purified *E. agallocha* leaf peptide

This Chapter has thus consolidated our attempts on purification of an antimicrobial cationic peptide from *E. agallocha*. The seasonal variation of proteinaceous content in the leaf extracts was studied in the crude as well as partially purified extracts. Highest protein concentration of  $4.6 \pm 0.033$  mg/g was recorded in the month of March (pre-monsoon season). The antimicrobially active peptide was further purified using Sephadex G-50 gel filtration chromatography. The basic nature of the peptide was ascertained by subjecting it to AU-PAGE, wherein cathodal migration of the single band was observed. Molecular weight of the peptide was confirmed by MALDI TOF analysis as 1904.615 Da.

**CHAPTER 6:**  
**Antimicrobial Cationic**  
**Peptides from Microbes**  
**from the Marine**  
**Environment**

## 6.1 Introduction

Marine waters have distinctive properties and may harbour biota native to coastal or open ocean regions. Coastal zones are generally rich in organic matter due to terrestrial runoff and are better endowed with primary productivity. These zones include unique niches such as coral reefs, mangroves and estuarine habitats, besides rocky as well as sandy beaches (Rämä et al., 2014; Zhou et al., 2016). Physicochemical parameters such as temperature, nutrient and salinity play an important role in determining the community composition and abundance of many organisms, which defines the functional characteristics of these ecosystems. Among the abiotic factors, salinity has been extensively quoted to be the major contributor influencing microbial community composition (Wasmund et al., 1999; Cognetti & Maltagliati, 2000; Kirchman et al., 2005; Kan et al., 2008; Ojaveer et al., 2010; Telesh & Khlebovich, 2010). There are many reports focusing either on determining the microbial diversity in marine environments (Miloslavich et al., 2010; Singh et al., 2012; Klindworth et al., 2014), or on the isolation of novel bioactive compounds from marine microorganisms (Blunt et al., 2014). However, only a few studies are available on bioactive metabolites obtained from different isolates in specific relation to their distinctive habitats. Due to unique habitat differences that display diversity in ambient environmental conditions (salinity, pressure, temperature, light, oxygen, pH and nutrient availability), organisms tend to develop various chemo-physiological processes for survival. Such metabolic processes of marine microbes encourage the synthesis of a range of bioactive molecules exhibiting activities such as antimicrobial, anticancer, antiviral and antifungal (Villa & Gerwick, 2010; Blunt et al., 2010). While their isolation was initially carried out from sites at random, there is now a growing recognition that the sampling location could play an important role in increasing the success rate of bioactivity discoveries (Burgess et al., 1999; Penesyan et al., 2009)

Marine microbes are known to produce various classes of metabolites such as polyketides, terpenes, peptides, and alkaloids which have been shown to exhibit antimicrobial and antiviral activity (Hughes & Fenical, 2010; Smith et al., 2010). As the conventional small molecules lose their potency quickly due to antimicrobial resistance (Gordana et al., 2012), one important class which has been drawing increasing attention is that of proteinaceous or peptidic antimicrobials from bacteria, also known as bacteriocins. It has the advantage of being minimally vulnerable to such resistance. Killing mechanisms adopted by bacteriocins are (a) formation of ion-permeable pores in the cytoplasmic membrane, (b) degradation of cellular

DNA, and (c) alteration in the structure of the 30S RNA subunit with subsequent degradation of rRNA or tRNA. These peptides have different structural diversity and properties and vary significantly in molecular weight, from small peptides (microcins, < 10 kDa) to large ones (colicins, > 80 kDa) (Andryukov et al., 2018).

Bacteriocins are majorly produced by Gram-positive bacteria and are divided into four classes. Class I includes lantibiotics, which are hydrophobic, thermostable, and pore-forming peptides with a low molecular weight (< 5 kDa). Class II peptides are also heat-stable and small in size (<10 kDa) and mainly induce destabilisation and permeabilization of the bacterial membranes or cause pore formation into the membrane. Class III bacteriocins are large peptides (> 30 kDa) and may be heat-labile, lytic or non-lytic. These bacteriocins have an antibacterial activity linked to enzymatic activity (such as endopeptidase), leading to the disruption of the bacterial cell wall. Class IV bacteriocins contain lipid or carbohydrate parts which disrupt the bacterial cell membrane. Due to their peculiar structure these peptides are sensitive to several enzymes (such as glycolytic or lipolytic enzymes) (Simons et al., 2020). Bacteriocins produced by Gram-negative bacteria are divided into four major classes: Colicins (10 kDa), Colicin-Like Bacteriocins (CLB), Phage Tail-Like Bacteriocins (PTLB), and Microcins (Riley & Wertz 2002).

Numerous microbial strains capable of producing protein/peptide antibiotics targeting prevalent bacterial pathogens have been discovered in different marine environments. For instance, Bacitracin, effective against many Gram-positive organisms, is derived from a marine bacterium, *Bacillus licheniformis* (McEvoy, 1993). Similarly, Enterocin, isolated from *Enterococcus faecium* found in the mangrove environment of the Vellar estuary on the east coast of India, exhibits antimicrobial activity against a wide range of Gram-positive and Gram-negative pathogens, including *S. aureus*, *B. subtilis*, *E. coli*, and *Vibrio sp.* (Annamalai et al., 2009). Pumiviticin, a bacteriocin derived from the marine bacterium *Bacillus pumilus* DR2 isolated from seawater samples from Ennore (Tamil Nadu, India), showed antimicrobial activity against *Lactobacillus jugurti*, *Lactobacillus casei*, *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Listeria monocytogenes* (Dasarathan et al., 2012). In addition to clinical applications, such peptides have a wide variety of applications such as in the food industry, animal husbandry, agriculture and aquaculture, and are also used to tackle biofilm and biofouling challenges (Huan et al., 2020; Patra et al., 2022). To the best of our knowledge, the present study is the first report on a possible role for ambient salinity in the antimicrobial

peptide production potential of bacteria sourced from varying salinity zones such as Divar mangrove area (fluctuating salinity zone), Miramar and Cakra beaches (less saline, near-estuarine regions) as well as Vagator and Anjuna beaches (coastal waters) of Goa, India. While some bacteria isolated from salinity zones have been reported to possess antibiotic activities, the work presented in this Chapter focuses majorly on proteinaceous antimicrobials (bacteriocins), as distinct from antibiotics as a class.

## 6.2 Materials and methods

### 6.2.1 Details of sampling sites

The coastline of Goa, India, is characterised by continuous stretches of sandy beaches, with several places intersected by estuarine river systems that empty into the Arabian Sea. For the isolation of marine bacteria, five of Goa's geographical areas of study were sampled (Fig. 6.1). The sites were selected for their salinity characteristics. The samples were collected in the month of January 2018 from estuarine waters near the Divar Mangrove (DM) area which is separated from the mainland by the Mandovi estuary, Miramar beach (ME) intertidal zones, the near-estuarine regions at Cakra (CE) beach, and the more typically saline waters of Vagator (VC) and Anjuna (AC) beaches which had minimal influence from river water discharge (Fig. 6.2).

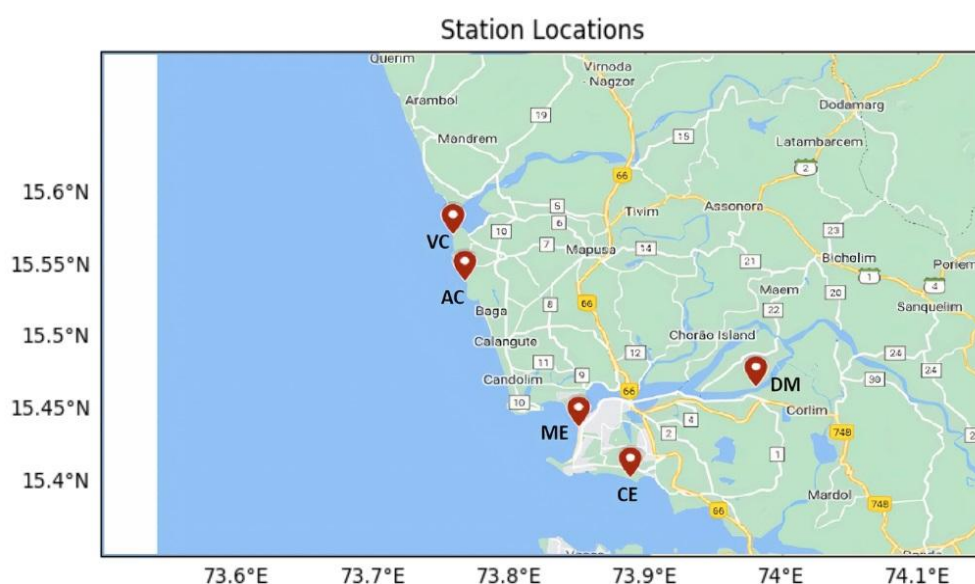


**Fig. 6.1:** Geographical location of study area and sampling sites: DM - Divar mangrove area, ME - Miramar beach, CE – Cakra beach, VC - Vagator beach, and AC - Anjuna beach.



**Divar mangrove area**



**Miramar beach**



**Cacra beach**



**Vagator beach**



**Anjuna beach**

**Fig. 6.2** The different sampling sites

### **6.2.2 Isolation and enumeration of bacteria**

Surface water sample from each sampling site (approx. 1 L) was collected in pre-sterilised screw-capped bottles, transferred on ice to the laboratory and processed within 24 h. The bacterial population in water samples was enumerated using the standard spread-plate technique (Pawsey 1974; Clesceri et al., 1998). One milliliter aliquots of water samples obtained from each location were serially diluted using sterile saline solution, and 0.1 mL of the diluted samples was spread onto Zobell Marine Agar (ZMA), Nutrient Agar (NA) and Seawater Agar (SWA) plates, which were then incubated at room temperature for 24 h, 48 h and 72 h, respectively, for enumeration of Total Viable (culturable) bacterial Counts (TVC). The ZMA and NA were procured from HiMedia, while for the SWA preparation, 3 g agar powder (HiMedia) was added to 100 mL aged and filtered seawater and autoclaved.

### **6.2.3 Growth and maintenance of the bacterial strains isolated**

The predominant bacterial isolates were singled out based on morphological characteristics and designated with codes DPU, MPU, CPU, VPU, APU corresponding to Divar, Miramar, Cacara, Vagator and Anjuna, respectively. The pure bacterial cultures were subcultured and maintained on their respective medium of isolation viz., ZMA or NA. All the media components and chemicals used were of analytical grade.

### **6.2.4 Cell morphology and Gram character**

Morphological characterization of the selected bacterial isolates was done with reference to colony morphology, pigmentation and opacity. The bacterial cultures were Gram stained according to Gram (1884) and morphologically characterized as cocci (spherical cells) or rods (cylindrical cells) as observed under the microscope.

### **6.2.5 Extraction and partial purification of proteinaceous exudates from colonies in culture**

Overnight grown cultures were centrifuged at 6,000 rpm for 10 min at 4°C to obtain the cell-free supernatants (CFS), which were then subjected to precipitation at 4°C with ammonium sulphate at 90% saturation in order to enrich the content of proteinaceous molecules. After

centrifugation at 10,000 rpm and 4°C for 30 min, the pellet obtained was suspended in 10 mM sodium acetate buffer (pH 5.6).

#### **6.2.6 Quantification of total protein by Bradford's method**

Ammonium sulphate precipitated samples suspended in 10 mM sodium acetate buffer were analysed for their protein content by the Bradford's method (Bradford, 1976), using Bovine Serum Albumin (BSA) as standard.

#### **6.2.7 Test pathogens**

Pure cultures of *Salmonella typhimurium* (MTCC 98), *Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (MTCC 443), *Candida albicans* (MTCC 227) and *Aeromonas hydrophila* (MTCC 1739) were used for the study.

#### **6.2.8. Antimicrobial assay (well diffusion assay)**

The test pathogen inocula, prepared by transferring a loopful of culture into 100 mL NB and incubating for 24 h on an orbital shaker, were swabbed onto NA plates. The isolates were allowed to grow in ZMB for 24 h on a rotary shaker. The broth culture was then centrifuged at 6,000 rpm for 10 min at 4 °C to obtain the Cell-Free Supernatant (CFS). The CFS was partially purified by 90% ammonium sulphate precipitation (as described in Section 6.2.5). Wells (6 mm) were punched onto the above NA plates using a borer, and 30 µL of CFS (crude or partially purified) was added into the wells. Antimicrobial activity was evaluated by the agar-well diffusion method as per Shanmugaraju et al. (2012), zones of inhibition being measured after incubation for 24 h at 37 °C.

#### **6.2.9. Analytical and purification techniques**

The procedures for SDS- and AU- PAGE, gel filtration and MALDI-TOF MS were as previously described under Section 5.2.

#### **6.2.10. Biochemical characterisation of the bacterial isolates**

A bacterial isolate (VPU003) was subjected to a series of physiological and biochemical identification tests. The tests were performed following the Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974).

#### **6.2.10.1 Spore staining**

The culture was stained using the Schaeffer-Fulton method (1933). The position of the spores was observed and recorded as central, sub-terminal, or terminal.

#### **6.2.10.2 Motility test**

The motility of the cultures was determined by the hanging drop method (Collins et al, 1984).

#### **6.2.10.3 Catalase test**

Bacterial cultures were grown on NA plates for 24 h. A loopful of the culture was placed on a glass slide, and 3% hydrogen peroxide (2 drops) was added. Effervescence production was observed as an indication of a positive catalase test (Vasanthakumari, 2009).

#### **6.2.10.4 Oxidase test**

Oxidase test was carried out by oxidase discs (HiMedia DD018). The bacterial culture (10  $\mu$ L) was inoculated on a disc and the colour change was observed. Microorganisms were documented as oxidase positive when the disc colour changed from white to purple within 5 to 10 s. Change beyond 10 seconds or no colour change was considered oxidase negative.

#### **6.2.10.5 Citrate utilization test**

Simmon citrate agar (Himedia) slants were prepared. Bacterial culture was inoculated onto the slants and the slants were incubated at 37°C for 48 h. After incubation, a culture was identified as citrate-positive when growth was observed on the surface of the Simmons citrate agar slant, accompanied by a deep Prussian blue coloration of the medium (Aditi et al., 2017).

#### **6.2.10. 6. Starch hydrolysis**

The starch hydrolysis test was conducted to determine if the microorganism could utilize starch as a source of carbon and energy for growth. Starch utilization is facilitated by the enzyme  $\alpha$ -amylase. A sterile plate of starch agar was inoculated and then incubated at 35-37°C for 48 hours. After incubation, iodine reagent was added to flood the surface of the plate. Areas where bacterial growth has utilized starch show clear halos amid the dark plate, indicating a positive result for  $\alpha$ -amylase activity, or starch hydrolysis (Chakraborty & Roy, 2011).

#### **6.2.11. 16S rRNA sequencing and BLAST search analysis**

The methodology began with the isolation of genomic DNA, followed by amplification of the 16S rRNA gene using Polymerase Chain Reaction (PCR). Subsequently, the amplified PCR product underwent DNA sequencing, which was performed by Eurofins Genomics India Pvt Ltd. The raw sequences were assembled into contigs using BioEdit software, version 7.2.5, and then aligned. These contigs were compared with the GenBank database using NCBI-BLAST for identification. Further confirmation was obtained through 16S rRNA sequencing, followed by a BLAST search as outlined by Altschul et al. (1990).

### **6.3. Results and Discussion**

#### **6.3.1 Physicochemical Analysis**

The physicochemical parameters recorded at each sampling site are summarised in Table 6.1. The pH of the sampled water, which represents its degree of ionisation, ranged from 7.36 to 8.5. The temperature of the surface water at these sites varied from 28 to 29.8 °C. Salinity variations were from 26.9 to 33.4 psu; Vagator (VC) and Anjuna (AC) beach sites exhibited the highest salinity (33.04 to 33.42 psu), as expected. The Miramar (ME) and Cacra (CE) sites were comparatively less saline, being influenced by the input of freshwater from the Mandovi and Zuari rivers. Salinity of estuarine waters around the Divar Mangrove area (DM) was 26.9 psu. Notwithstanding interannual variabilities and seasonal influences, these values are in agreement with the data from earlier reports (Alkawri & Ramaiah, 2011; Poharkar et al., 2016), where salinity values ranging from 31 to 35 psu were reported in coastal beach waters and from 24 to 27 psu in mangrove areas of Goa.

**Table 6.1** Physicochemical parameters at the sampling location **DM**- Near Divar mangrove area; **ME**- Miramar beach; **CE**- Cacara beach; **VC**- Vagator beach; **AC**- Anjuna beach.

| <b>Location:<br/>Station (&amp;<br/>code)</b> | <b>pH</b> | <b>Temp.<br/>(°C)</b> | <b>DO<br/>(mg/L)</b> | <b>Salinity<br/>(psu)</b> | <b>Conductivity<br/>(ms)</b> | <b>TDS<br/>(ppt)</b> |
|-----------------------------------------------|-----------|-----------------------|----------------------|---------------------------|------------------------------|----------------------|
| <b>Divar<br/>(DM)</b>                         | 7.36±0.07 | 28±0                  | 6.81±0.37            | 26.95±0.18                | 43.35±0.93                   | 28.86±2.81           |
| <b>Miramar<br/>beach (ME)</b>                 | 8.31±0    | 29.8±0                | 6.01±0               | 31.96±0.15                | 48.95±0.21                   | 34.29±0.26           |
| <b>Cacara<br/>beach (CE)</b>                  | 8.35±0.01 | 28±0                  | 5.97±0.32            | 32.14±0.08                | 49.18±0.12                   | 34.44±0.08           |
| <b>Vagator<br/>beach (VC)</b>                 | 8.41±0.01 | 29.8±0                | 6.68±0.11            | 33.04±0.21                | 50.42±0.28                   | 35.31±0.20           |
| <b>Anjuna<br/>beach (AC)</b>                  | 8.50±0    | 29.8±0                | 6.70±0.12            | 33.42±0.18                | 50.93±0.25                   | 35.63±0.12           |

In our study, Miramar and Cacara beach areas have been categorised as representative of ‘near-estuarine’ regions and the mangrove habitat as ‘estuarine’ environment, while the Vagator and Anjuna sites better conformed to ‘coastal ocean’ environments. All these typically exemplify the dynamic environmental conditions that characterise the estuarine and marine waters around Goa. Dissolved oxygen (DO), being one of the limiting factors for the growth and functioning of marine microorganisms, becomes a very useful parameter to gauge water quality. The observed DO values ranged between 5.97 and 6.7 mg/L. Total Dissolved Solids (TDS) comprise of inorganic salts (bicarbonates, chlorides and sulphates of calcium, potassium, magnesium and sodium) and some amount of organic matter. The measured TDS values ranged from 28.8 to 35.6 ppt. Electrical conductivity, which depends upon the quantity of dissolved salts in the water, ranged from 43.3 to 50.9 ms. Maximum and minimum values for pH, DO, salinity, conductivity and TDS were recorded at AC and DM sites, respectively, in the month of January. Although, in general, abiotic parameters such as salinity, nutrient concentration and organic matter would all influence the composition of natural microbiota (Carlson et al., 2002; Schloss et al., 2004), salinity, in particular, influences the osmoregulatory functions of organisms and would therefore be a dominant factor in shaping microbial diversity and population in the marine waters (Oren, 2011). Previous studies have shown the influence of salinity on microbial community composition (Silveira et al., 2011). There are, however, very few reports on bacterial abundance over a range of salinities especially in relation to their bioactivities. The focus of our study was hence directed to evaluate the influence of salinity

ranges of estuarine and marine habitats on the total viable bacterial count and the production of peptidic antimicrobials.

### **6.3.2 Total viable count (TVC)**

Bacterial abundance in the estuarine and coastal waters of Goa was gauged by the TVC, which would provide an estimate of their culturable load in these waters (Fig. 6.3). The values were highest at location DM, as  $660 \times 10^3$  cfu/mL and  $210 \times 10^2$  cfu/mL, respectively, upon incubation on ZMA and NA media for 24 h. In all cases, only a slight increase in TVC was observed after 48 h of incubation, which remained unchanged even at 72 h, indicating that the exponential phase of growth was complete even at 24 h of incubation. No colonies were observed on SWA at 24 h, and even after 48 h of incubation, the highest TVC recorded was only  $5 \times 10^2$  cfu/mL at VC. Although a continued increase to  $9.2 \times 10^2$  cfu/mL was recorded in SWA medium at 72 h, this was markedly lower than the values in richer media such as ZMA and NA. This slow growth may be attributed to autoclaving of the medium, which could have affected the natural nutrient availability, as against filtration, which might have been a better choice. The more significant values of TVC as recorded for growth in ZMA and NA media at 48 h were assessed in relation to ambient salinity at the sampling sites. It was observed that the values were higher in samples from a lower salinity environment (mangrove area) and decreased with increasing ambient salinity (Fig. 6.3, a & b). Other parameters at the sampling sites besides salinity might also have contributed to the TVC differences, considering the trend of variations of pH, temperature, conductivity and TDS (Table 6.1).

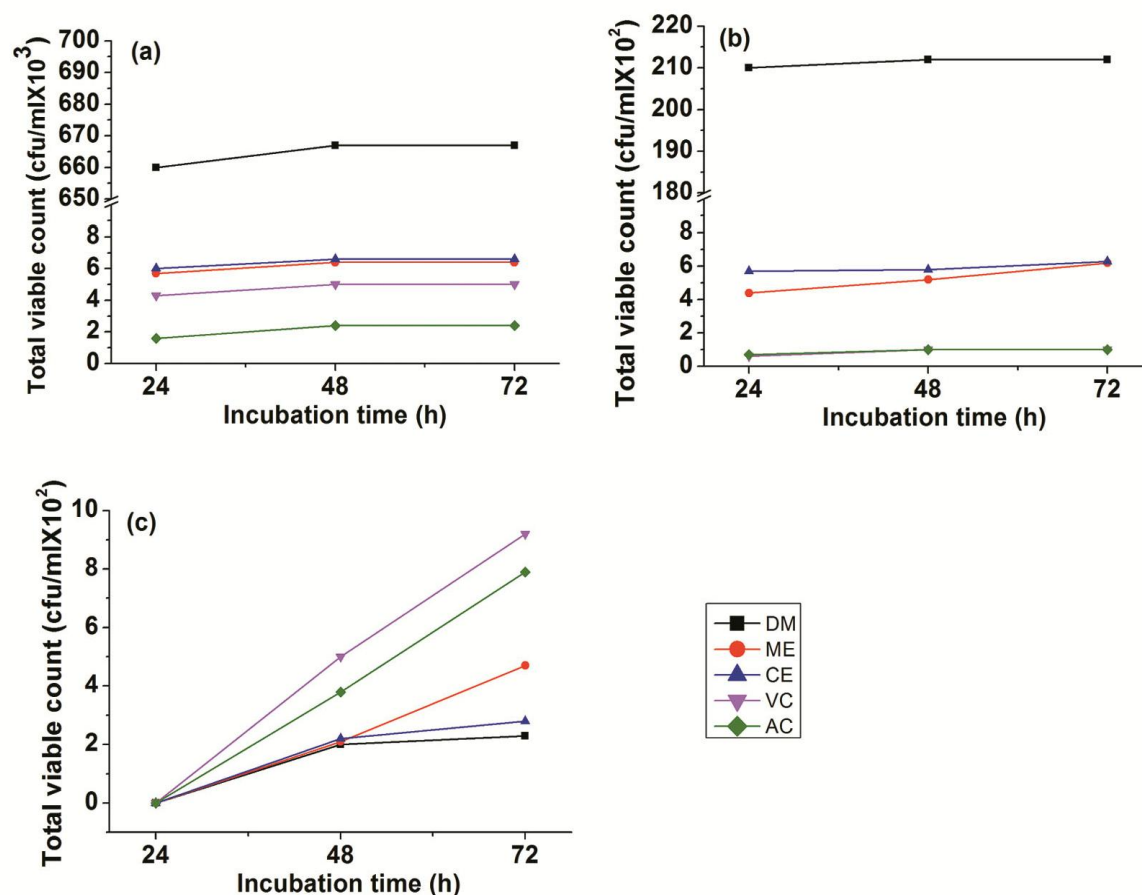


**Fig. 6.3** Total viable count at the sampling locations of various salinities on (a) Zobell marine agar, (b) Nutrient agar, and (c) Natural seawater agar. DM - Divar mangrove area; ME - Miramar beach; CE - Cacara beach; VC - Vagator beach; and AC - Anjuna beach

### 6.3.3 Cell morphology and Gram characteristic

A total of 82 unique bacterial isolates were selected based on differences in colony morphology and Gram staining characteristics. A representation of the distinct colony types obtained is depicted in Fig. 6.4, while detailed colony characteristics of each isolate are presented in Tables 6.2 to 6.6.

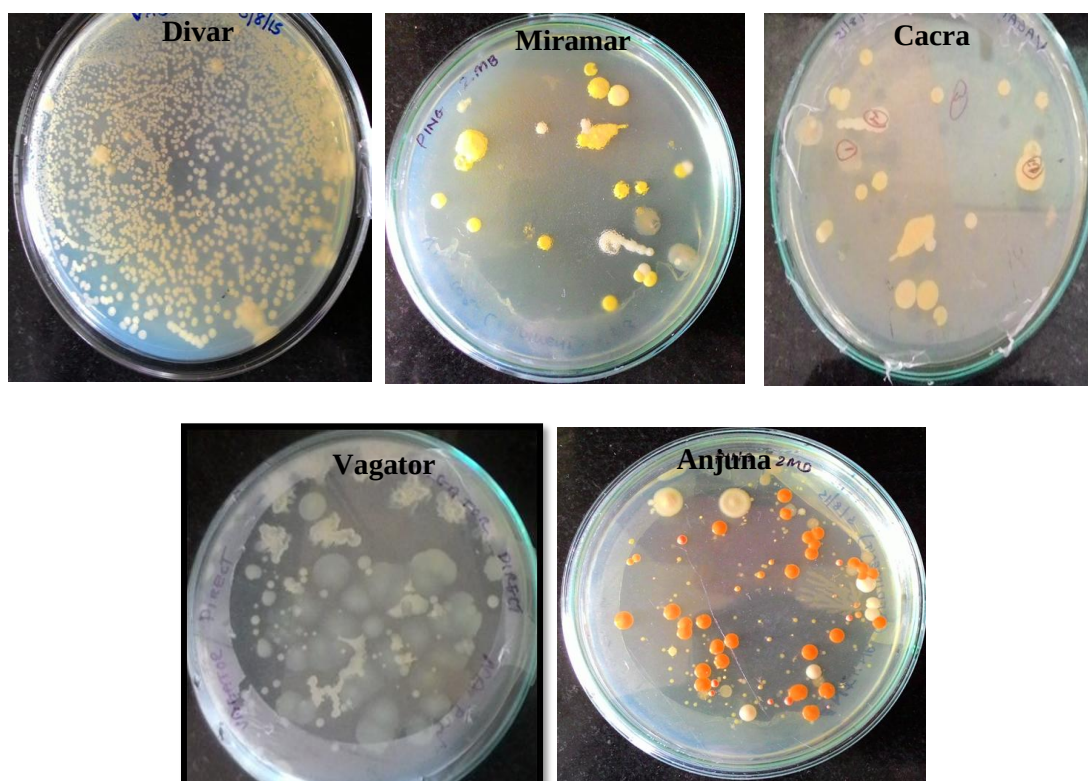


**Fig. 6.4:** Bacterial colonies isolated from various locations, when cultured on Zobell marine agar.

**Table 6.2:** Colony characteristics of isolates from Divar mangrove region

| Culture | Elevation | Margin    | Configuration               | Opacity     | Pigmentation    |
|---------|-----------|-----------|-----------------------------|-------------|-----------------|
| DPU001  | Umbonate  | Smooth    | Round with raised margin    | Opaque      | Yellow          |
| DPU002  | Umbonate  | Smooth    | Round with raised margin    | Opaque      | Light orange    |
| DPU003  | Flat      | Smooth    | Round                       | Translucent | No pigmentation |
| DPU004  | Flat      | Smooth    | Round                       | Opaque      | Yellow          |
| DPU005  | Flat      | Irregular | Irregular Spreading         | Opaque      | No pigmentation |
| DPU006  | Flat      | Smooth    | Round With Scalloped Margin | Transparent | No pigmentation |

|        |          |             |                             |             |                     |
|--------|----------|-------------|-----------------------------|-------------|---------------------|
| DPU007 | Flat     | Smooth      | Round                       | Translucent | No pigmentation     |
| DPU008 | Flat     | Smooth      | Round                       | Translucent | Orange              |
| DPU009 | Flat     | Lobate      | Complex                     | Transparent | No pigmentation     |
| DPU010 | Flat     | Thread like | Irregular Spreading         | Translucent | No pigmentation     |
| DPU011 | Unbonate | Smooth      | Round                       | Opaque      | Pink                |
| DPU012 | Flat     | Smooth      | Concentric                  | Opaque      | No pigmentation     |
| DPU013 | Convex   | Smooth      | Round                       | Opaque      | Pink                |
| DPU014 | Convex   | Smooth      | Round                       | Opaque      | Yellow              |
| DPU015 | Raised   | Lobate      | Irregular Spreading         | Opaque      | No pigmentation     |
| DPU016 | Raised   | Smooth      | Round                       | Opaque      | No pigmentation     |
| DPU017 | Convex   | Smooth      | Round with scalloped margin | Opaque      | Brick red           |
| DPU018 | Raised   | Wavy        | Irregular Spreading         | Opaque      | Brownish            |
| DPU019 | Umbonate | Smooth      | Round                       | Opaque      | Pink                |
| DPU020 | Flat     | Wavy        | Round                       | Opaque      | No pigmentation     |
| DPU021 | Flat     | Lobate      | Fliliform                   | Translucent | No pigmentation     |
| DPU022 | Flat     | Smooth      | Round                       | Transparent | No pigmentation     |
| DPU023 | Raised   | Smooth      | Round                       | Opaque      | No pigmentation     |
| DPU024 | Umbonate | Smooth      | Round                       | Translucent | Brown (Central Dot) |
| DPU025 | Convex   | Smooth      | Round                       | Opaque      | Pink                |
| DPU026 | Convex   | Smooth      | Round                       | Opaque      | No Pigmentation     |
| DPU027 | Convex   | Smooth      | Round                       | Translucent | Faint pink          |
| DPU028 | Convex   | Smooth      | Round                       | Opaque      | No pigmentation     |
| DPU029 | Convex   | Smooth      | Round                       | Opaque      | No Pigmentation     |

|        |          |        |       |             |                 |
|--------|----------|--------|-------|-------------|-----------------|
| DPU030 | Convex   | Smooth | Round | Opaque      | No pigmentation |
| DPU031 | Flat     | Smooth | Round | Transparent | No pigmentation |
| DPU032 | Flat     | Smooth | Round | Opaque      | Bright yellow   |
| DPU033 | Flat     | Smooth | Round | Opaque      | Pink            |
| DPU034 | Flat     | Smooth | Round | Opaque      | Orange          |
| DPU035 | Umbonate | Smooth | Round | Opaque      | Orange          |
| DPU036 | Flat     | Smooth | Round | Translucent | No pigmentation |
| DPU037 | Flat     | Smooth | Round | Opaque      | Reddish pink    |
| DPU038 | Umbonate | Smooth | Round | Translucent | Red             |
| DPU039 | Flat     | Smooth | Round | Translucent | No pigmentation |
| DPU040 | Flat     | Smooth | Round | Translucent | No pigmentation |

**Table 6.3:** Colony characteristics of isolates from Miramar

| <b>Culture</b> | <b>Elevation</b> | <b>Margin</b> | <b>Configuration</b> | <b>Opacity</b> | <b>Pigmentation</b> |
|----------------|------------------|---------------|----------------------|----------------|---------------------|
| MPU001         | Raised           | Smooth        | Round                | Translucent    | No pigmentation     |
| MPU002         | Raised           | Smooth        | Round                | Opaque         | Light pink          |
| MPU003         | Raised           | Smooth        | Round                | Transparent    | No pigmentation     |
| MPU004         | Convex           | Smooth        | Round                | Opaque         | Orange              |
| MPU005         | Punctate         | Smooth        | Round                | Opaque         | Yellow              |
| MPU006         | Raised           | Irregular     | Irregular spreading  | Opaque         | No pigmentation     |
| MPU007         | Raised           | Smooth        | Round                | Translucent    | No pigmentation     |
| MPU008         | Flat             | Lobate        | Concentric           | Opaque         | No pigmentation     |
| MPU009         | Raised           | Smooth        | Round                | Opaque         | Yellow              |
| MPU010         | Flat             | Wavy          | Irregular spreading  | Translucent    | No pigmentation     |

|        |          |        |                     |             |                 |
|--------|----------|--------|---------------------|-------------|-----------------|
| MPU011 | Convex   | Smooth | Round               | Transparent | No pigmentation |
| MPU012 | Flat     | Smooth | Round               | Translucent | No pigmentation |
| MPU013 | Convex   | Smooth | Round               | Opaque      | Yellow          |
| MPU014 | Flat     | Wavy   | Irregular spreading | Transparent | No pigmentation |
| MPU015 | Flat     | Wavy   | Irregular spreading | Opaque      | No pigmentation |
| MPU016 | Umbonate | Smooth | Round               | Translucent | No pigmentation |
| MPU017 | Umbonate | Smooth | wrinkled            | Opaque      | No pigmentation |
| MPU018 | Convex   | smooth | Round               | Opaque      | No pigmentation |

**Table 6.4:** Colony characteristics of isolates from Cacra

| <b>Culture</b> | <b>Elevation</b> | <b>Margin</b> | <b>Configuration</b> | <b>Opacity</b> | <b>Pigmentation</b> |
|----------------|------------------|---------------|----------------------|----------------|---------------------|
| CPU001         | Raised           | Smooth        | Round                | Opaque         | No pigmentation     |
| CPU002         | Convex           | Smooth        | Round                | Opaque         | Pink                |
| CPU003         | Raised           | Smooth        | Round                | Translucent    | No pigmentation     |
| CPU004         | Convex           | Wavy          | Round                | Opaque         | Yellow              |
| CPU005         | Umbonate         | Wavy          | Concentric           | Opaque         | No pigmentation     |
| CPU006         | Flat             | Smooth        | Round                | Opaque         | No pigmentation     |
| CPU007         | Flat             | Wavy          | Filliform            | Translucent    | No pigmentation     |
| CPU008         | Convex           | Smooth        | Round                | Translucent    | No pigmentation     |
| CPU009         | Raised           | Smooth        | Round                | Opaque         | Pink                |
| CPU010         | Raised           | Smooth        | Round                | Opaque         | Yellow              |
| CPU011         | Raised           | Smooth        | Round                | Opaque         | No pigmentation     |
| CPU012         | Convex           | Smooth        | Round                | Opaque         | No pigmentation     |

|        |        |           |                     |        |                 |
|--------|--------|-----------|---------------------|--------|-----------------|
| CPU013 | Flat   | Irregular | Irregular Spreading | Opaque | No pigmentation |
| CPU014 | Raised | Smooth    | Round               | Opaque | No pigmentation |
| CPU015 | Raised | Smooth    | Round               | Opaque | Light yellow    |

**Table 6.5:** Colony characteristics of isolates from Vagator

| <b>Culture</b> | <b>Elevation</b> | <b>Margin</b> | <b>Configuration</b> | <b>Opacity</b> | <b>Pigmentation</b> |
|----------------|------------------|---------------|----------------------|----------------|---------------------|
| VPU001         | Raised           | Smooth        | Round                | Opaque         | No pigmentation     |
| VPU002         | Raised           | Smooth        | Round                | Opaque         | Yellow              |
| VPU003         | Raised           | Smooth        | Round                | Opaque         | Brownish            |
| VPU004         | Flat             | Smooth        | Round                | Opaque         | No pigmentation     |
| VPU005         | Raised           | Smooth        | Round                | Opaque         | No pigmentation     |
| VPU006         | Umbonate         | Irregular     | Complex              | Opaque         | Pink                |
| VPU007         | Convex           | Smooth        | Round                | Transparent    | No pigmentation     |
| VPU008         | Raised           | Smooth        | Round                | Opaque         | No pigmentation     |

**Table 6.6** Colony characteristics of isolates from Anjuna

| <b>Culture</b> | <b>Elevation</b> | <b>Margin</b> | <b>Configuration</b> | <b>Opacity</b> | <b>Pigmentation</b> |
|----------------|------------------|---------------|----------------------|----------------|---------------------|
| APU001         | Raised           | Smooth        | Round                | Opaque         | Pink                |
| APU002         | Umbonate         | Smooth        | L Form               | Translucent    | No pigmentation     |
| APU003         | Raised           | Smooth        | Round                | Opaque         | Dark pink           |
| APU004         | Raised           | Smooth        | Round                | Opaque         | No pigmentation     |
| APU005         | Raised           | Wavy          | Complex              | Translucent    | No pigmentation     |
| APU006         | Raised           | Smooth        | Round                | Translucent    | Orangish            |

|        |          |           |                          |             |                 |
|--------|----------|-----------|--------------------------|-------------|-----------------|
| APU007 | Convex   | Smooth    | Round                    | Transparent | No pigmentation |
| APU008 | Convex   | Smooth    | Round                    | Transparent | No pigmentation |
| APU009 | Flat     | Wavy      | Concentric               | Opaque      | No pigmentation |
| APU010 | Umbonate | Irregular | Complex                  | Opaque      | Brown           |
| APU011 | Convex   | Smooth    | Round                    | Opaque      | Light orange    |
| APU012 | Umbonate | Smooth    | Round                    | Opaque      | No pigmentation |
| APU013 | Convex   | Smooth    | Round with raised margin | Opaque      | No pigmentation |
| APU014 | Convex   | Irregular | Irregular                | Translucent | No pigmentation |
| APU015 | Umbonate | Smooth    | Round with raised margin | Opaque      | No pigmentation |
| APU016 | Convex   | Smooth    | Round                    | Opaque      | Red             |
| APU017 | Convex   | Smooth    | Round                    | Opaque      | Orangish        |
| APU018 | Convex   | Smooth    | Round                    | Transparent | No pigmentation |
| APU019 | Flat     | Lobate    | Irregular and spreading  | Opaque      | No pigmentation |
| APU020 | Raised   | Wavy      | Round with raised margin | Translucent | No pigmentation |
| APU021 | Raised   | Wavy      | Round with raised margin | Translucent | No pigmentation |

Estuarine samples showed higher diversity of colonies than marine samples. The majority of isolates (71–77 %) obtained from the five sampling sites were Gram-negative (Fig. 6.5b, hatched bars, *cf.* Fig. 6.5a, hatched bars), in concurrence with a recent article that cites Gram-positive bacteria contributing to 14% of the total bacterial count in seawater (Lima-Mendez et al., 2015).

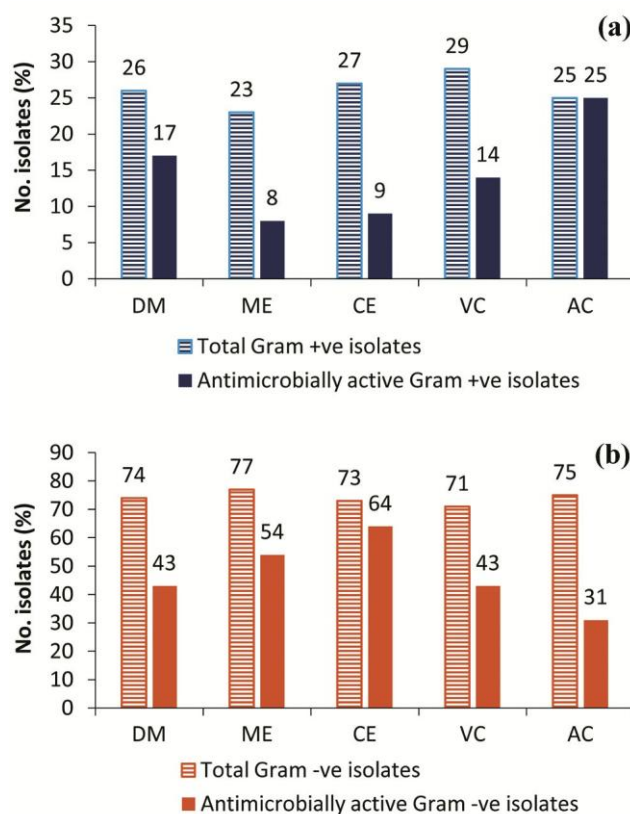


**Fig. 6.5** Percentage of (a) Gram-positive, and (b) Gram-negative isolates amongst the culturable isolates obtained during the sampling. The solid bars represent the relative percentage of Gram-positive and Gram-negative isolates that showed antimicrobial activity. The respective percent values (rounded up) are indicated above each bar.

Among the Gram-positive bacteria, a higher percentage of isolates obtained from estuarine and coastal waters were antimicrobially active (solid bars, Fig. 6.5a), while among the Gram-negative isolates, those exuding antimicrobially active compounds were predominant in the near-estuarine regions (solid bars, Fig. 6.5b). Unlike those introduced by runoff from terrestrial ecosystems, indigenous producers of antimicrobial peptides from the marine environment were mainly strains of Gram-positive marine bacteria, despite the relative abundance of Gram-negative species (Andryukov et al., 2019). Although Gram-positive bacteria are basically abundant in soils and could get introduced into near-shore marine environments, their survival rates are low in competition with the indigenous better-adapted Gram-negative species (Jensen & Fenical, 1995). Besides, on account of their membrane porosity characteristics, Gram-positive organisms would be more sensitive to deleterious compounds in the vicinity. Such compounds that may be effectively transported across the thick peptidoglycan layer of Gram-positive bacteria lack, in many cases, the appropriate chemical properties to cross the vastly

different glycolipid layer and membranes characteristic of Gram-negative bacteria (Austin, 1989; Hughes & Fenical, 2010).

### 6.3.4 Antimicrobial activity in proteinaceous molecules from the bacterial isolates

The search for proteinaceous or peptide antimicrobials from marine microbes as an alternative to conventional antibiotics being the focus of the current study, bioprospecting for antimicrobial activity was performed using the well diffusion assay (Fig. 6.6). Partial purification by ammonium sulphate precipitation would serve to extract proteinaceous material more specifically, besides also concentrating such active molecules. It is interesting to note that taken as a whole, about 61 % of the culturable isolates had antimicrobial activity associated with proteinaceous material (Table 6.7). The isolates displayed broad-spectrum inhibitory effects on the five test pathogens used, and the antimicrobial activity in partially purified CFS samples was higher than in the crude CFS preparations. The significantly enhanced activity upon partial purification is not surprising, considering that antimicrobial peptides and proteins are generally not generated in high amounts by the producer strain (Pingitore et al., 2007). In fact, the proteinaceous content in these active preparations ranged from 0.7 to 2 mg/mL (Table 6.7).

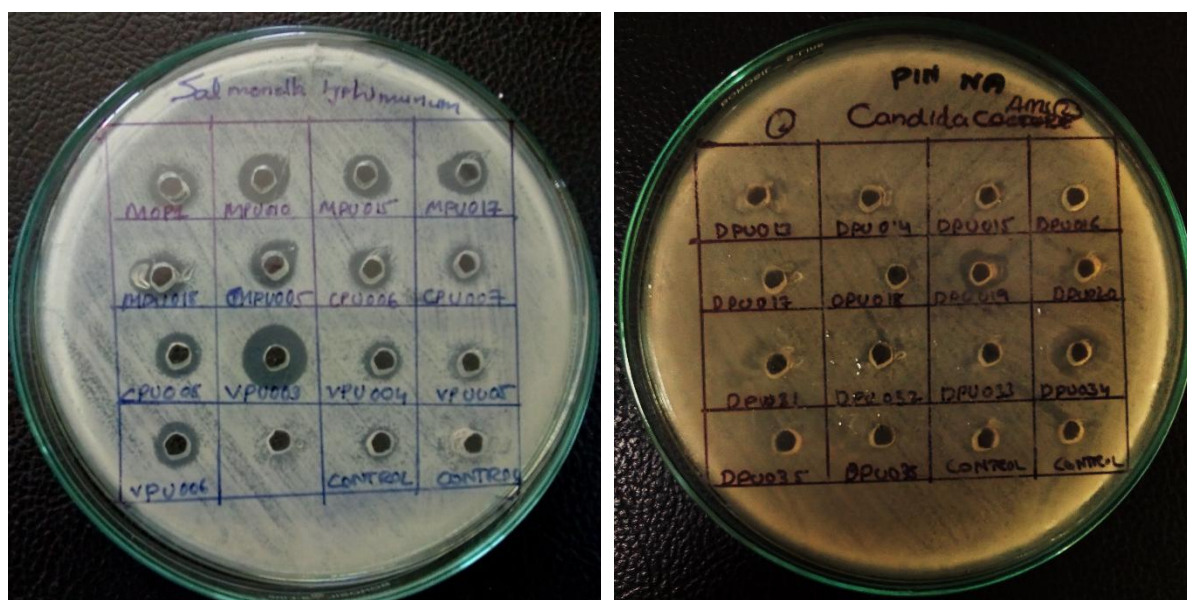


**Fig. 6.6:** Antimicrobial susceptibility testing using the well diffusion method

**Table 6.7:** Antimicrobial activity exhibited by partially purified extract against Gram positive bacteria (*S. aureus*), Gram negative bacteria (*S. typhimurium*, *E. coli* and *A. hydrophila* ) and fungi (*C. albicans* ).

| Marine isolate | Gram character | Antimicrobial activity<br>(clearance zone, mm) |                    |                |                  |                      | Protein content (mg/mL) |
|----------------|----------------|------------------------------------------------|--------------------|----------------|------------------|----------------------|-------------------------|
|                |                | <i>S. typhimurium</i>                          | <i>C. albicans</i> | <i>E. coli</i> | <i>S. aureus</i> | <i>A. hydrophila</i> |                         |
| DPU02          | -ve            | 10                                             | 0                  | 0              | 0                | 0                    | 0.09                    |
| DPU03          | -ve            | 9                                              | 0                  | 0              | 0                | 0                    | 0.08                    |
| DPU04          | -ve            | 9                                              | 0                  | 0              | 0                | 0                    | 0.07                    |
| DPU05          | -ve            | 9                                              | 0                  | 0              | 0                | 0                    | 0.07                    |
| DPU08          | -ve            | 9                                              | 0                  | 0              | 0                | 0                    | 0.08                    |
| DPU09          | +ve            | 6                                              | 0                  | 0              | 0                | 0                    | 0.11                    |
| DPU10          | +ve            | 9                                              | 0                  | 0              | 0                | 0                    | 0.09                    |
| DPU11          | +ve            | 9                                              | 0                  | 0              | 0                | 0                    | 0.11                    |
| DPU12          | -ve            | 9                                              | 0                  | 0              | 0                | 0                    | 0.11                    |
| DPU13          | -ve            | 0                                              | 0                  | 0              | 6                | 0                    | 0.11                    |
| DPU14          | -ve            | 0                                              | 0                  | 0              | 6                | 0                    | 0.1                     |
| DPU15          | -ve            | 6                                              | 0                  | 0              | 6                | 0                    | 0.1                     |
| DPU16          | -ve            | 0                                              | 0                  | 0              | 6                | 0                    | 0.11                    |
| DPU19          | +ve            | 0                                              | 9                  | 0              | 0                | 0                    | 0.15                    |
| DPU25          | +ve            | 6                                              | 0                  | 6              | 6                | 0                    | 0.14                    |
| DPU26          | -ve            | 6                                              | 0                  | 0              | 0                | 0                    | 0.13                    |
| DPU29          | -ve            | 6                                              | 0                  | 0              | 0                | 0                    | 0.1                     |
| DPU31          | +ve            | 0                                              | 0                  | 0              | 6                | 0                    | 0.13                    |
| DPU34          | -ve            | 6                                              | <b>12</b>          | 0              | 0                | 0                    | 0.14                    |

|       |     |           |           |           |           |           |      |
|-------|-----|-----------|-----------|-----------|-----------|-----------|------|
| DPU38 | -ve | 0         | 0         | 0         | 10        | 0         | 0.17 |
| DPU42 | -ve | 9         | 0         | 0         | 0         | 0         | 0.14 |
| MPU04 | -ve | 15        | 12        | 0         | 17        | 13        | 0.09 |
| MPU05 | -ve | 13        | 0         | 0         | 0         | 0         | 0.04 |
| MPU09 | -ve | 12        | 0         | 0         | 0         | 0         | 0.12 |
| MPU10 | +ve | 10        | 0         | 0         | 15        | 0         | 0.07 |
| MPU15 | -ve | 8         | 0         | 0         | 10        | 0         | 0.11 |
| MPU17 | -ve | 8         | 0         | 0         | 10        | 0         | 0.11 |
| MPU18 | -ve | 8         | 0         | 8         | 10        | 0         | 0.07 |
| CPU02 | -ve | <b>20</b> | 0         | 0         | 0         | 0         | 0.13 |
| CPU04 | -ve | 10        | 0         | 0         | 0         | 0         | 0.11 |
| CPU05 | -ve | 9         | 0         | 0         | 15        | 0         | 0.12 |
| CPU06 | -ve | 0         | 0         | 0         | 11        | 0         | 0.12 |
| CPU07 | -ve | 0         | 0         | 0         | 11        | 0         | 0.14 |
| CPU08 | +ve | 0         | 0         | 10        | 10        | 0         | 0.11 |
| CPU10 | -ve | 0         | 0         | 0         | 0         | <b>16</b> | 0.2  |
| CPU12 | -ve | 16        | <b>12</b> | <b>12</b> | 0         | 0         | 0.1  |
| VPU02 | -ve | 0         | 0         | 0         | 0         | 13        | 0.14 |
| VPU03 | +ve | 11        | 0         | 11        | <b>20</b> | 0         | 0.1  |
| VPU04 | -ve | 0         | 0         | 9         | 11        | 0         | 0.12 |
| VPU05 | -ve | 0         | 0         | 9         | 11        | 0         | 0.1  |
| APU02 | -ve | 7         | 0         | 0         | 7         | 0         | 0.1  |
| APU03 | +ve | 6         | 0         | 0         | 9         | 0         | 0.09 |
| APU04 | -ve | 6         | 0         | 0         | 9         | 0         | 0.06 |
| APU05 | +ve | 0         | 0         | 0         | 0         | 0         | 0.1  |
| APU06 | +ve | <b>20</b> | 0         | 0         | 0         | 0         | 0.1  |

|       |     |    |    |           |    |    |      |
|-------|-----|----|----|-----------|----|----|------|
| APU09 | +ve | 6  | 6  | 6         | 0  | 10 | 0.09 |
| APU11 | -ve | 12 | 11 | 10        | 18 | 10 | 0.08 |
| APU15 | -ve | 7  | 0  | 0         | 0  | 0  | 0.1  |
| APU21 | -ve | 13 | 11 | <b>12</b> | 0  | 0  | 0.16 |

As detailed above, a marked difference was observed in the microbial load from estuarine to coastal marine ecosystems and in their production of antimicrobial peptides/proteins. The comparative analysis using the partially purified proteinaceous samples is presented in Fig. 6.7, in relation to the different sampling sites and test pathogens used. In the estuarine environment, high bacterial abundance contrasting with low antimicrobial activity was observed against all five pathogens tested. A possible explanation could be that many marine and freshwater microflora that entered into the estuarine mangrove environment (mixing zone) were no longer active because of the rapid salinity fluctuations encountered. In contrast, the seawater salinity tolerant microbes appear to be a better-adapted community and are metabolically active.

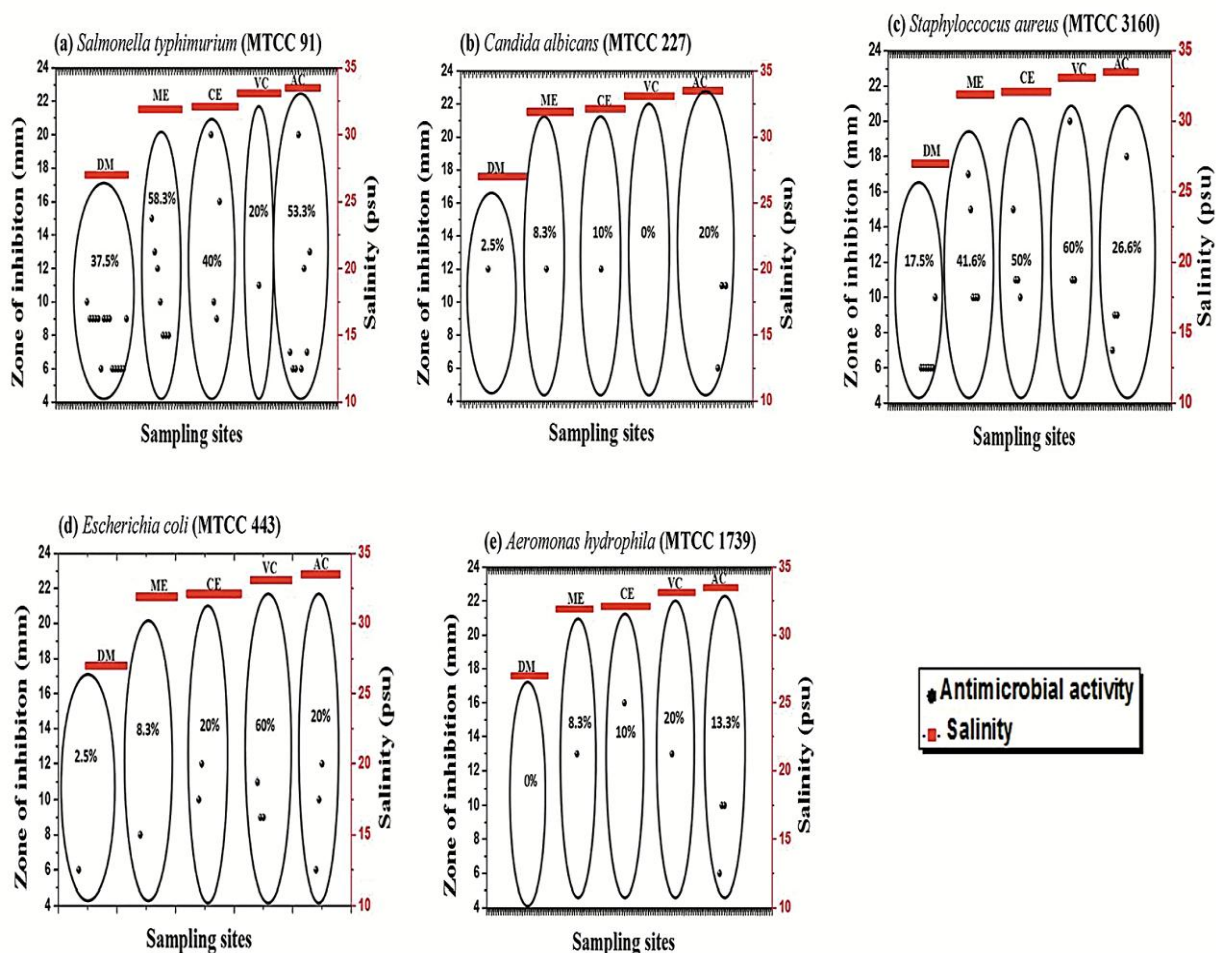


**Fig. 6.7:** Antimicrobial activity in partially purified exudates of the isolates, against five pathogenic test cultures, as viewed in relation to sampling site salinity variations

The results of the present study have indicated that as compared to estuarine bacteria, those in other areas showed production of more potent antimicrobial peptides that were probably used to counter the high competition for resources. These bacteria could therefore be a more promising source for bacteriocin-like compounds.

Five isolates selected based on their broad antimicrobial activity were subcultured and maintained on ZMA (Fig. 6.8).

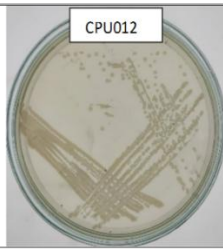
|  |  |  |  |  |              |                |
|-----------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------|----------------|
| Culture                                                                           | Elevation                                                                         | Margin                                                                            | Configuration                                                                      | Opacity                                                                             | Pigmentation | Gram Character |
| MPU004                                                                            | Convex                                                                            | Smooth                                                                            | Round                                                                              | Opaque                                                                              | Orange       | -ve short rods |
| CPU012                                                                            | Convex                                                                            | Smooth                                                                            | Round                                                                              | Opaque                                                                              | No colour    | -ve rods       |
| VPU003                                                                            | Flat                                                                              | wavy                                                                              | Irregular                                                                          | Translucent                                                                         | No colour    | +ve rods       |
| APU009                                                                            | Flat                                                                              | smooth                                                                            | Concentric                                                                         | Opaque                                                                              | No           | -ve rods       |
| APU011                                                                            | Convex                                                                            | Smooth                                                                            | Round                                                                              | Opaque                                                                              | Creamish     | -ve rods       |

**Fig. 6.8:** Isolates that showed potent antimicrobial activity

### 6.3.5 SDS-PAGE analysis of the bacterial extracts

The selected isolates (MP004, CPU012, VPU003, APU009, APU011) were grown in ZMB for 48 h at 37°C. The cell-free supernatant obtained was subjected to 90% ammonium sulphate precipitation to collect the total protein, which was then analysed by SDS-PAGE using a 15% separating gel (Fig. 5).

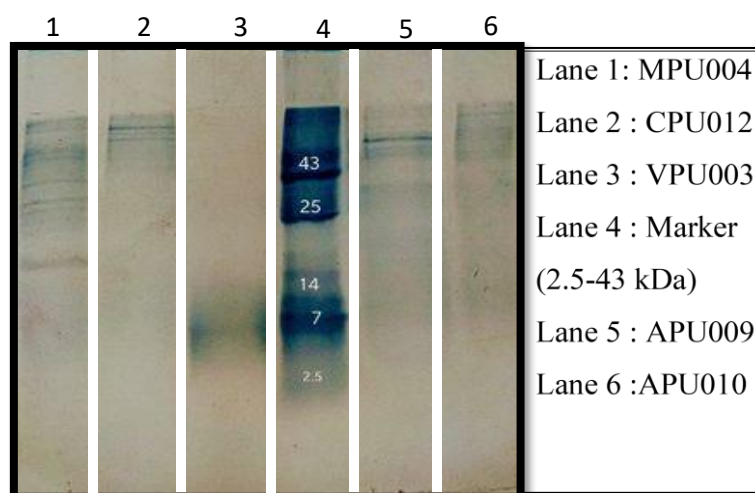


**Fig. 6.9** SDS-PAGE protein profile of the extracts

While isolates MPU004 and APU010 extracts showed the presence of proteins/peptides in a wide range of molecular weights, the protein bands from isolates CPU012 and APU009 were predominantly of higher masses. Isolate VPU003 extract, on the other hand, showed a strong low molecular weight band falling the size range of ‘peptides’. This sample was hence subjected to further purification.

### 6.3.6 Purification by gel filtration chromatography

The ammonium sulphate suspension of isolate VPU003 extract was loaded on a pre-equilibrated Sephadex G-50 column. While the proteinaceous material eluted as a minor higher molecular weight peak ( $V_e = 26$  mL) followed by a significantly broad peak ( $V_e = 62$  mL) as monitored by the 220 nm absorbance of the fractions (Fig. 6), the antimicrobial activity was confined to the later fractions, which eluted much after the elution volumes evidenced for cyt *c* and aprotinin as depicted in chapter 5 section., confirming that the bioactive peptide of interest was of low molecular weight. Fraction numbers 59 to 80 were pooled and concentrated by lyophilisation.

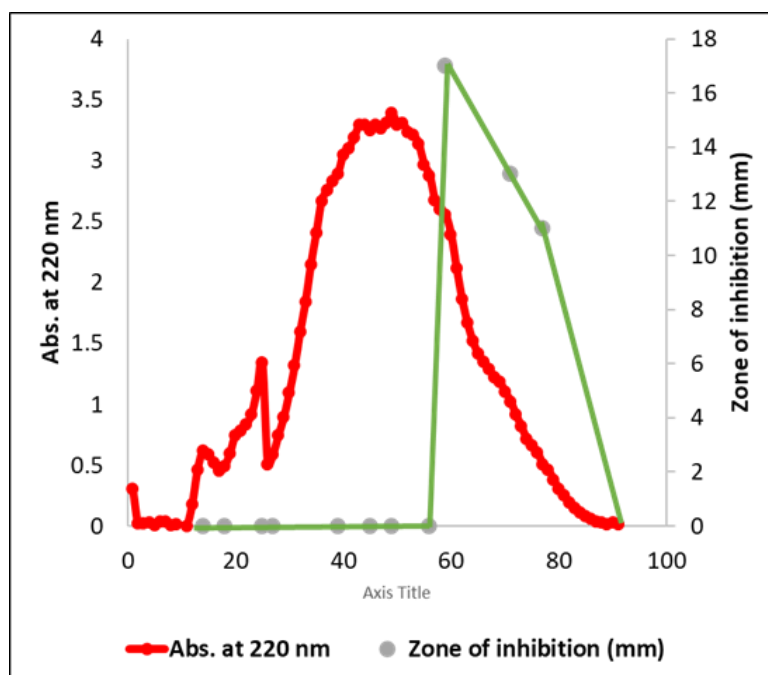


**Fig 6.10:** Elution profile of VPU003 extract on the Sephadex G-50 column

### 6.3.7 SDS-PAGE

The concentrated antimicrobially active peptide fraction was subjected to SDS-PAGE, together with standard low molecular weight protein markers (Fig. 6.11). A single broad band of low molecular weight was observed just above the 2.5 kDa band of the standard marker peptide.

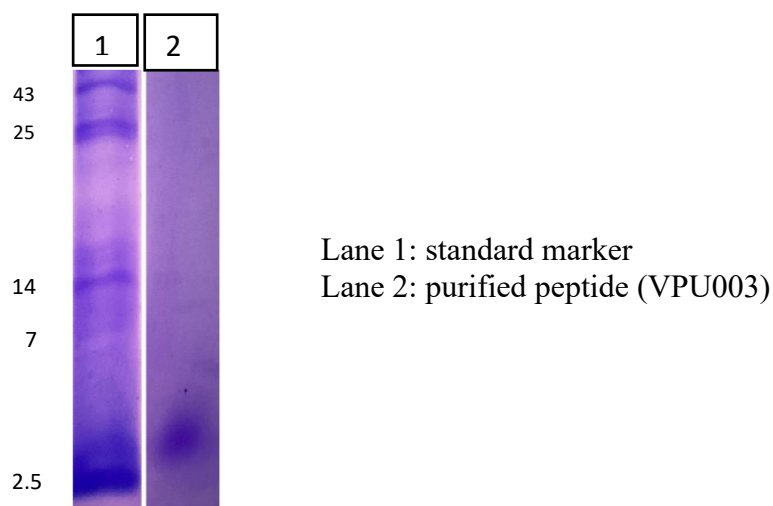


**Fig 6.11:** SDS profile of purified peptide fromVPU003

### 6.3.8 AU-PAGE

Acid-urea PAGE resolves peptides and proteins based on their cationic charge density and is highly suited for the analysis of antimicrobial peptides. The concentrated pool of column fractions which exhibited antimicrobial activity was analysed by AU-PAGE, with standard lysozyme as a marker (Fig. 6.12). Such a cathodal migration additionally confirmed that our purified antimicrobial peptide was cationic.

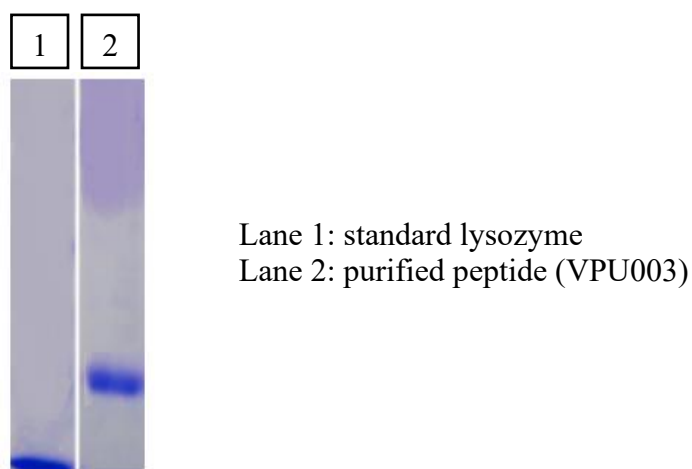


**Fig 6.12:** AU PAGE of purified peptide fromVPU003

### 6.3.9 MALDI-TOF MS Analysis

The molecular weight of the purified peptide was determined by MALDI-TOF-MS analysis, as per which the intact molecular mass of the peptide was 3351.45 Da (Fig. 6.13).

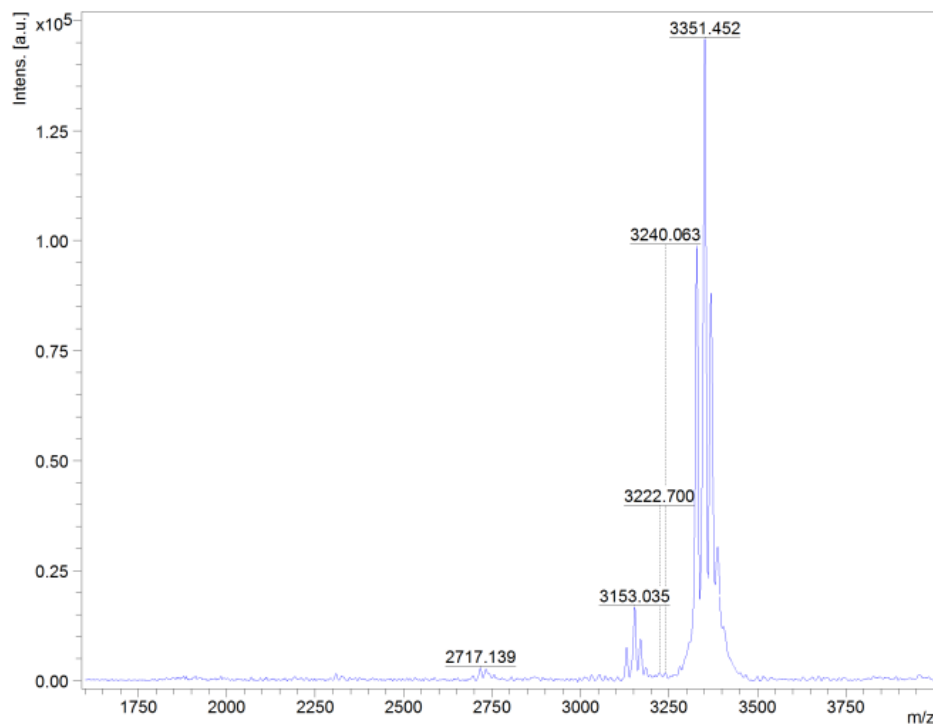


**Fig. 6.13:** MALDI-TOF MS analysis of purified VPU003 peptide

### 6.3.10 Characterization of isolate VPU003 based on biochemical characteristics

Out of all 82 cultures, isolate VPU003 that produced a low molecular weight cationic peptide was selected for further identification based on biochemical characteristics, as given below:

Morphological and biochemical characteristics of the selected isolates

| Test            | Result        |
|-----------------|---------------|
| Grams staining  | Gram +ve rods |
| Spore formation | +ve           |
| Motility        | +ve           |

### Biochemical tests used for preliminary identification of the selected isolates

| Test                | Result |
|---------------------|--------|
| Oxidase             | +ve    |
| Citrate utilization | +ve    |
| Catalase            | +ve    |
| Starch hydrolysis   | +ve    |

### 6.3.11 Microbial identification of isolate VPU003 based on 16S rRNA sequencing and BLAST search analysis

The DNA sequence was obtained through Eurofins Genomics India, Pvt. Ltd.. The raw sequences obtained were used to assemble the contigs using BioEdit software, version 7.2.5 and aligned. The contigs were matched with GenBank database using NCBI-BLAST.

#### Molecular identification details of isolate VPU003

| Sr. no | Culture code | Length of the sequence | Closest match                          | Percent similarity |
|--------|--------------|------------------------|----------------------------------------|--------------------|
| 1.     | VPU003       | 1528 bp                | <i>Bacillus subtilis</i> strain RHMR 7 | 99.89 %            |

Isolate VPU003 showed 99 % similarity with *Bacillus subtilis* strain RHMR 7, and was submitted to GenBank with the accession number OQ271371.

To summarise the contents of this Chapter, 82 bacterial isolates were screened from distinct coastal environs of Goa. Bacterial abundance, antimicrobial potential of the isolates and their structuring in relation to the ambient salinity variations has been specifically investigated. There was a progressive increase in TVC from estuarine to higher salinity marine waters. Isolates from more saline coastal regions had stronger antimicrobial potential than those from the estuarine habitats. This bioactivity could be largely attributed to peptidic molecules. Isolate VPU003 from the Vagator beach area had shown superior potential of producing an antimicrobial peptide of molecular mass 3351.452 Da. The isolate was identified as *Bacillus subtilis*. The present study adds to the knowledge of distribution of marine bacteria with antimicrobial potential for furthering biotechnological applications to address and alleviate

rising concerns about antibiotic resistance. More work on the isolation and characterisation of such antimicrobial peptides would open up avenues in the search for novel antimicrobial peptides and with that, more such molecules would continue to enter clinical trials. Also, novel compounds with different chemical structures and biological activities would become available to counter the ever-growing reality of antimicrobial resistance.

# **CHAPTER 7:**

## **Potential Applications of Two Cationic Peptides**

## 7.1 Introduction

Dermal wounds, characterized by the disruption of the skin epithelium due to various factors such as thermal or physical damage, represent a significant healthcare concern globally (Dai et al., 2020). Treatment and management of dermal wounds are paramount in healthcare systems, with substantial financial resources being allocated to address the challenges associated with wound healing and tissue regeneration. Both acute wounds, typically healing within 8 to 12 weeks, as well as chronic wounds, characterized by delayed healing processes, pose distinct challenges in clinical practice (Dhivya et al., 2015).

The healing of dermal wounds involves a complex interplay of biological processes, including inflammation, tissue repair and remodelling, all of which are influenced by factors such as local pH conditions and the presence of infections (Guo & Dipietro, 2010). The pH environment of skin wounds, ranging from 5 to 9, plays a crucial role in modulating the healing process and influencing the efficacy of wound care interventions. Effective wound management strategies are essential to minimize the risk of infections, promote tissue regeneration, and accelerate the healing of both acute and chronic wounds.

In the context of wound care, the selection of appropriate wound dressing materials is critical for providing a conducive environment for healing while preventing complications such as infections and tissue damage. Traditional wound dressings, although widely used, often lack optimal moisture balance and oxygen exchange, leading to issues such as skin maceration and delayed healing (Borda et al., 2016; Shi et al., 2020). The demand for advanced wound dressing materials with improved properties, including enhanced skin compatibility, absorption capacity, and local drug delivery capabilities, has driven research efforts towards developing innovative solutions for wound management.

Natural polymers such as cellulose, alginate, hyaluronic acid and chitosan have been favoured for wound dressing applications due to their biocompatibility; however, their commercial viability is hindered by limitations in mechanical properties and cost-effectiveness compared to synthetic polymers (Marroquin-Garcia et al., 2022).

In view of the complex nature of dermal wounds and the challenges associated with their management, innovative approaches in materials science and biomedical engineering are essential to advance the field of wound care and improve patient outcomes. The exploration of

novel wound dressing materials holds great potential for addressing the unmet needs in wound management and enhancing the quality of care for individuals with dermal wounds.

### **7.1.1 Hydrogel bandages as wound dressing**

Hydrogels are crosslinked networks of the same or different types of polymers with a high capacity for water retention. They can be constituted by the assembly of natural polymers such as collagen, gelatin, cellulose, alginate or chitosan, or produced synthetically using polymers such as polyethylene glycol (PEG), polyvinyl alcohol (PVA) or polyglycolic acid (PGA). Providing a moist wound environment facilitating cell migration and absorption of some exudate are the primary attributes of a good hydrogel. Another advantage of hydrogel dressings lies in autolytic debridement without harm to epithelial cells. Novel hydrogels with stronger and more stable properties are thus constantly in demand and remain an important direction for research (Ávila-Salas et al., 2019).

### **7.1.2 Chitosan hydrogels incorporated with cationic peptides?**

Chitosan, found abundantly in nature, serves as a renewable resource. It can be obtained from various sources, including the shells of marine arthropods such as shrimps and crabs, molluscs, insects and the cell walls of higher plants, and subsequently processed through deacetylation (Tian et al., 2020). Chitosan can be chemically and physically cross-linked to prepare a variety of chitosan-based hydrogels for wound repair.

Chitosan-based hydrogels are increasingly recognized as a promising material for next-generation dressings due to their excellent biocompatibility and wetting properties. Extensive studies have highlighted their effectiveness in facilitating the healing of acute wounds. However, the approach to treating chronic wounds differs significantly. The choice of dressings for chronic wounds depends on the specific characteristics of each wound. As a result, managing chronic wounds requires specialized dressings. The limited effectiveness of some chitosan hydrogels in promoting the healing of chronic wounds has restricted their widespread use as wound dressings. To address this limitation, researchers have investigated the combination of therapeutic drugs with chitosan-based hydrogels (Che et al., 2024), so as to enhance their efficacy to treat chronic wounds such as diabetic foot ulcers (DFUs).

The DFUs are a serious complication arising from diabetes mellitus and often entail a heightened risk of morbidity and premature mortality. Globally affecting approximately 40-60 million individuals with diabetes, DFUs are marked by the development of persistent wounds. These wounds result from a combination of metabolic irregularities, nerve impairment, impaired blood circulation, and biomechanical alterations in the lower extremities. Over time, these factors culminate in chronic tissue damage and susceptibility to infections (Güiza-Argüello et al., 2022).

Most diabetic wounds end up with bacterial infections because the hyperglycaemia of the wound provides an ideal environment for growth of microorganisms. The bacteria can then develop into a biofilm after attachment, which drives wounds into a long-lasting inflammatory phase. Once biofilms form, it is a difficult challenge to clear infections due to the robust resistance toward treatments, especially those involving small molecule conventional antibiotics. It is reported that more than 78% of chronic wounds had biofilms. In a small-scale clinical study of chronic wounds, out of 50 wound specimens from patients, 30 were found to contain biofilms (Afonso et al., 2021). The persistently infected chronic wounds in diabetic patients can often lead to undesirable outcomes, 14–24% of patients with diabetic ulcers being estimated to eventually require amputation (Burgess et al., 2021).

Chronic infection gives rise to antimicrobial-resistant pathogens and the increasing failure of conventional antibiotics to repair such wounds calls for the development of new antimicrobial agents with novel targets against antibiotic-resistant microbial pathogens. In this context, the search for bioactive substances from natural resources is a common practice for the development of new agents. Antimicrobial peptides of plant and animal origin are an excellent source in the search for a new antibacterial wound dressing agent (Pfalzgraff et al., 2018).

Peptides, as amphoteric molecules derived from amino acids, are readily absorbable, have low energy requirements for production and demonstrate notable attributes such as high affinity, specificity, and minimal toxicity. Previous research has indicated the capacity of peptides to facilitate wound healing (Pickart, 2008). In order to tackle the urgent concern of antimicrobial resistance, Rezaei, et al. (2020) developed a hydrogel utilizing a chitosan-based carrier incorporated with both the antibacterial agent hydrogen peroxide and antibacterial peptides. This hydrogel was applied directly to wounds, effectively targeting biofilm-related bacteria,

biofilms and infections. Their findings demonstrated the hydrogel's inhibitory activity against methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria.

This Chapter outlines our first attempts to gauge the feasibility of preparing chitosan hydrogels incorporating the purified cationic peptides Ealp and Vpup, isolated from the mangrove plant *E. agallocha* and from the bacterium *Bacillus subtilis*, respectively.

## **7.2 Materials and methods**

### **7.2.1 Reagents**

All reagents were purchased from HiMedia Laboratories Pvt. Ltd., India, unless otherwise stated.

### **7.2.2 Preparation of chitosan-based hydrogel films**

Chitosan solution (4%, w/v) was prepared by dissolving chitosan powder in 2% (v/v) acetic acid aqueous solution at 60°C. Glycerol, as a plasticizer, was added to the chitosan solution at a concentration of 30 wt% of chitosan, with 1 mg/mL of peptide Ealp (from *Excoecaria agallocha*) or peptide Vpup (from the bacterial isolate VPU003). These were then mixed, cast, and left for drying at 50°C for 24 h. The hydrogel obtained was neutralised with 5M NaOH and kept in a chamber at room temperature (28 ± 2°C) for 48 h before measurements (Konwar et al., 2016; Wang et al., 2017; Ahsan et al., 2020; Chopra et al., 2022).

### **7.2.3 Determination of moisture content**

Moisture content (MC) of the films was determined by measuring weight loss of the hydrogel upon drying. Each hydrogel pad was cut into 8 mm discs, put individually in petri dishes and the initial weight ( $W_0$ ) was measured. At different time intervals of 24, 48 and 78h, the petri dishes maintained at 37°C were weighed ( $W_t$ ). The MC was arrived at from the water loss rate (Wang et al., 2017):

$$MC (\%) = W_t / W_0 \times 100$$

### **7.2.4 Swelling index measurement**

The stability of the hydrogels at pH 3, pH 5 and pH 9 was evaluated in triplicate, for 3 days. Each hydrogel was placed in a 2 mL Eppendorf tube loaded with 1 mL of the solution. Sample

incubations were uniformly carried out at 37 °C and 100 rpm. Weight of the sample was noted and the pH solutions were refreshed from time to time. The mean weight change was calculated (Ahsan et al., 2020) according to the eqn:

$$\text{Weight change (\%)} = W_{\text{wet}} / W_{\text{dry}} \times 100$$

### **7.2.5 *In Vitro* drug release studies**

The drug-release profiles of the different samples were evaluated at pH values of 3, 5, and 9. Solutions at different pH were prepared by addition of required quantities of 0.5M HCl or 1 wt % NaOH to distilled water. Each sample was suspended in 1 mL of the solution of the specific pH and incubated at 37 °C and 100 rpm. Aliquots were then withdrawn at different time intervals (24, 48 and 72 h) and analyzed at 280 nm on a UV-Vis spectrophotometer. The volume of water was then replenished with the respective fresh solutions (of the specific pH) for continuation of the incubation (Ahsan et al., 2020).

### **7.2.6 Enzymatic biodegradation**

Biodegradation of the hydrogels was performed using lysozyme (a common polymer-degrading enzyme found in wound exudates) or trypsin. The samples were incubated in lysozyme/PBS or trypsin/PBS solution (1 mg/mL) at 37 °C for up to 3 days and the degradation behaviour at different time points quantified using the weight change equation (Ahsan et al., 2020).

### **7.2.7 Antimicrobial activity**

Antimicrobial activity was tested for Chitosan-Ealp and Chitosan-Vpup hydrogels on *S. aureus* in culture, using the disc diffusion method as per Piegat et al. (2020). Overnight-grown bacterial culture was swabbed onto Mueller-Hinton agar (MHA) medium. Hydrogel discs of 8 mm were cut out and placed on to the inoculated MHA plates which were then incubated at 37 °C for 24 h. The diameter of the zone of clearance was then recorded.

## **7.3 Results and discussion**

Researchers have developed hydrogels with diverse properties using both physical and chemical cross-linking methods, effectively catering to the treatment needs of different

wounds. Currently, there exists a variety of hydrogel dressings, with optimization of new hydrogel materials ongoing, leading to increasingly enhanced functionalities. However, there remains a gap in achieving the ideal hydrogel dressing, particularly in addressing chronic wounds. Both natural and synthetic polymers exhibit favourable biological activity and versatile applications in these materials. Notably, the synergistic use of different polymers highlights their individual contributions and accelerates the wound healing process (Su et al., 2021).

This Chapter reports the synthesis of chitosan-based hydrogels incorporated with the antimicrobial peptides Ealp or Vpup for potential wound dressing applications.

### 7.3.1 Visual evaluation of the hydrogel

The chitosan hydrogel was prepared as described in Section 7.2.2. The prepared hydrogel was slightly yellow but transparent at the experimental temperature (25 °C), proving that the added ingredients were homogeneously mixed. It was observed that until the end of the experiment (*i.e.*, after 72 h) there was no significant colour change in both the Chitosan-Ealp as well as the Chitosan-Vpup hydrogels when stored at 25°C (Fig. 7.1), suggesting that the preparations were stable. Had they not met the set standards, changes in visual appearance of the hydrogels would have occurred, indicating that the material might not be stable anymore and could be nearing the end of its shelf-life (Roy et al., 2013).

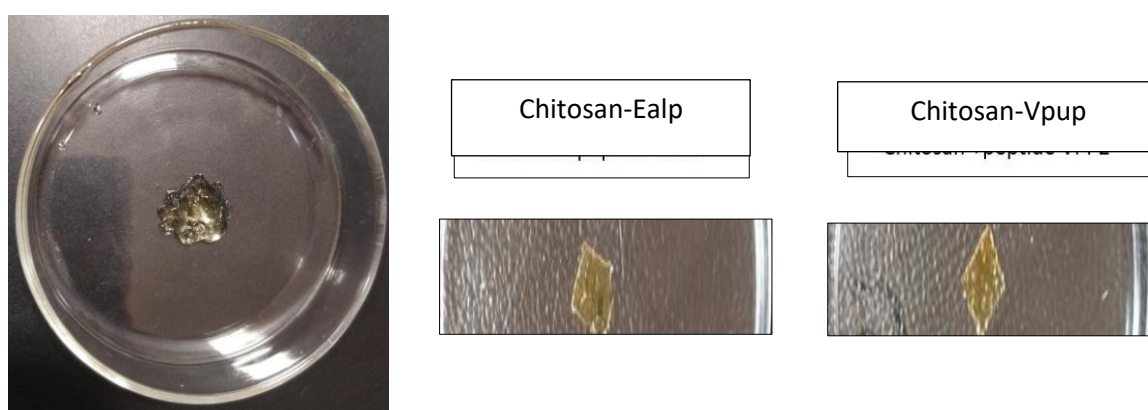


**Fig. 7.1** Chitosan hydrogel with the antimicrobial peptides

### 7.3.2 Moisture retention potential

Being an important factor of the wound dressing, moisture retention capability of the hydrogels was investigated. Maintaining increased humidity in wounds covered with such a hydrogel ensures a suitable environment for faster healing (Roy et al., 2011). It can be seen from Fig. 7.2 that at 37°C both the hydrogels lost water in 24 h, but only in a very nominal amount of up to ~5% for the Chitosan-Vpup hydrogel; a higher water loss of ~25% was observed in the Chitosan-Eapl hydrogel during this period. Both hydrogels did not exhibit further moisture loss of significance after 72 h of incubation. A study by Boateng et al. (2008) on hydrogel wound dressings emphasized that an ideal dressing should retain at least 70% moisture for over 24 hours to promote wound healing. Our hydrogels meet this criterion, especially Chitosan-Vpup, which exhibits minimal dehydration.

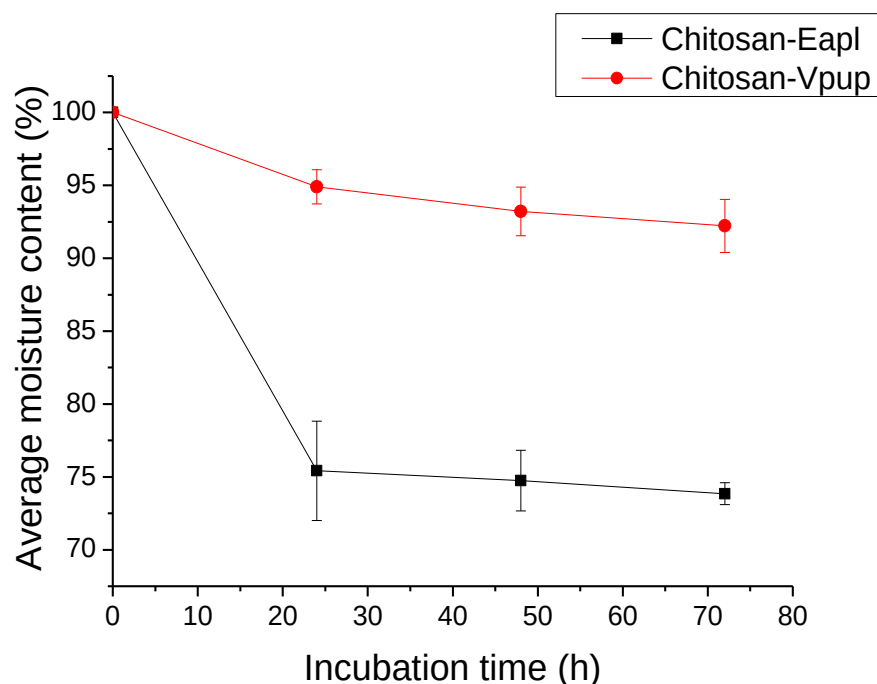


**Fig 7.2** Moisture content studies of Chitosan-Eapl and Chitosan-Vpup hydrogels with time

### 7.3.3 Swelling index evaluation

Swelling is described as a phenomenon in which water / biological fluids are absorbed and retained in a polymer network. The capability of a hydrogel to swell depends on its chemical structure and the characteristics of its medium (Singh et al., 2006).

Physiological conditions of transdermal wounds typically lie between pH 5 and 9 (Marroquin-Garcia et al., 2022). Swelling studies of chitosan-based hydrogels were performed at 37 °C and at different pH values of 3, 5 and 9 (Fig. 7.3).

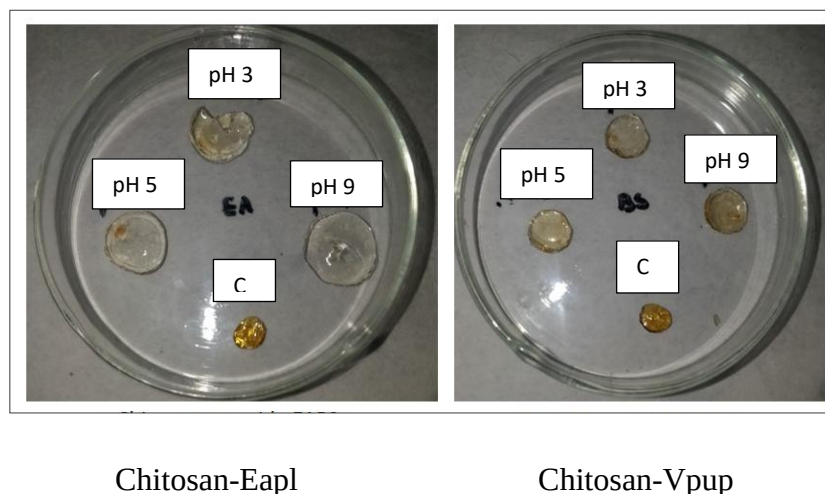
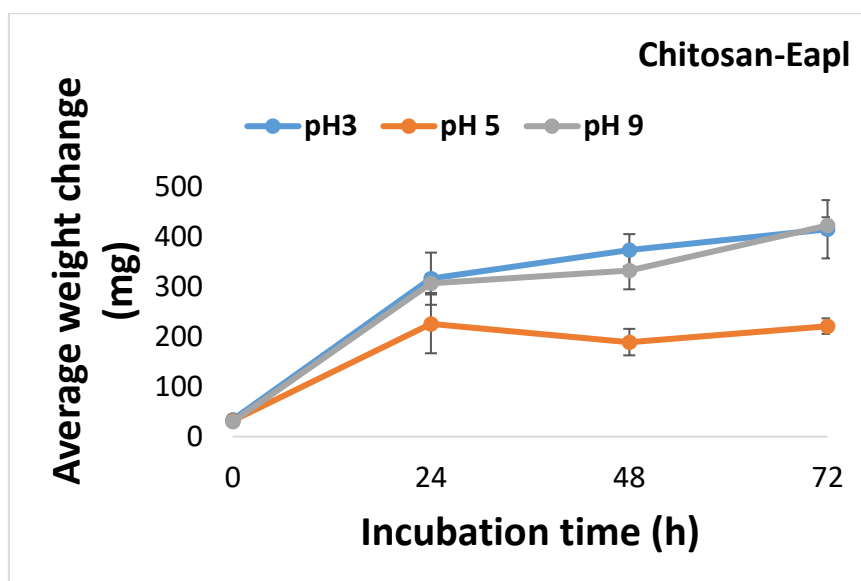


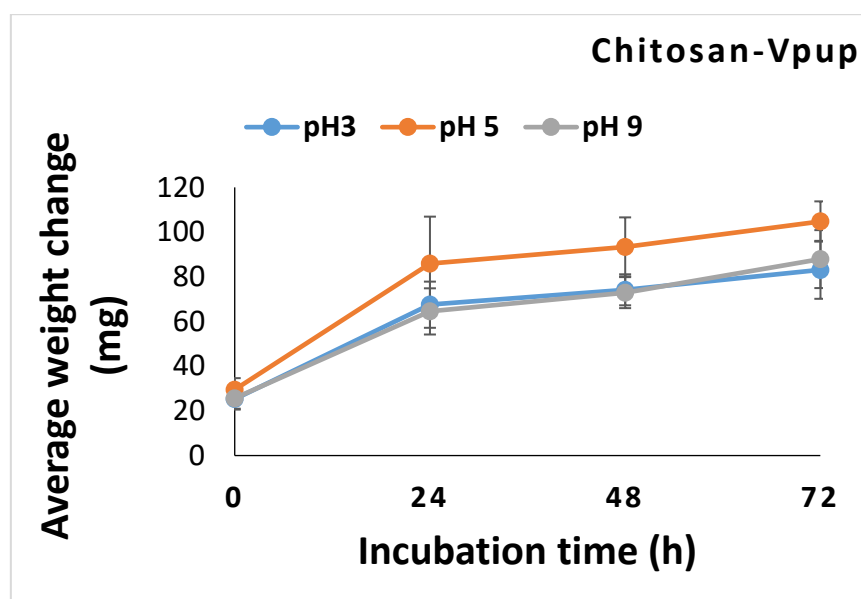
**Fig. 7.3** Swelling studies on Chitosan-Eapl and Chitosan-Vpup hydrogels at pH 3, 5 and 9 at 37 °C.

The weight of both the hydrogels gradually increased over the 3 days of the study (Fig. 7.4). The Chitosan-Eapl hydrogel exhibited swelling of approximately 10-14 times its weight after 72 h at pH 3 and pH 9. At pH 5, it swelled to about 7 times its weight after 24 h, with no significant increase observed after 48 or 72 h of incubation. Chitosan-Vpup hydrogel swelled up to approximately 2.5-3.5 fold of its weight at these pH values.

The Chitosan-Eapl displayed maximum swelling at acidic pH and lower swelling at pH 5, while at pH 9 the hydrogel again started to swell. Our observations are similar to the results obtained by Ghauri et al. (2021) with an ONB-chitosan hydrogel. On the other hand, in the case of the Chitosan-Vpup hydrogel, higher swelling was observed at pH 5 as compared to pH 3 & 9. In acidic conditions, the swelling of chitosan occurred because the amino groups ( $-\text{NH}_2$ ) were protonated, forming ammonium ions ( $-\text{NH}_3^+$ ). Additionally, the higher concentration of ionic groups (ammonium ions) resulted in increased charge repulsion and reduced osmotic pressure, leading to enhanced swelling. Conversely, when the pH increased, deprotonation of ammonium ions occurred, causing a reduction in swelling due to decreased interactions between polymer chains (Ghauri et al., 2021).



(a)



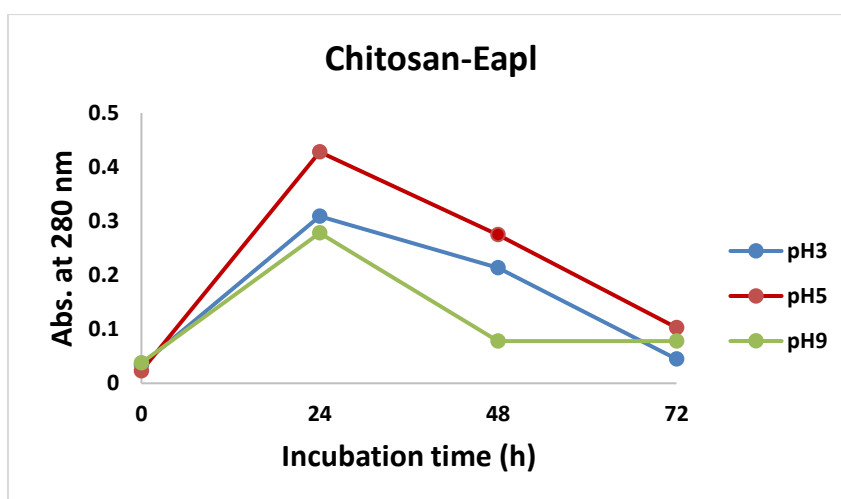
(b)

**Fig. 7.4** Swelling studies on (a) Chitosan-Eapl and (b) Chitosan-Vpup hydrogels at 37 °C at different pH values.

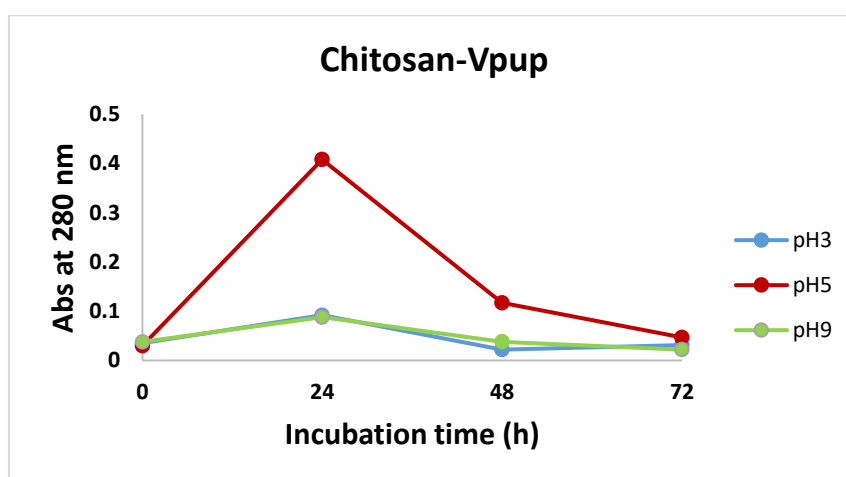
Chitosan-Eapl hydrogel would thus offer great swellability and stability in wound-like conditions (pH 3–9) as compared to Chitosan-Vpup hydrogel. Overall, both hydrogels showed good swelling potential, which would render them ideally suited for (prolonged) wound-dressing applications.

### 7.3.4 *In vitro* drug release studies

As described above, our hydrogels displayed excellent features of water retention and stability. To further investigate the suitability of our preparations, (a) Chitosan-Eapl and (b) Chitosan-Vpup were tested for drug release over time, under different pH conditions. Design of the wound dressing must be carried out judiciously such that the materials remain stable under the applied conditions, and release drugs gradually to treat the wound. The cumulative drug release of antimicrobial cationic peptides (ACPs) as monitored by their absorbance at 280 nm profiles is demonstrated in Fig. 7.5. As observed, the drugs eluted gradually over time (24- 48 h), which projects the hydrogels suitably for prolonged wound treatment.



(a)



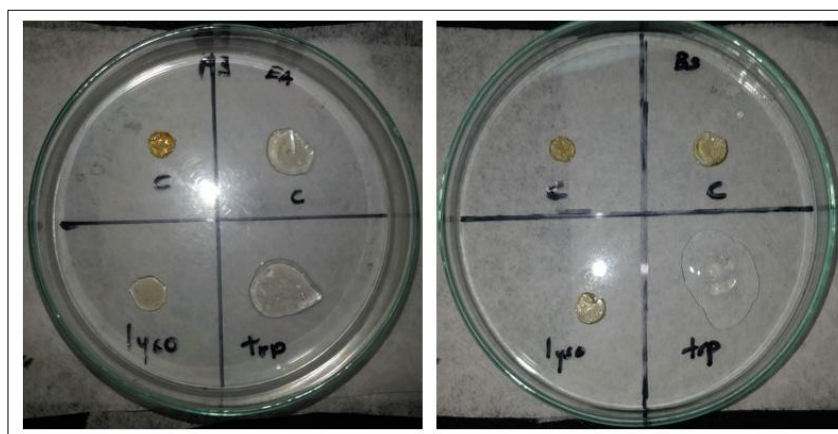
(b)

**Fig. 7.5.** Antimicrobial peptide release monitored by Abs at 280 nm, from (a) Chitosan-Eapl and (b) Chitosan-Vpup hydrogels as a function of time, under different pH conditions.

The sustained release of ACPs from the Chitosan-Eapl hydrogels lasted for 72 h, as evidenced by the UV absorption profiles at 280 and 220 nm. In comparison, sustained release of ACPs from Chitosan-Vpup was observed for 48 h. At pH 5, both Chitosan-Eapl and Chitosan-Vpup hydrogels exhibited better release of ACPs as compared to pH 3 and 9. Fasiku et al. (2021) had reported comparable findings in their investigation of antimicrobial drug (hydrogen peroxide) release from chitosan hydrogels.

### **7.3.5 Stability to enzymatic biodegradation**

Lysozyme plays an important role in preventing wound infections as it breaks down the glycoside bonds of certain bacterial cell walls. However, recent studies also suggest that it degrades chitosan by cleaving the 1,4-beta-glycosidic bond in chitosan (Kim et al. 2018). Yang et al. (2021) demonstrated that tyrosinase could induce a transition from a gel to a sol state in the Ac-YYYpT-OMe hydrogel. (Gao et al., 2011). Trypsin is a proteolytic enzyme which selectively hydrolyzes peptide linkages involving the carboxyl groups of lysine or arginine. It is widely recognized that peptides, including those in hydrogel form, typically degrade within hours or days upon exposure to proteases, which poses a significant limitation to their clinical application. The proteolytic stability of the peptide component of the hydrogel significantly enhanced its longevity in biological settings (Liang et al., 2009). The susceptibility to enzymatic degradation of the hydrogels had therefore to be evaluated using lysozyme or trypsin in PBS solution at 37°C. Over a 72 h period, both our hydrogels displayed a considerable increase in weight. They were unaffected by lysozyme as it was unable to significantly break the diester bond in our materials. (Fig. 7.6). The observed cleavage by trypsin was not totally unexpected in view of the higher abundance of basic residues in cationic peptides. This suggests proper peptide incorporation within the chitosan matrix. Trypsin, a serine protease, cleaves peptide bonds at lysine and arginine residues, disrupting the hydrogel network and reducing its stability and water retention. In contrast, lysozyme targets glycosidic bonds in chitosan, causing slower degradation than trypsin's rapid proteolytic activity (Das & Das, 2021). Future research could work out means to overcome this issue.



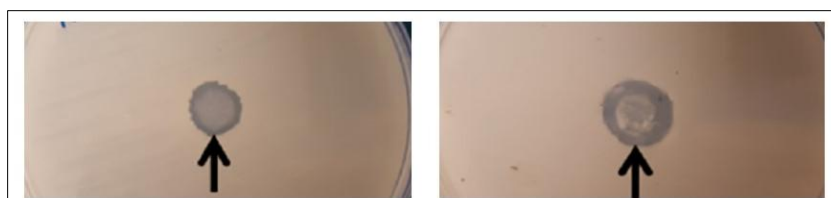
Chitosan-Eapl

Chitosan-Vpup

**Fig. 7.6** Enzymatic biodegradation of Chitosan-Eapl and Chitosan-Vpup hydrogels at pH 3, 5 and 9 at 37 °C.

### 7.3.6 Antimicrobial activity of the prepared hydrogels

The antimicrobial zone of clearance against *S. aureus* was 9 mm for the Chitosan-Eapl hydrogel and 10 mm for the Chitosan-Vpup hydrogel. No activity was observed with chitosan only (control), as observed in **Fig. 7.7**.



Chitosan-Eapl

Chitosan-Vpup

(a)

(b)



Chitosan (Control)

(c)

**Fig. 7.7** Antimicrobial activity of (a) Chitosan-Eapl (b) Chitosan-Vpup and (c) Chitosan (control) hydrogels on *S. aureus*.

In summary, this Chapter has focussed on laboratory studies involving the preparation of hydrogels infused with antimicrobial peptides, intended for use as wound dressings. Unlike traditional gauze and cotton dressings which can cause additional harm upon removal, hydrogel dressings offer benefits across various wound types. Both our hydrogel preparations experienced low water loss within 24 h, with Chitosan-Vpup hydrogel losing ~5%, while Chitosan-Eapl hydrogel exhibited a water loss of ~25%. The synthesized hydrogels facilitated an increase of up to 3.5-fold and 14-fold in the swelling properties of Chitosan-Vpup and Chitosan-Eapl, respectively. Moreover, the hydrogels were hydrolytically stable under wound-like pH and enzymatic conditions when treated with lysozyme, although degradation occurred upon treatment with trypsin. Finally, the hydrogels were able to release both the antimicrobial peptides (Ealp and Vpup) in a prolonged and controlled manner in different wound-like pH conditions. The Chitosan-Vpup hydrogel showed a higher antimicrobial activity as compared to Chitosan-Eapl. Needless to say, more issues need to be resolved with follow-up research on these preliminary, yet encouraging, results.

## Summary and Conclusions

The overuse of antibiotics to treat various diseases has led to the emergence of antimicrobial resistance in pathogens (Wagenlehner & Dittmar, 2022). With bacteria developing resistance to existing antibiotics and their analogs, the search for a new class of antibiotics with a more unique mode of action has become increasingly important (Lei et al., 2019).

Antimicrobial cationic peptides (ACPs) represent such a class, demonstrating broad-spectrum antimicrobial activity against various pathogens. These peptides carry a cationic charge ranging from +2 to +7 under physiological pH conditions (Hancock, 2001) and are typically less than 10 kDa in size.

Mangroves and marine waters are unique environments that host diverse plants and microbial communities, including halotolerant and halophilic bacteria (Kathiresan & Rajendran, 2005; Troussellier et al., 2002). These sources, however, remain relatively underexplored with regard to the production of novel antimicrobial peptides. The present study aims to explore such sources for ACPs, and to purify and characterize potential candidates.

### **Screening mangrove plants for cationic peptides: optimizing crude extraction procedures and assessing their bioactivities**

- ❖ Leaf, stem and root extracts of *Sonneratia alba*, *Excoecaria agallocha*, *Rhizophora mucronata* and *Acanthus ilicifolius*, mangrove plant species which had earned a distinct space in traditional/indigenous systems of medicine, were screened for antimicrobial cationic peptides.
- ❖ Extraction was carried out using acetic acid, which favored selective isolation of soluble cationic peptides due to their basic nature.
- ❖ Percent homogenate (w/v) and solvent strength (v/v) standardization experiments were conducted, from which a 10% concentration proved to be efficacious in both cases.

- ❖ Seasonal fluctuations in the peptidic content of crude extracts from the leaves, stems, and roots of the four plants were examined. The peptide content was observed to be higher during the pre- and post-monsoon seasons, with values significantly decreasing during the monsoon. The influence of seasonal variations in physicochemical parameters was found to be significant in the production of antimicrobial peptides.
- ❖ Phytochemical examination of crude and partially purified extracts (precipitated with 90% ammonium sulfate) revealed the continued presence of flavonoids and tannins despite the partial purification process.
- ❖ Charcoal-based adsorption of polyphenols (flavonoids, tannins etc.) from the crude extracts was carried out, and the process parameters derived through the OFAT method optimized using Response Surface Methodology (RSM). The optimal values determined through RSM analysis were as follows: (A) 25.81 mg/L charcoal, (B) 20 min, and (C) 28°C, resulting in a maximum decolorization efficiency of 89.15% and a protein recovery of 44.41%.
- ❖ Modelling of the decolorization process of *E. agallocha* leaf extract was carried out by fitting the decolorization data into Langmuir and Freundlich equations. A linear plot was obtained for the Freundlich equation over the studied concentrations, suggesting that the adsorption process of these polyphenols exhibited a Freundlich behavior, which confirms multilayer adsorption on activated charcoal.
- ❖ The study further attempted to explore the bioactivities of cationic peptides extracted from the selected mangrove plants; these included antimicrobial activity (evaluated through a well diffusion assay), the DPPH scavenging assay (antioxidant activity, following Brand-Williams et al., 1995), and alpha-amylase inhibition assay (to assess the potential antidiabetic activity, based on Bernfeld, 1995). All tested extracts from the mangrove plants exhibited antimicrobial activity, with that from *E. agallocha* leaf demonstrating notably higher and broad-spectrum activity compared to the others. Leaf, stem and root extracts from all the four mangrove plants elicited significant radical (DPPH) scavenging activity, but very low alpha amylase inhibitory activity.

### **Purification and characterization of a cationic peptide from *Excoecaria agallocha***

- ❖ The chromatographic separation of the 0-90% ammonium sulfate fraction of *Excoecaria agallocha* leaf extract through a Sephadex G-50 gel filtration column, coupled with SDS-PAGE and Acid-urea-PAGE analyses confirmed the purification of a small peptide (<3.5 kDa) which resolved cathodally on the acid-urea gel. The peptide was designated 'Eapl'. The mass spectrum obtained upon MALDI-TOF analysis established the intact molecular weight of the purified peptide as 1904.6 Da.

### **Screening of marine microbes for production of antimicrobial cationic peptides**

- ❖ Microbes from the marine environment of estuarine and coastal beach waters of Goa were explored for bioactive cationic peptide production. The physicochemical parameters were recorded at each sampling site.
- ❖ Total viable counts of  $660 \times 10^3$  cfu/mL and  $210 \times 10^2$  cfu/mL, respectively, upon incubation on ZMA and NA media for 24 h, indicated higher bacterial load in estuarine water environments than in the higher salinity waters, suggesting a role of salinity variation as a driver of community composition in these habitats. On seawater agar (SWA) medium, growth was observed after 72 h, with total viable count of  $9.2 \times 10^2$  cfu/mL, which was significantly lower than that observed in richer media such as ZMA and NA.
- ❖ In all, 82 bacterial isolates were singled out, and the overall proteinaceous content in their exudates screened for antimicrobial activity. Of these, 51.8 % showed activity against *S. typhimurium*, 8.8 % against *C. albicans*, 13.9 % against *E. coli*, 30.3 % against *S. aureus* and 7.5 % against *A. hydrophila*.
- ❖ Five of the active isolates were selected on the basis of broad-range antimicrobial activity against pathogens.
- ❖ Their exudate samples were partially purified by ammonium sulphate precipitation at 90% saturation and the total proteins obtained analyzed on 15% SDS-PAGE, of which the fraction from isolate VPU003 showing a low molecular weight band (~ 3.5 kDa) was of interest.

### **Purification and characterization of a cationic peptide from bacterial isolate VPU003**

- ❖ The proteinaceous pellet obtained from isolate VPU003 exudate was purified on a Sephadex G-50 column. While a broad peptide peak was obtained, the antimicrobial activity was associated only with lower molecular weight fractions therein. The pooled and concentrated active fractions showed the presence of single band on SDS-PAGE and AU-PAGE analyses, and MALDI-TOF MS analysis of this peptide designated 'Vpup' revealed a major peak at 3351.4 Da.

### **Potential Applications of Two Cationic Peptides**

- ❖ This study involved encapsulating of peptide Eapl from *E.agallocha* and peptide Vpup from the bacterial isolate VPU003 within a Chitosan hydrogel matrix and studying their properties for potential wound dressing applications.
- ❖ Moisture retention potential of the hydrogel was evaluated. Both hydrogels experienced water loss within 24 h, but the Chitosan-Vpup hydrogel lost only a minimal amount, approximately 5%. In contrast, the Chitosan-Ealp hydrogel showed a higher water loss of approximately 25% during this period. However, neither hydrogel exhibited significant moisture loss after 72 h of incubation.
- ❖ Swelling index measurement experiments were conducted at pH values of 3, 5, and 9, and at a temperature of 37°C. The average weight change was calculated. Chitosan-Eapl hydrogel could hold up to 10-14 times fold of its weight in water at pH 5, pH 7 and pH 9 and Chitosan-Vpup hydrogel up to 2.5-3.5 fold of its weight in water at these pH values.
- ❖ The hydrogels were further tested for drug release over time, under different pH conditions. The cumulative drug release (Abs at 280 nm) profiles demonstrated that the drugs eluted gradually over time (24- 48 h).
- ❖ The enzymatic degradation stability of the hydrogels was evaluated using lysozyme as well as trypsin in PBS solution at 37°C, over a 72 h period. All hydrogels displayed a

considerable increase in weight during the experiment. It can be observed that they were unaffected by lysozyme but cleaved by trypsin. That lysozyme was unable to significantly break the diester bond in our materials, was suggestive of enzymatic stability.

- ❖ Antimicrobial activity of Chitosan-Eapl and Chitosan-Vpup hydrogel was tested on *Staphylococcus aureus* bacterial culture by the disc diffusion method. An antimicrobial zone of clearance of 9 mm was observed for Chitosan-Eapl hydrogel and 10 mm for Chitosan-Vpup hydrogel.

To conclude, this thesis was all about “Exploring Mangrove Plants and Marine Microbes for Potent Cationic Peptides with Biotechnological Applications”. The salient achievements of this research have been covered under the five sub-heads elaborated above.

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## *Appendix*

### APPENDIX – I Media preparations

#### **Nutrient Agar**

|                         |         |
|-------------------------|---------|
| Nutrient agar (HiMedia) | 28 g    |
| Distilled water         | 1000 mL |

Dissolve 28.0 grams in 1000 mL of purified/distilled water and heat until boiling to ensure complete dissolution. Sterilize the solution by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow it to cool to 45-50°C. Thoroughly mix the solution and pour it into sterile Petri plates.

#### **Nutrient Broth**

|                          |         |
|--------------------------|---------|
| Nutrient broth (HiMedia) | 13 g    |
| Distilled water          | 1000 mL |

Dissolve 13 grams in 1000 mL of distilled water, heat if required to achieve complete dissolution and sterilize it by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

#### **Zobell Marine Agar**

|                                   |         |
|-----------------------------------|---------|
| Zobell Marine Agar 2216 (Himedia) | 55.25g  |
| Distilled water                   | 1000 mL |

Dissolve 55.25 grams in 1000 mL of purified/distilled water by heating until boiling to ensure thorough dissolution. Sterilize the solution by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Once sterilized, allow the solution to cool to a temperature range of 45-50°C. Mix the solution well and then pour it into sterile Petri plates.

### **Zobell Marine Broth**

|                                    |       |
|------------------------------------|-------|
| Zobell Marine Broth 2216 (Himedia) | 40.25 |
|------------------------------------|-------|

|                 |         |
|-----------------|---------|
| Distilled water | 1000 mL |
|-----------------|---------|

Suspend 40.25 grams in 1000 mL purified / distilled water. Heat if necessary to dissolve the medium. Dispense into tubes or flasks as desired. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

### **Seawater Agar**

|                |    |
|----------------|----|
| Agar (Himedia) | 25 |
|----------------|----|

|                   |         |
|-------------------|---------|
| Filtered seawater | 1000 mL |
|-------------------|---------|

Dissolve 25 grams of the medium in 1000 mL of purified or distilled water. Sterilize by autoclaving at 15 pounds pressure (121°C) for 15 minutes. After sterilization, allow the solution to cool within the temperature range of 45-50°C. Thoroughly mix the solution before pouring it into sterile Petri plates.

### **Mueller-Hinton Agar**

|                               |      |
|-------------------------------|------|
| Mueller Hinton agar (HiMedia) | 38 g |
|-------------------------------|------|

|                 |         |
|-----------------|---------|
| Distilled water | 1000 mL |
|-----------------|---------|

Dissolve 38.0 grams in 1000 mL of purified/distilled water by heating until boiling to ensure full dissolution. Sterilize the solution by autoclaving at 15 lbs pressure (121°C) for 15 minutes. After sterilization, cool the solution to a temperature range of 45-50°C. Thoroughly mix the solution and pour it into sterile Petri plates.

## APPENDIX – II Chemical Reagents Preparation

### **Sodium acetate buffer - 0.2 M Stock (pH 5.6)**

Solution A contains 0.2 M acetic acid, while Solution B contains 0.2 M sodium acetate. X mL of Solution A and Y mL of Solution B were combined and then diluted to a total volume of 100 mL using distilled water (D/W).

| <b>Solution A (mL)</b> | <b>Solution B (mL)</b> | <b>pH</b> |
|------------------------|------------------------|-----------|
| 46.3                   | 3.7                    | 3.6       |
| 44.0                   | 6.0                    | 3.8       |
| 41.0                   | 9.0                    | 4.0       |
| 36.8                   | 13.2                   | 4.2       |
| 30.5                   | 19.5                   | 4.4       |
| 25.5                   | 24.5                   | 4.6       |
| 14.8                   | 35.2                   | 4.8       |
| 10.5                   | 39.5                   | 5.0       |
| 8.8                    | 41.2                   | 5.2       |
| 4.8                    | 45.2                   | 5.6       |

It provides a suitable environment for maintaining a stable pH level, particularly in the slightly acidic range. Storage of the buffer can be done at room temperature or refrigerated at 4°C, depending on convenience and frequency of use.

### **Bradford Protein Reagent**

The Bradford protein reagent is prepared at the following concentrations of its different components in distilled water (Bradford, 1976):

Coomassie Brilliant Blue G-250                      100 mg

|                           |        |
|---------------------------|--------|
| Methanol 95% (v/v)        | 50 mL  |
| Phosphoric acid 85% (v/v) | 100 mL |
| Glycerol                  | 100 mL |
| Distilled water           | 750 mL |

Combine all the listed ingredients. Filter the mixture through Whatman filter paper No. 1 and store it in an amber-colored glass bottle at 4°C.

### **SDS-PAGE reagents**

#### **Resolving gel 15%**

|                                    |        |
|------------------------------------|--------|
| 1.5 M Tris-HCl (pH 8.8)            | 2.6 mL |
| Deionized distilled water          | 2.2 mL |
| 10% (w/v) SDS                      | 100 µL |
| 30% (w/v) Acrylamide/bisacrylamide | 5.0 mL |
| 10% (w/v) APS                      | 100 µL |
| TEMED                              | 10 µL  |

#### **4% Stacking gel**

|                                    |          |
|------------------------------------|----------|
| 0.5 M Tris-HCl (pH 6.8)            | 1.25 mL  |
| Deionized distilled water          | 2.975 mL |
| 10% (w/v) SDS                      | 50 µL    |
| 30% (w/v) Acrylamide/bisacrylamide | 0.67 mL  |
| 10% (w/v) APS                      | 50 µL    |
| TEMED                              | 5 µL     |

Tank buffer (5X Stock, pH 8.3)

|           |     |
|-----------|-----|
| Tris base | 15g |
|-----------|-----|

|               |         |
|---------------|---------|
| Glycine       | 72g     |
| SDS           | 5g      |
| Deionized D/W | 1000 ml |

#### Sample loading dye (5X Stock)

|                             |        |
|-----------------------------|--------|
| 0.5% (w/v) Bromophenol blue | 0.6 mL |
| 0.5 M Tris-HCl (pH 6.8)     | 1 mL   |
| 10% (w/v) SDS               | 1.6 mL |
| $\beta$ -mercaptoethanol    | 0.4 mL |
| Glycerol                    | 8 mL   |
| Deionized distilled water   | 4 mL   |

Store the sample loading dye in a dark colored bottle at 4 °C.

#### Coomassie Brilliant Blue stain

|                                 |        |
|---------------------------------|--------|
| Ammonium sulphate               | 50 g   |
| Deionized distilled water       | 4 mL   |
| 5% (w/v) Coomassie blue G-250   | 12 mL  |
| Orthophosphoric acid 100% (v/v) | 50 mL  |
| Methanol                        | 100 mL |

Filter the solution through Whatman filter paper and refrigerate at 4 °C.

### **Acid-Urea PAGE reagents**

#### **15% Resolving gel**

|                                    |             |
|------------------------------------|-------------|
| Urea                               | 3.6 g       |
| 30% (w/v) Acrylamide/bisacrylamide | 5 mL        |
| Glacial acetic acid                | 500 $\mu$ L |

|                           |        |
|---------------------------|--------|
| Deionized distilled water | 4.1 mL |
| 10% (w/v) APS             | 140 µL |
| TEMED                     | 60 µL  |

#### **Sample buffer (1X)**

|                           |         |
|---------------------------|---------|
| Urea                      | 3.6 g   |
| Glacial acetic acid       | 50 µL   |
| 0.2% (w/v) Methylene blue | 100 µL  |
| Glycerol                  | 300 µL  |
| Deionized distilled water | 300 µL` |

#### **Running buffer**

|                      |                      |
|----------------------|----------------------|
| 5% (v/v) Acetic acid | 5% (v/v) Acetic acid |
|----------------------|----------------------|

#### **DPPH assay reagent**

##### **DPPH Solution (5 Mm stock)**

|                                      |           |
|--------------------------------------|-----------|
| DPPH (1,1-diphenyl-2-picrylhydrazyl) | 1.9716 mg |
| Methanol                             | 1 mL      |

#### **Dinitrosalicyclic acid (DNSA reagent)**

|                            |            |
|----------------------------|------------|
| 3,5-dinitrosalicyclic acid | 1g         |
| 1 M NaOH                   | 50 mL      |
| Sodium sulphite            | 0.05%, w/v |
| phenol                     | 0.2% (v/v) |

|                    |      |
|--------------------|------|
| potassium tartrate | 20 g |
|--------------------|------|

|                           |       |
|---------------------------|-------|
| Deionized distilled water | 50 mL |
|---------------------------|-------|

All the chemicals were added with with continuous stirring finally to the solution and volume was made up to 100 mL with D/W.

### **Mayers reagent**

|                   |        |
|-------------------|--------|
| Mercuric chloride | 1.36 g |
|-------------------|--------|

|                  |    |
|------------------|----|
| Potassium iodide | 5g |
|------------------|----|

|                           |        |
|---------------------------|--------|
| Deionized distilled water | 100 mL |
|---------------------------|--------|

## *List of Abbreviation*

|                    |                                                 |
|--------------------|-------------------------------------------------|
| %                  | Percentage                                      |
| °C                 | Degrees Centigrade                              |
| µg                 | Micrograms                                      |
| µg/mL              | Micrograms per milliliter                       |
| µL                 | Microlitre                                      |
| µmol               | Micromole                                       |
| µmol/L             | Micromoles per litre                            |
| AC                 | Anjuna beach                                    |
| ACN                | Acetonitrile                                    |
| ACPs               | Antimicrobial cationic peptides                 |
| Al                 | <i>Acanthus ilicifolius</i>                     |
| AMPs               | Antimicrobial peptides                          |
| AMR                | Antmicrobial peptides                           |
| ANOVA              | Analysis of variance                            |
| AU                 | Acid-Urea                                       |
| BHT                | Butylated hydroxytoluene                        |
| BLAST              | Basic Local Alignment Search Tool               |
| BLIS               | Bacteriocin-Like Inhibitory Substances          |
| BSA                | Bovine serum albumin                            |
| <i>C. albicans</i> | <i>Candida albicans</i>                         |
| CCD                | Central Composite Design                        |
| CE                 | Cacra beach                                     |
| CFU/mL             | Colony forming unit per mililitre               |
| CLB                | Colicin-Like Bacteriocins                       |
| CLSI               | The Clinical and Laboratory Standards Institute |
| cm                 | Centimetre                                      |
| CuSO <sub>4</sub>  | Copper sulphate                                 |
| DFUs               | Diabetic Foot Ulcers                            |
| DM                 | Divar Mangrove                                  |
| DM                 | Divar mangrove area                             |
| DNA                | Deoxyribonucleicacid                            |

|                |                                                                 |
|----------------|-----------------------------------------------------------------|
| DO             | Dissolved oxygen                                                |
| DPPH           | 2,2-Diphenyl-1-picrylhydrazyl                                   |
| <i>E. coli</i> | <i>Escherichia coli</i>                                         |
| EA             | <i>Excoecaria agallocha</i>                                     |
| g              | Grams                                                           |
| g/L            | Gram per litre                                                  |
| GAEE           | Gallic Acid Equivalent                                          |
| GAP            | Global action plan                                              |
| h              | Hour(s)                                                         |
| HDPs           | Host Defense Peptides                                           |
| kDa            | kiloDalton                                                      |
| L              | Litre                                                           |
| LC-MS          | Liquid chromatography mass spectrometry                         |
| LTP            | Lipid transfer proteins                                         |
| M              | Molar                                                           |
| MALDI-TOFF     | Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass |
| MS             | Spectrometry                                                    |
| MC             | Moisture Content                                                |
| ME             | Miramar beach                                                   |
| mg             | Milligram(s)                                                    |
| mg/L           | Milligram per litre                                             |
| mg/mL          | Milligram per millilitre                                        |
| MIC            | Minimum inhibitory concentration                                |
| min            | minute(s)                                                       |
| mL             | milliliter(s)                                                   |
| mM             | Millimolar                                                      |
| MRSA           | Methicillin resistant <i>Staphylococcus aureus</i>              |
| MTCC           | MicrobialType CultureCollection                                 |
| N              | Normality                                                       |
| NA             | Nutrient Agar                                                   |
| NaCl           | Sodium chloride                                                 |
| NB             | Nutrient Broth                                                  |
| NCBI           | National Center for Biotechnology Information                   |

|          |                                                           |
|----------|-----------------------------------------------------------|
| NCL      | National Chemical Laboratory                              |
| nm       | Nanometre                                                 |
| NMR      | Nuclear magnetic resonance                                |
| OD       | Optical density                                           |
| PCR      | Polymerase chain reaction                                 |
| pH       | Hydrogen ion concentration                                |
| ppm      | Parts per million                                         |
| ppt      | Parts per thousand                                        |
| psu      | Percentile salinity units                                 |
| PTLB     | Phage Tail-Like Bacteriocins                              |
| RCI      | Remaining Colour Intensity                                |
| RM       | Rhizhophora mucronata                                     |
| RNA      | Ribonucleic acid                                          |
| rpm      | Rotations per minute                                      |
| rRNA     | Ribosomal ribonucleic acid                                |
| RSM      | Response Surface Methodology                              |
| s        | Second(s)                                                 |
| SA       | <i>Sonneratia alba</i>                                    |
| SD       | Standard deviation                                        |
| SDS PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| sp.      | Species                                                   |
| subsp.   | Subspecies                                                |
| SWA      | Seawater Agar                                             |
| TDS      | Total dissolved solids                                    |
| TPC      | Total Phenolic Content                                    |
| TVC      | Total viable count                                        |
| U/mg     | Units per milligram                                       |
| UV-Vis   | Ultraviolet-Visible                                       |
| VC       | Vagator beach                                             |
| WHA      | World Health Assembly                                     |
| WHO      | World Health Organization                                 |
| ZMA      | Zobell marine agar                                        |

## ***Publications***

### **Publications in journal:**

- ❖ Naik, P. G., & Muraleedharan, U. D. (2023). Production of peptidic antimicrobials by bacterial populations from diverse coastal habitats of Goa, India. *Indian Journal of Geo-Marine Sciences*, 52, 292-299.

### **Papers presented at Conferences:**

- ❖ Presented a paper entitled, “Bioactive peptides from mangrove plant sources” at the National Conference on Recent Trends in Biotechnology held on 19–21 Oct 2016 at NIOT, Chennai. (Poster presentation).
- ❖ Presented a paper entitled, “Decolourization and partial purification of antimicrobial peptides from acid extracts of *Excoecaria agallocha* using activated charcoal” at the International Conference on Impact of Innovations In Science and Technology for Societal Development (IISTSD– 2019) held on 19-21 Sept 2019 at Kongunadu College, Coimbatore (Oral presentation).
- ❖ Presented a paper entitled, “Response Surface Methodology for optimization of activated charcoal treatment in the study of peptide antimicrobials from *Excoecaria agallocha* extracts” at the National Seminar, New Vistas in Botany held on 13-14 Feb 2020 at Goa University, Goa (Poster presentation).

## Production of peptidic antimicrobials by bacterial populations from diverse coastal habitats of Goa, India

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With conventional antibiotics rapidly losing their efficacy, marine microorganisms found in diverse salinity conditions are being explored for new antibacterial agents. Salinity is a major contributing factor to the distribution of biota in aquatic systems, and little is known about the production of antimicrobials by microbial communities in response to stressful environmental conditions. This work adds to the comprehension of bacterial abundance in estuarine and coastal beach waters of Goa and population structuring concerning their antimicrobial activity as specifically conferred by antimicrobial peptides. Total viable counts indicated higher bacterial load in estuarine water environments than in the higher salinity waters, suggesting a role of salinity variation as a driver of community composition in these habitats. In total, 82 bacterial isolates were selected, and the overall proteinaceous content in their exudates were screened for antimicrobial activity against *Salmonella typhimurium* (MTCC 91), *Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (MTCC 443), *Candida albicans* (MTCC 227) and *Aeromonas hydrophila* (MTCC 1739), by the agar well diffusion method. Of these, 51.8 % showed activity against *S. typhimurium*, 30.3 % against *S. aureus*, 13.9 % against *E. coli*, 8.8 % against *C. albicans*, and 7.5 % against *A. hydrophila*. Compared with estuarine bacteria, those from more saline waters showed higher production of potent antimicrobial peptides, which were probably used to counter the high competition for resources. Such sites could, therefore, be potential niches for bioprospecting of microbes producing bacteriocin-like compounds.

[**Keywords:** Antimicrobial activity, Bacterial isolates, Peptidic antimicrobials, Salinity]

### Introduction

Marine waters have distinctive properties and may harbour biota native to coastal or open ocean regions. Coastal zones are generally rich in organic matter due to terrestrial runoff and are better endowed with primary productivity. These zones include unique niches such as coral reefs, mangroves and estuarine habitats, besides rocky as well as sandy beaches<sup>1,2</sup>. Physico-chemical parameters such as temperature, nutrient content and salinity play an important role in determining the community composition and abundance of many organisms, which in turn defines the functional characteristics of these ecosystems. Among the abiotic factors, salinity has been extensively quoted to be the major contributor influencing the microbial community composition<sup>3-8</sup>. There are many reports focusing either on determining the microbial diversity in marine environments<sup>9-11</sup>, or on the isolation of novel bioactive compounds from marine microorganisms<sup>12</sup>. However, only a few studies are available on bioactive metabolites obtained from different isolates in specific relation to their distinctive habitats. Due to

unique habitat differences that display diversity in ambient environmental conditions (salinity, pressure, temperature, light, oxygen, pH and nutrient availability), organisms tend to develop various chemo-physiological processes for survival. Such metabolic processes of marine microbes encourage the synthesis of a range of bioactive molecules exhibiting activities such as antimicrobial, anticancer, antiviral and antifungal<sup>13,14</sup>. While their isolation was initially carried out from sites at random, there is now a growing recognition that the sampling location could play an important role in increasing the success rate of bioactivity discoveries<sup>15,16</sup>.

Marine microbes are known to produce various classes of metabolites such as polyketides, terpenes, peptides, and alkaloids which have been shown to exhibit antimicrobial and antiviral activity<sup>17,18</sup>. As the conventional small molecules lose their potency quickly due to antimicrobial resistance<sup>19</sup>, one important class which has been drawing increasing attention is that of proteinaceous or peptidic antimicrobials from bacteria, also known as bacteriocins. It has the advantage of being minimally

vulnerable to such resistance. Killing mechanisms adopted by bacteriocins are (a) formation of ion-permeable pores in the cytoplasmic membrane, (b) degradation of cellular DNA, and (c) alteration in the structure of the 30S RNA subunit with subsequent degradation of rRNA or tRNA. These peptides have different structural diversity and properties and vary significantly in molecular weight, from small peptides (microcins, < 10 kDa) to large ones (colicins, > 80 kDa)<sup>20</sup>. Bacteriocins are majorly produced by Gram-positive bacteria and are divided into four classes. Class I includes lantibiotics, which are hydrophobic, thermostable, and pore-forming peptides with a low molecular weight (< 5 kDa). Class II peptides are also heat-stable and small in size (< 10 kDa) and mainly induce destabilisation and permeabilisation of the bacterial membranes or cause pore formation into the membrane. Class III bacteriocins are large peptides (> 30 kDa) and may be heat-labile, lytic or non-lytic. These bacteriocins have an antibacterial activity linked to enzymatic activity (such as endopeptidase), leading to the disruption of the bacterial cell wall. Class IV bacteriocins contain lipid or carbohydrate parts which disrupt the bacterial cell membrane. Due to their peculiar structure these peptides are sensitive to several enzymes (such as glycolytic or lipolytic enzymes)<sup>21</sup>. Bacteriocins produced by Gram-negative bacteria are divided into four major classes: colicins (10 kDa), Colicin-Like Bacteriocins (CLB), Phage Tail-Like Bacteriocins (PTLB), and microcins<sup>22</sup>.

Microbes thriving under varying salinity conditions can be explored further to obtain natural products. A large number of the strains known to produce protein/peptide antibiotics against the most common pathogenic bacteria have been isolated from various marine environments. Bacitracin, which showed inhibitory activity against many Gram-positive organisms, is produced from a marine bacterium *Bacillus licheniformis*<sup>23</sup>. Enterocin isolated from *Enterococcus faecium* found in the mangrove environment of the Vellar estuary on the east coast of India showed antimicrobial activity against a broad range of Gram-positive and Gram-negative pathogens viz., *S. aureus*, *B. subtilis*, *E. coli* and *Vibrio* sp.<sup>24</sup>. Pumivitticin, a bacteriocin derived from the marine bacterium *Bacillus pumilus* DR2 isolated from seawater samples from Ennore (Tamil Nadu, India), showed antimicrobial activity against *Lactobacillus jugurti*, *Lactobacillus casei*, *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Listeria monocytogenes*<sup>25</sup>. In addition to clinical applications,

such peptides have a wide variety of applications such as in the food industry, animal husbandry, agriculture, aquaculture and are also used to tackle biofilm and biofouling challenges<sup>26,27</sup>.

To the best of our knowledge, this is the first report on a possible role of the ambient salinity in antimicrobial peptide production potential of bacteria sourced from varying salinity zones such as Divar mangrove area (fluctuating salinity zone), Miramar and Cakra beaches (less saline, near-estuarine regions) as well as Vagator and Anjuna beaches (coastal waters) of Goa, India. While some bacteria isolated from salinity zones have been reported to show antibiotic activities, the present work focuses majorly on proteinaceous antimicrobials (bacteriocins), as distinct from antibiotics as a class.

## Materials and Methods

### Sites and sample collection

The coastline of Goa, India, is characterised by continuous stretches of sandy beaches, with several places intersected by estuarine river systems that empty into the Arabian Sea. For the isolation of marine bacteria, five of Goa's geographical areas of study were sampled (Fig. 1). The sites were selected for their salinity characteristics. The samples were collected in the month of January 2018 from estuarine waters near the Divar Mangrove (DM) area, which is separated from the mainland by the Mandovi estuary; Miramar beach (ME) intertidal zones; the near-estuarine regions at Cakra (CE) beach; and the more typically saline waters of Vagator (VC) and Anjuna (AC) beaches which had minimal influence from river water discharge. Surface water sample from each site

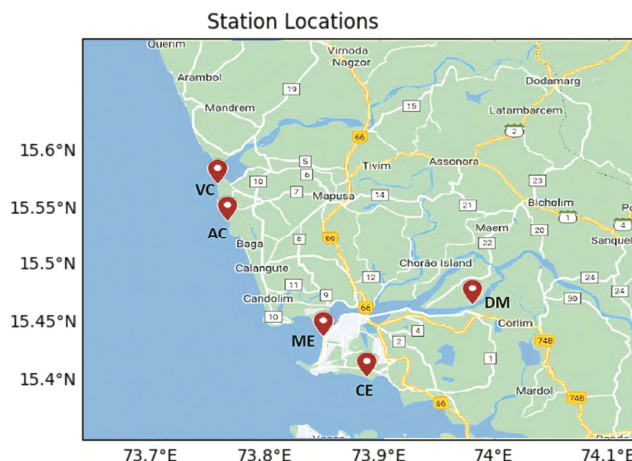


Fig. 1 — Geographical location of study area and sampling sites: DM - Divar mangrove area, ME - Miramar beach, CE - Cakra beach, VC - Vagator beach, and AC - Anjuna beach

(approx. 1 L) was collected in pre-sterilised screw-capped bottles, transferred on ice to the laboratory and processed within 24 h.

#### Measurement of physico-chemical parameters at sampling sites

Salient parameters of the water samples, such as temperature, salinity, conductivity, Total Dissolved Solids (TDS) and Dissolved Oxygen (DO), were determined on the field using a multiparameter instrument (CyberScan PC 650, Eutech Instruments, Thermo Fisher Scientific, India) and pH was measured on a digital pH meter (Eutech pH meter-700, Thermo Fisher Scientific, India).

#### Isolation and enumeration of bacteria

The bacterial population in water samples was enumerated using the standard spread-plate technique<sup>28,29</sup>. Water sample (1 mL) collected from each location was serially diluted using sterile saline and 0.1 mL spread-plated on Zobell Marine Agar (ZMA), Nutrient Agar (NA) and Seawater Agar plates (SWA) which were then incubated at room temperature for 24 h, 48 h and 72 h, respectively, for enumeration of Total Viable (culturable) bacterial Counts (TVC). The ZMA and NA were procured from HiMedia, while, for the SWA preparation, 3 g agar powder (HiMedia) was added to 100 mL aged and filtered seawater and autoclaved.

#### Test pathogens used for screening of antagonistic activity

Pure cultures of *Salmonella typhimurium* (MTCC 98), *Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (MTCC 443), *Candida albicans* (MTCC 227) and *Aeromonas hydrophila* (MTCC 1739) were used for the study. These bacterial and fungal strains obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India were maintained on nutrient agar, prior to use.

#### Antimicrobial assay

Antimicrobial activity of the isolates was evaluated by the agar-well diffusion method, as per

Shanmugaraju *et al.*<sup>30</sup>. The predominant isolates were selected based on morphological characteristics, subcultured and maintained on ZMA. The test pathogen inocula, prepared by transferring a loopful of culture into 100 mL nutrient broth and incubating for 24 h on an orbital shaker, were swabbed onto NA plates. The isolates were allowed to grow in Zobell marine broth for 24 h on a rotary shaker. The broth culture was then centrifuged at 6,000 rpm for 10 min at 4 °C to obtain the Cell-Free Supernatant (CFS). Solid ammonium sulphate was added to 90 % (w/v) saturation, and the CFS was stirred for 3 to 4 h at 4 °C to maximally precipitate proteinaceous molecules. Centrifugation was then carried out at 10,000 rpm for 30 min and 4 °C. The pellet obtained was re-suspended in 10 mM sodium acetate buffer (pH 5.6). Wells (6 mm) were punched onto the test pathogen NA plates using a borer, and 30 µl of CFS (crude or partially purified) was added into the wells. Zones of inhibition were measured after incubation for 24 h at 37 °C.

#### Protein estimation

Ammonium sulphate precipitated samples suspended in 10 mM sodium acetate buffer, pH 5.6 were analysed for protein content by the Bradford method<sup>31</sup>, using Bovine Serum Albumin (BSA) as standard.

## Results and Discussion

The physico-chemical parameters recorded at each sampling site are summarised in Table 1. The pH value, which represents the degree of ionisation of the sampled water, ranged from 7.36 to 8.5. The temperature of the surface water at these sites varied from 28 to 29.8 °C. Salinity variations were from 26.9 to 33.4 psu; Vagator (VC) and Anjuna (AC) beach sites exhibited the highest salinity (33.04 to 33.42 psu), as expected. The Miramar (ME) and Cakra (CE) sites were comparatively less saline, being influenced by the input of freshwater from the Mandovi and Zuari rivers. Salinity of estuarine waters around the Divar Mangrove area (DM) was 26.9 psu. Notwithstanding interannual variabilities and

Table 1 — Variations of ambient parameters in the surface water samples collected from different sampling locations: DM - Divar mangrove area, ME - Miramar beach, CE - Cakra beach, VC - Vagator beach, and AC - Anjuna beach

| Location (& Station code) | pH        | Temperature (°C) | DO (mg/L) | Salinity (psu) | Conductivity (ms) | TDS (ppt)  |
|---------------------------|-----------|------------------|-----------|----------------|-------------------|------------|
| Divar (DM)                | 7.36±0.07 | 28±0             | 6.81±0.37 | 26.95±0.18     | 43.35±0.93        | 28.86±2.81 |
| Miramar beach (ME)        | 8.31±0    | 29.8±0           | 6.01±0    | 31.96±0.15     | 48.95±0.21        | 34.29±0.26 |
| Cakra beach (CE)          | 8.35±0.01 | 28±0             | 5.97±0.32 | 32.14±0.08     | 49.18±0.12        | 34.44±0.08 |
| Vagator beach (VC)        | 8.41±0.01 | 29.8±0           | 6.68±0.11 | 33.04±0.21     | 50.42±0.28        | 35.31±0.2  |
| Anjuna beach (AC)         | 8.50±0    | 29.8±0           | 6.70±0.12 | 33.42±0.18     | 50.93±0.25        | 35.63±0.12 |

seasonal influences, these values are in agreement with the data from earlier reports<sup>32,33</sup>, where salinity values ranging from 31 to 35 psu were reported in coastal beach waters and from 24 to 27 psu in mangrove areas of Goa. In the present study, Miramar and Cakra beach areas were categorised as ‘near-estuarine’ regions and the mangrove habitat as ‘estuarine’ environment, while the Vagator and Anjuna sites better conformed to ‘coastal ocean’ environments. All these typically exemplify the dynamic environmental conditions that characterise the estuarine and marine waters around Goa.

Dissolved oxygen (DO), being one of the limiting factors for the growth and functioning of marine microorganisms, becomes a very useful parameter to gauge water quality. The observed DO values ranged between 5.97 and 6.7 mg/L. Total Dissolved Solids (TDS) comprise of inorganic salts (bicarbonates, chlorides and sulphates of calcium, potassium, magnesium and sodium) and some amount of organic matter. The measured TDS values ranged from 28.8 to 35.6 ppt. Electrical conductivity depends upon the quantity of dissolved salts in the water, and it ranged from 43.3 to 50.9 ms. Maximum and minimum values for pH, DO, salinity, conductivity and TDS were

recorded at AC and DM sites, respectively, in the month of January.

Although, in general, abiotic parameters such as salinity, nutrient concentration and organic matter would all influence the composition of natural microbiota<sup>34,35</sup>, salinity, in particular, influences the osmoregulatory functions of organisms and would therefore be a dominant factor in shaping microbial diversity and population in the marine waters<sup>36</sup>. Previous studies have shown the influence of salinity on microbial community composition<sup>37</sup>. There are, however, very few reports on bacterial abundance over a range of salinities especially in relation to their bioactivities. The focus of this study was hence directed to evaluate the influence of salinity ranges of estuarine and marine habitats on the total viable bacterial count and the production of peptidic antimicrobials.

#### Total viable count

Bacterial abundance in the estuarine and coastal waters of Goa was gauged by the TVC, which would provide an estimate of their culturable load in these waters (Fig. 2). The values were highest at location DM, as  $660 \times 10^3$  cfu/mL and  $210 \times 10^2$  cfu/mL,

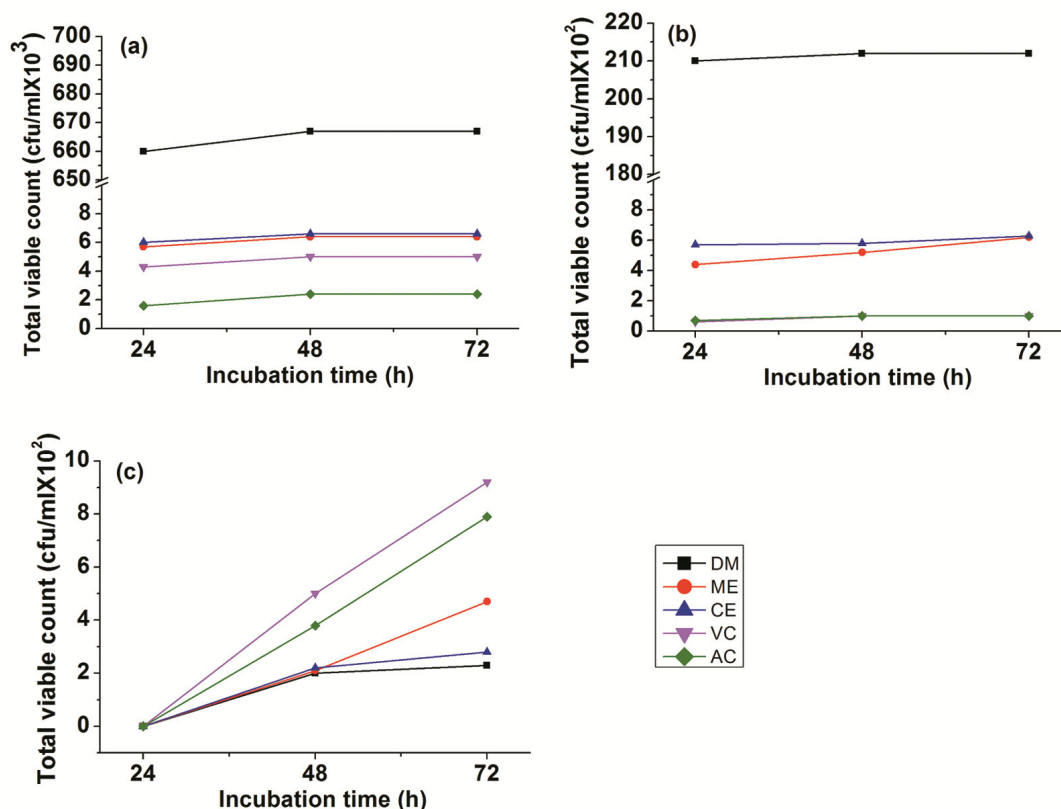


Fig. 2 — Total viable count at the sampling locations of various salinities on (a) Zobell marine agar, (b) Nutrient agar, and (c) Natural seawater agar. DM - Divar mangrove area; ME - Miramar beach; CE - Cakra beach; VC - Vagator beach; and AC - Anjuna beach

respectively, upon incubation on ZMA and NA media for 24 h. In all cases, only a slight increase in TVC was observed after 48 h of incubation, which remained unchanged even at 72 h, indicating that the exponential phase of growth was complete even at 24 h of incubation. No colonies were observed on SWA at 24 h, and even after 48 h of incubation, the highest TVC recorded was only  $5 \times 10^2$  cfu/mL at VC. Although a continued increase to  $9.2 \times 10^2$  cfu/mL was recorded in SWA medium at 72 h, this was markedly lower than the values in richer media such as ZMA and NA. This slow growth may be attributed to autoclaving of the medium, which could have affected the natural nutrient availability, as against filtration, which might have been a better choice.

The more significant values of TVC as recorded for growth in ZMA and NA media at 48 h were assessed in relation to ambient salinity at the sampling sites. It was observed that the values were higher in samples from a lower salinity environment (mangrove area) and decreased with increasing ambient salinity (Fig. 2a & b). Other parameters at the sampling sites besides salinity might also have contributed to the TVC differences, considering the trend of variations of pH, temperature, conductivity and TDS (Table 1).

A total of 82 distinct bacterial isolates, as obtained on three different media, were selected based on differences in colony morphology and Gram character. Estuarine samples showed more diversity of colonies than marine samples. The majority of isolates (71 – 77 %) obtained from the five sampling sites were Gram-negative (Fig. 3b, hatched bars, *cf.* Fig. 3a, hatched bars), in concurrence with a recent article that cites Gram-positive bacteria contributing to 14 % of the total bacterial count in seawater<sup>38</sup>.

Among the Gram-positive bacteria, a higher percentage of isolates obtained from estuarine and coastal waters were antimicrobially active (solid bars, Fig. 3a), while among the Gram-negative isolates, those exuding antimicrobially active compounds were predominant in the near-estuarine regions (solid bars, Fig. 3b). Unlike those introduced by runoff from terrestrial ecosystems, indigenous producers of antimicrobial peptides from the marine environment were mainly strains of Gram-positive marine bacteria, despite the relative abundance of Gram-negative species<sup>39</sup>. Although Gram-positive bacteria are basically abundant in soils and could get introduced into near-shore marine environments, their survival rates are low in competition with the indigenous

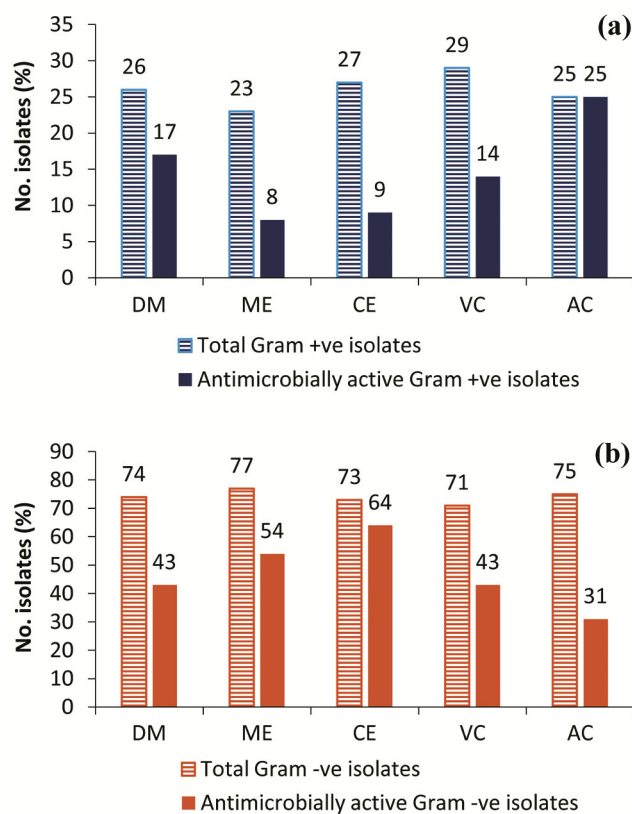


Fig. 3 — Percentage of (a) Gram-positive, and (b) Gram-negative isolates amongst the culturable isolates obtained during the sampling. The solid bars represent the relative percentage of Gram-positive and Gram-negative isolates that showed antimicrobial activity. The respective percent values (rounded up) are indicated above each bar

better-adapted Gram-negative species<sup>40</sup>. Besides, on account of their membrane porosity characteristics, Gram-positive organisms would be more sensitive to deleterious compounds in the vicinity. Such compounds that may be effectively transported across the thick peptidoglycan layer of Gram-positive bacteria lack, in many cases, the appropriate chemical properties to cross the vastly different glycolipid layer and membranes characteristic of Gram-negative bacteria<sup>41,42</sup>.

#### Antimicrobial activity in proteinaceous molecules

The search for proteinaceous or peptide antimicrobials from marine microbes as an alternative to conventional antibiotics being the focus of the current study, bioprospecting for antimicrobial activity was performed using the well diffusion assay (Fig. 4). Partial purification by ammonium sulphate precipitation would serve to extract proteinaceous material more specifically, besides also concentrating such active molecules. It is interesting to note that

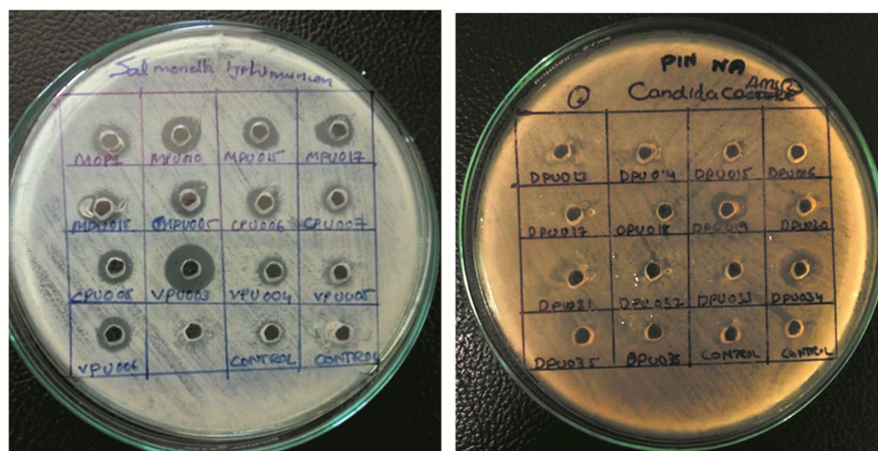
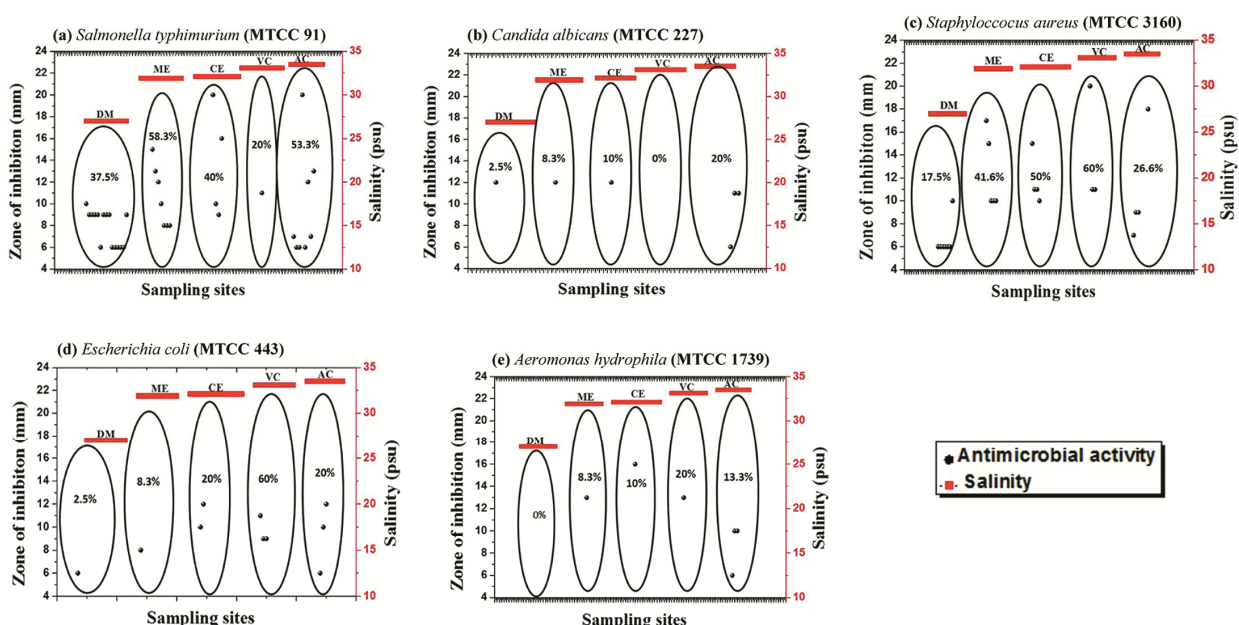


Fig. 4 — Antimicrobial susceptibility testing using the well diffusion method


 Fig. 5 — Antimicrobial activity in partially purified exudates of the isolates as against five pathogenic test cultures [(a) *Salmonella typhimurium* (MTCC 91), (b) *Candida albicans* (MTCC 227), (c) *Staphylococcus aureus* (MTCC 3160), (d) *Escherichia coli* (MTCC 443), and (e) *Aeromonas hydrophila* (MTCC 1739)], and viewed in relation to sampling site salinity variations

taken as a whole, about 61 % of the culturable isolates had antimicrobial activity associated with proteinaceous material (data not shown). The isolates displayed broad-spectrum inhibitory effects on the five test pathogens used, and the antimicrobial activity in partially purified CFS samples was higher than in the crude CFS preparations. The significantly enhanced activity upon partial purification is not surprising, considering that antimicrobial peptides and proteins are generally not generated in high amounts by the producer strain<sup>43</sup>. In fact, the proteinaceous content in these active preparations ranged from 0.7 to 2 mg/mL (data not shown).

#### Salinity variations and antimicrobial activity

As detailed above, a marked difference was observed in the microbial load from estuarine to coastal marine ecosystems and in their production of antimicrobial peptides/proteins. The comparative analysis using the partially purified proteinaceous samples is presented in Figure 5, in relation to the different sampling sites and test pathogens used. In the estuarine environment, high bacterial abundance contrasting with low antimicrobial activity was observed against all five pathogens tested. A possible explanation could be that many marine and freshwater microflora that entered into the estuarine mangrove

environment (mixing zone) were no longer active because of the rapid salinity fluctuations encountered. In contrast, the seawater salinity tolerant microbes appear to be a better-adapted community and are metabolically active<sup>44</sup>. Results of the present study indicated that as compared to estuarine bacteria, those in other areas showed the production of more potent antimicrobial peptides probably used to counter the high competition for resources. Hence, these could be a more promising source for bacteriocin-like compounds.

### Conclusion

Eighty two bacterial isolates were screened from distinct coastal environs of Goa. Bacterial abundance, antimicrobial potential of the isolates and their structuring in relation to the ambient salinity variations has been specifically investigated. There was a progressive increase in TVC from estuarine to higher salinity marine waters. Isolates from more saline coastal regions had stronger antimicrobial potential than those from the estuarine habitats. This bioactivity could be largely attributed to peptidic molecules. The present study adds to the knowledge of distribution of marine bacteria with antimicrobial potential for furthering biotechnological applications to address and alleviate rising concerns about antibiotic resistance. Further work on the isolation and characterisation of such antimicrobial peptides would open up avenues in the search for novel antimicrobial peptides and thereby, more such molecules would continue to enter clinical trials. Also, novel compounds with different chemical structures and biological activities would become available to counter the ever-growing antimicrobial resistance.

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### Conflict of Interest

The authors declare no conflict of interest.

### Author Contributions

PGN: Laboratory and field investigations, formal analysis, use of software and writing original draft of the manuscript; and UDM: Conceptualisation, supervision, and review & editing of the manuscript.

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