Research Article

Screening of Protease Inhibitors with Antibacterial Property from Mangroves of Kerala

Sapna Kesav[1], Basim Karayil Saidalavi[2], Manzur Ali Pannippara[2,*], Ammanamveetil Abdulla Mohamed Hatha[1,*]

[1]Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Cochin, Kerala, INDIA.
[2]Department of Biotechnology, MES College, Marampally, Aluva, Kerala, INDIA.

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ABSTRACT

Plants have long been used as a source of therapeutic agents. They are thought to be important ingredients in many prophylactic medicines. The aim of this study was to screen mangrove plants of Kerala for protease inhibitors (PI) and antibacterial activity. Biological ingredients from the bark and leaves of five different species of mangrove plants collected from Puthuvypin Station of Cochin coast, Kerala were extracted in phosphate buffer and fractionated using ammoniumsulphate. Out of 30 fractions assayed using α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), 12 were found to be inhibitory against the protease produced by the bacterium *Bacillus* sp. MESVP01 isolated from the mangrove rhizosphere sediment. The PI activity was also proved by qualitative caseinolytic plate assay. The mangrove extracts were further analyzed for antibacterial activity against 9 different standard test bacterial cultures. Bark extracts of mangroves; *Avicennia officinalis* Ao (B), *Avicennia marina* Am (B), *Eexcoecaria agallocha* Ea (B) and leaf extract of *Sonneratia caseolaris* Sc (L) were shown to inhibit the growth of *Pseudomonas aerogenosa*. The leaf extracts of *Rhizophora mucronata* Rm (L), *Sonneratia caseolaris* Sc (L) and *Avicennia marina Am* (L) inhibited the growth of *Staphylococcus aureus*. Results indicate that many mangrove species present in Kerala coast are potential source of protease inhibitors and antibacterial agents, which could be turned out in to valuable therapeutic agents.

Key words: Protease inhibitor, Mangroves, Antibacterial activity, Protease, Therapeutic agents.

[1]**Correspondence:**
Dr. Manzur Ali P P,
Department of Biotechnology, MES College Marampally Aluva-683 107, Kerala, INDIA.
Phone no: +91 9447587335
Email id: manzur@mesmarampally.org

[2]**Correspondence:**
Dr. A A Mohamed Hatha,
Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Cochin-682 016, Kerala, INDIA.
Phone no: +91 9446866050
Email id: mohamedhatha@gmail.com

INTRODUCTION

Protease inhibitors are a group of proteins that hinder the function of proteases, the enzymes that are capable of catalyzing the breakdown of proteins via a process known as proteolysis.[1] Proteases are involved in many life processes and have pivotal role in modulation of protein-protein interactions, generation of new bioactive molecules, regulation of activities of other proteins, contribution to the processing of cellular information and magnification of molecular signals.[2-4] Hence any dysregulation in their action may lead to serious pathological conditions.[5] Many proteases are linked to the life processes of pathogenic organisms too. Therefore selective and specific inhibition of these target proteases can be used as a strategy for drug designing and can be used as diagnostic or therapeutic agents for viral, bacterial, fungal and parasitic diseases as well as for treating cancer, immunological, neurodegenerative and cardiovascular diseases.[6-8] Protease inhibitors may within the next several years become available as a powerful accompaniment to now-standard cancer therapies.[8] The search for potent antiviral agents and antibacterial agents are urgent in view of the dramatic situation of the global pandemics.[9] In this situation, more attention

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should be paid to the search for more effective therapeutic agents present in natural products.

Plants have been identified as potential source for protease inhibitors with significant therapeutic potential.[10] Many types of plant protease inhibitors have been isolated and purified from various plants across the globe to assess their nutritional and medicinal properties.[11] It has been identified that plant protease inhibitors are effective for inactivating target proteases that are involved in pathogenesis of various human diseases such as cardiovascular disorders, cancers, HIV-AIDs, neurodegenerative diseases, inflammatory conditions, ulcerative colitis, pancreatitis and osteoporosis.[8,12-14] Diverse plant protease inhibitors are known to be efficient against cardiovascular disorders, inflammatory conditions, osteoporosis, neurodegenerative diseases and many viral and parasitic infections.[15,8]

Mangrove plants show great potential for use in the discovery of bioactive compounds.[16] Due to the abiotic factors and severe environmental conditions that impose adaptive pressures they produce substances that function in the defense of these plants and promote their effective development and survival in harsh environments. Extracts from mangrove plants are mainly used in folk medicines as insecticides, pesticides, astringents, or tonics for healing sores, dysentery and fever.[17] Therefore, the search for protease inhibitors from mangroves remains open to further investigation. In this juncture, the present work targets on the protease inhibitory as well as antibacterial property of mangroves of Cochin Coast of Kerala, India.

MATERIALS AND METHODS

Collection of mangroves

The mangrove plant materials were collected from Puthuvypin Station of Cochin Coast, Kerala and kept at 4°C until use. *Avicennia officianlis* (Ao), *A. marina* (Am), *Excoecaria agallocha* (Ea), *Rhizophora mucronata* (Rm) *and Sonneratia caseolaris* (Sc) were used for the screening. Leaves (L) and barks (B) of the plants were used for the study.

Screening of protease producing bacterium from mangrove rhizosphere and isolation of protease

Sediment sample was collected from mangrove rhizosphere from Puthuvypin station of Cochin Coast, Kerala. Serial dilutions were prepared and pour plated using Nutrient Agar (Hi-Media) medium. The isolated pure colonies were screened for protease production potential. For that the overnight grown culture in nutrient broth were centrifuged and a suitable dilution of crude cultures supernatant was added in the wells made in skimmed milk (1%) agar plates. The plates were incubated at 37°C. The hydrolysis of casein by bacterial protease was indicated by the formation of clearance zone around the well.

Identification of the selected protease producing bacterium

Molecular identification of the selected bacterium MESVP01 was performed by PCR amplification of 16S rDNA sequences. This was carried out using two degenerate primers.

Primer sequence

Forward primer : AGAGTTTGATCCTGGCTACAG
Reverse primer : ACGGCTACCTTGTTACGACTT

PCR amplification product was subjected to sequencing by the ABI Prism 310 genetic analyser by using the big dye Terminator kit (Applied Bio-systems). The sequenced PCR products were analysed online using BLAST software (http://www.ncbi.nlm.nih.gov/blast) and the identity of the sequences were determined.[18]

Extraction and recovery of protease inhibitor from mangrove plant materials

Plant materials (leaves and bark) were washed in distilled water and extracts were prepared in 0.01 M phosphate buffer (pH 7.5) by homogenizing 50 g of leaves in 50 mL and 20 g of bark in 80 mL of buffer. The slurry is then filtered through cheesecloth and centrifuged at 10,000 g for 10 min at 4°C for removing any cell debris. The clear supernatant represents the crude extract, which was kept at -20°C until used for the study.

Ammonium sulphate precipitation

Ammonium sulphate precipitation of the prepared sample was done.[19] The fractionation using ammonium sulphate precipitation has the advantage of intermediate removal of unwanted proteins with the simultaneous concentration of the protein of interest. Ammonium sulphate (SRL, India) required to precipitate the protease inhibitor was optimized by adding varying concentrations (initially at 30%, then, 60% and up to 90% saturations) to the crude extract.

Dialysis

The precipitated protein was resuspended in minimum quantity of 0.1 M phosphate buffer (pH 7.5). The solution was taken in the pre-treated dialysis tube (Sigma-Aldrich, cut off value 12 kDa) against 0.01 M solution of phosphate buffer pH 7.5 for 24 h at 4°C with frequent changes of buffer. The sample after dialysis

was assayed for protease inhibitory activity, protein content and specific activity.

Acetone precipitation

The dialyzed samples were further subjected to precipitation of protein with four times volume of cold acetone (at -20°C for 1 h) for the removal of any contaminating salts or lipids present. Then centrifuge at 10,000 g for 10 min at 4°C. Resulting pellets were air dried to evaporate all the acetone in it and dissolved in minimum quantity of 0.01 M phosphate buffer (pH 7.5) and kept at -20°C for further use.

Protein estimation

Protein content was estimated according to the method of Bradford (1976) using bovine serum albumin (BSA) as the standard and the concentration was expressed in mg/mL.[20]

Protease inhibitor assay using BAPNA

Protease inhibitor activity was also measured using the synthetic substrate α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA, Sigma).[21] Three hundred and seventy-five microlitre of the inhibitory protein diluted with phosphate buffer (pH 7.5) was incubated with suitable dilution of 25 µL of bacterial protease in 0.01 M phosphate buffer pH 7.5 for 10 min at 37°C. Then 50 µL, 2 mM freshly prepared BAPNA was added and incubated at 37°C for 30 min. The reaction was stopped by the addition of 500 µL of 30% acetic acid. The optical absorbance of *p*-nitroaniline released by the reaction was read at 410 nm in a UV-Visible spectrophotometer (Shimadzu, Japan). The difference in OD was calculated by assaying trypsin activity in the absence and presence of inhibitor.

One unit of inhibitory activity (U) was defined as the amount of inhibitor required to inhibit the release of 1 µmol of *p*-nitroaniline per mL per min at pH 7.5 and at 37°C. The amount of protein present in each step was estimated.

Specific activity

Specific activity of the sample was calculated by dividing the inhibitory activity units with the protein content and expressed as Units /mg protein.

Protease inhibition assay using casein agar plate

The plate assay for the detection of protease inhibitor was performed in skimmed milk agar plate (1% of skimmed milk added to nutrient agar medium) with wells made in the center and on the periphery at a distance of 1 cm from the central well. Twenty microlitre of suitable dilution of bacterial protease in phosphate buffer was added in the central well.

A suitable dilution of mangrove extract was added in one of the peripheral wells and 0.01 M sterile phosphate buffer (pH 7.5) in the other to serve as a negative control. The plate was incubated at 37°C. The inhibition of hydrolysis of casein by trypsin was indicated by the absence of clearance zone around the well containing the inhibitor.

Antibacterial activity of mangrove extract by well diffusion method

Inhibition of growth of bacteria by bark and leaf extracts of mangroves *A. officinalis, A. marina, E. agallocha, R. mucronata* and *S. caseolaris* were studied using agar well diffusion assay.[22] The bacterial strains *Escherichia coli* (NCIM 5051), *Pseudomonas aeruginosa* (NCIM 2863), *Klebsiella pneumoniae* (NCIM 2957), *Proteus vulgaris* (NCIM 2027), *Clostridium perfringens* (NCIM 2677), *Staphylococcus aureus* (NCIM 2127), *Bacillus cereus* (NCIM2155), *Bacillus coagulans* (NCIM 2030) and *Bacillus pumilus* (NCIM 2189) were purchased from National Collection of Industrial Microorganisms (NCIM), NCL Pune, India and used as test bacteria.

Test bacteria freshly grown in Nutrient Broth (HiMedia) for 12 h were used for the study. Antibacterial analysis was performed in Petri plates containing Nutrient Agar (HiMedia) in which each of the test bacterium was swab-inoculated. Well was cut aseptically in the test bacterial plate and 20 µL (5 mg/mL) of mangrove extract was added in it. Plates were incubated for 24 h at 37°C and observed for the clearance zones of inhibited bacterial growth.

RESULTS

Isolation of bacteria for protease activity from mangrove rhizophore sediment.

Based on the formation of clearance zone on skimmed milk agarplate, four protease producing bacterial isolates were screened and one strain MESVP01 with high production potential (large clearance zone) was selected as source of protease for further study (Figure 1).

Molecular identification of the selected bacterial strain MESVP01

The molecular identification of the bacterial strain MESVP01 was done by ribotyping using partial gene sequences of 16S rRNA gene. A portion of the 16S rRNA gene (~1085 bp, Figure 2) was amplified from the genomic DNA and the amplicon was subjected to sequencing followed by homology search analysis.

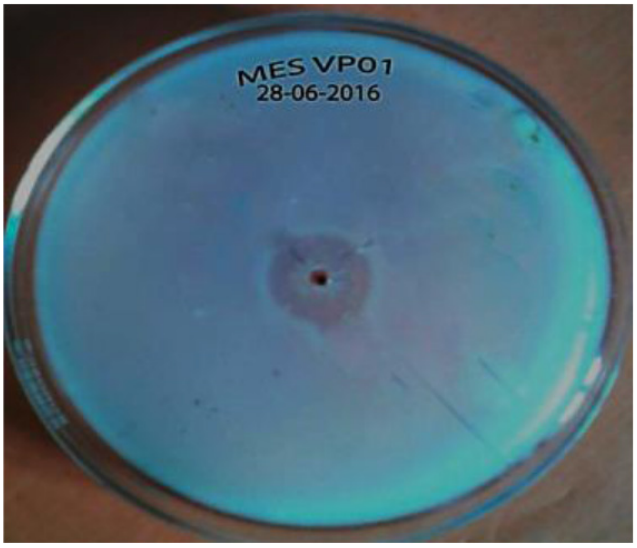


Figure 1: Caseinolytic plate assay for protease production.

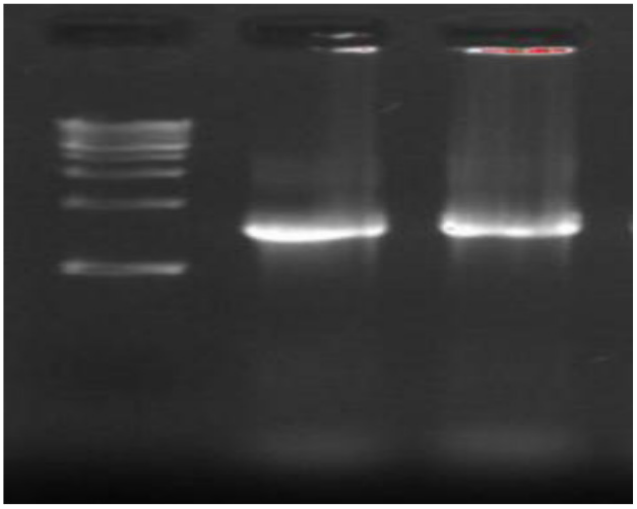


Figure 2: 16s rDNA amplification. Lane 1:1Kb Ladder. Lane 2 and 3: amplicon.

The bacterial strain MESVP01 was identified as *Bacillus* sp. with 85% identity to the reported rRNA gene sequence of *Bacillus gaemokensis*.

Inhibition of bacterial protease by mangrove extract

Mangrove plant materials (leaf and bark) of five different species; Ao, Am, Ea, Rm and Sc collected from Puthuvypin Station of Cochin Coast, Kerala were screened for their protease inhibitory activity against bacterial protease.

Protease inhibitor assay using BAPNA as substrate

Crude mangrove plant extracts prepared from the leaves as well as barks were subjected to partial purification by ammonium sulphate fractionation. The precipitates obtained after each fractionation were dissolved in minimum quantity of phosphate buffer (0.01 M, pH 7.5) and evaluated for inhibitory activity by BAPNA liquid assay. Results of mangrove extracts which showed considerable protease inhibition activity are presented in Table 1. Among the total 30 fractions assayed, 12 fractions including six 0-30% fractions three each from leaf and bark extracts, three 30-60% fractions of leaf extracts and three 60-90% fractions of leaf extracts showed protease inhibition activity.

Table 1: Protease inhibition of ammonium sulphate precipitated active fractions of mangrove extracts.

Sl. No.	Mangrove Plant Extract	Saturation of ammonium Sulphate (%)	Inhibitory activity (U)	Specific inhibitory activity (U/mg protein)
1	Ao(L)	0-30	40.2	9.9
2	Am (L)		72.4	28.06
3	Sc(L)		39	11.4
4	Ea(B)		40	12.08
5	Am(B)		82	20.75
6	Ao(B)		81	20.87
7	Ao(L)	30-60	72	13.84
8	Rm(L)		80	19.05
9	Sc(L)		82	33.33
10	Rm(L)		82.2	54.8
11	Ea(L)	60-90	83.3	41.24
12	Sc(L)		82	25

Protease inhibition by caseinolytic plate assay

Twelve ammonium sulphate fractions of the mangrove extracts were recognized as positive for the presence of protease inhibitor in BAPNA assay. They were subjected to further protease inhibition analysis employing caseinolytic plate assay.

Results presented in Figure 3 demonstrate the inhibition of proteolysis by the ammonium sulphate fractions of mangrove extracts in skimmed milk agar plate, Central well: Protease 20 µL, Peripheral wells: Ammonium sulphate fractions of mangrove extracts. In Figure 3(a), peripheral wells are, 0-30% fractions of, Ao leaf extract, L1; Am leaf extract, L2; Sc leaf extract L5 and Am bark extract, B2. In Figure 3(a), the inhibition by, 0-30% fractions of; Sc leaf extract, L5 and Am bark extract are evident by the absence of clearing zone, where hydrolysis of casein was inhibited due to the presence of inhibitors in the extract. Inhibition of protease enzyme by 60-90% ammonium sulphate fractions of;

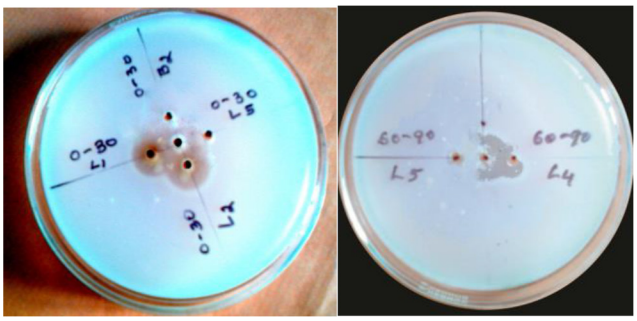
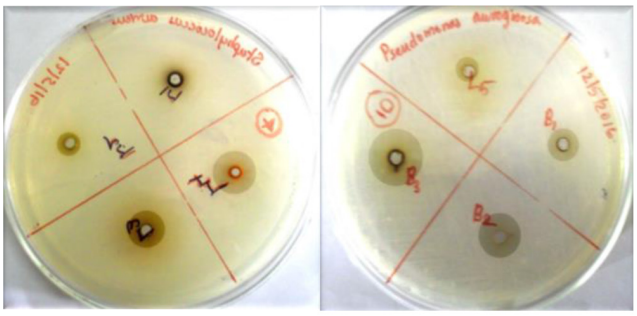


Figure 3: Caseinolytic plate assay: The zone of inhibition of the hydrolysis of casein.



4(a) **4(b)**

Figure: 4 Antibacterial activity of mangrove plant extracts. (a) Leaf extracts of Am (L), Am (L), Ea(L) and *Rm* (L) against *S. aureus* 4(b) Bark extracts of Ao (B), Am (B), Ea (B) and leaf extract of Sc (L) against *P. aeroginosa*

Sc leaf extract L5 and Rm leaf extract L4, were clearly visible in Figure 3(b).

Antibacterial potential of mangrove plants

Mangrove extracts were checked for its ability to inhibit bacterial growth by growth inhibition method in nutrient agar plates. The inhibitory activity was evaluated against nine different standard bacterial cultures. Among them the inhibition towards *P. aeroginosa* by bark extracts of mangroves Ao(B), Am(B), Ea(B) and leaf extract of Sc(L) (Figure 4a) and inhibition towards *S. aureus* by leaf extracts of Rm(L), Sc(L) and Am(L) were evident (Figure 4b) by the presence of growth inhibiting clearing zones in culture plates.

DISCUSSION

Medicinal plant biotechnology has been recognised as an emerging procedure which is useful to induce the formation and accumulation of desirable compounds and eventually develop the therapeutic product.[23] Mangroves are plants growing usually in the salty damp marsh places, adjacent to the highly polluted back waters. So, they are expected to have some defensive mechanisms to thrive over the harsh environmental conditions. In the present study the bark and leaves of five different species of mangroves were used to prepare extracts in 0.01 M phosphate buffer and fractionated using ammonium sulphate (0-30%, 30-60% and 60-90% saturation) and dialyzed. The representative serine protease used for inhibition studies was from *Bacillus* sp. MESVP01, which was isolated from the mangrove rhizosphere sediment. Plant PIs are usually considered to be endogenous proteinases regulators and also function as plant defence agents blocking the insect and microbial proteinases present in the surroundings.[24]

Among the thirty ammonium sulphate precipitate fractions of mangrove extracts analyzed, twelve showed the presence of inhibitor against the protease of *Bacillus* sp. MESVP01 in BAPNA liquid assay. The 0-30% and 30-60% fractions of leaf extracts and 0-30% bark extract of *A. officinalis (*Ao) exhibited protease inhibition. All the three ammonium sulphate fractions of the leaf extract of *S. caseolaris,* Sc (L) were found to be inhibitory. Both the leaf and bark extracts of *A. marina* 0-30% fractions showed inhibitory potential. 0-30% bark extract fraction and 60-90% leaf extract fraction of *E. agllocha,* Ea (L and B) and 30-60% and 60-90% leaf extract fractions of *R. mucronata*, Rm (L) possessed protease inhibitory potential. The inhibitory potential of these mangrove plant extracts could be demonstrated effectively in skimmed milk agar plates. The zone of inhibition of casein hydrolysis was evident as white patch around the well in the presence of inhibitor, whereas the clearance zone was present around the uninhibited wells.

Mangrove extracts are potential antibacterial agents too. That may be due to the effect of inhibitor on vital physiological activities of test bacteria where protease perform important role.[25] In the present study, the growth of *P. aeroginosa* was inhibited by three bark extract samples and one leaf extract sample of mangroves in phosphate buffer. Bacterial inhibition by four mangrove leaf extracts inhibited the *S. Aureus* growth in culture plates.[27] Protease inhibitors from plants have been identified as potent antibacterial agents.[28,29] Dried parts of 37 out of 64 Indonesian mangrove plant extracts in water, ethanol, ethyl acetate and hexane showed antibacterial activity.[26] A protease inhibitor 'fistulin' isolated from the leaves of *Cassia fistula,* exhibited antibacterial activity against *E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis* and *K. pneumonia.* Protease inhibitor CGPIs extracted from *Coccinia grandis* leaves have exhibited a significant inhibitory effect for chymotrypsin and bovine pancreatic trypsin.[30] However there has been no report on the antibacterial PI, from mangroves of Kerala.

CONCLUSION

Presence of protease inhibitors and antibacterial agents in five different mangrove species of Ernakulam district in Kerala were evidenced. In the current situation it is important to explore new plant protease inhibitors useful in the treatment and control of human diseases.

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CONFLICT OF INTEREST

The authors declare that there are no conflict of interests associated to this manuscript.

ABBREVIATIONS

PI: Protease Inhibitor; **OD:** Optical Density; **BAPNA:** N-Benzoyl-DL-arginine-4-nitroanilide hydrochloride; **BSA**: Bovine Serum Albumin.

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