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ISOLATION AND PHYLOGENETIC ANALYSIS OF MARINE FUNGUS *PENICILLIUM SP.* SDBF1 AND PARTIAL CHARACTERIZATION OF ITS CYSTEINE PROTEASE INHIBITOR

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ABSTRACT:

The marine fungus *Penicillium sp.* SDBF1 was isolated from the mangrove leaves of *Avicennia marina.* Phylogenetic analyses were performed based on internal transcribed spacer sequencing. The sequence was submitted to NCBI gene bank, under the accession number FJ403589. This fungal species produces a cysteine protease inhibitor (CPI) having single peak as analyzed on rp-HPLC and mass spectrometry (ESI-MS) showed a Mr of 437 Da. This CPI was stable in a broad range of pH (2-11) and temperature (37-100°C). A very high inhibitory activity of CPI was recorded against papain. However, this inhibitor did not show any inhibitory activity against other proteases like trypsin, chymotrypsin and pepsin. Intracellular fraction showed antibacterial activity against human pathogen *Serratia marcescens* and plant pathogen *Bacillus circulans.*

Keywords: *protease inhibitor, phylogenetic analysis, marine fungus, sequencing, mangrove, antibacterial*

[I] INTRODUCTION

Fungi isolated from mangroves are the second largest group among the marine fungi [1]. A large variety of new bioactive compounds have recently been isolated from the marine fungi, especially the mangrove fungi [2, 3]. Cysteine proteases are important therapeutic targets because of their role in several diseases, such as cathepsin B in tumor growth and cathepsins K in osteoporosis. Calpains are involved in a variety of disease states, such as Alzheimer's disease, multiple sclerosis, muscular dystrophy, type 2 diabetes, traumatic brain, spinal cord injury and cerebral ischemia [4, 5]. Recent studies have shown that they play significant role in plant pathogen interactions [6, 7]. Therefore, there is continuous demand for new chemical scaffolds for cysteine protease inhibitors. Nature continues to be one of the most significant sources of pharmacologically active compounds in the quest for drugs. *Penicillium* species are widely distributed in nature and often found living on foods and in indoor environments. They are the source of several β-lactam antibiotics, most significantly penicillin. Other secondary metabolites of *P. chrysogenum* include various different penicillins, roquefortine C, meleagrin, chrysogine, xanthocillins, secalonic acids, sorrentanone, sorbicillin and PR-toxin [8]. Several un-described, marine-derived *Penicillium sp.* have been recently isolated from a variety of substrates such as mollusks, sponges, algae and sands. These *Penicillium* species are important producers of new metabolites such as the

Sculezonones A & B [9]. During the past decade, despite the rational drug discovery taking the movement away from the development of enzyme inhibitors by screening of natural products, the biodiversity widespread in soil, water, insects, tropical plants and marine sources still have tremendous potential for the isolation of novel and effective enzyme inhibitors. There has been plethora of reports of synthetic cysteine protease inhibitors from various sources. However, there is a paucity of reports of such inhibitors from microorganisms. The present paper describes isolation, identification of a salt tolerant *Penicillium sp.* SDBF1 strain from the mangrove leaves of *Avicennia marina* and purification and biochemical characterization of a cysteine protease inhibitor from this fungus.

[II] MATERIALS AND METHODS

Lysozyme, SDS, EDTA, chloroform, ethanol, agarose, ethidium bromide, proteinase K, RNase, deoxyribonucleoside triphosphates (dNTPs) and *Taq* DNA polymerase were from Sigma Chem. Co. (USA). ITS rDNA primers were commercially obtained from Life technologies, (India). NaCl, malt extract, glucose, yeast extract, peptone, ultra marine salt, Tris and agar were purchased from Himedia and Qualigens (India). Papain, N α-Benzoyl-DL-Arginine p-Nitroanilide (BAPNA), Trifluoroacetate (TFA) and other chromogenic substrates were purchased from Sigma Chem. Co. (USA) and reverse phase matrix for FPLC (Fast protein liquid chromatography) was from GE healthcare (India). All other chemicals were of analytical grade.

2.1. Isolation, identification and phylogenetic analysis of the fungus

The marine fungus was isolated in the laboratory from the mangrove plant *Avicennia marina*. The plant leaves were collected from Mandovi estuaries, Goa, India. The culture was purified by a single colony plating technique on MGYP agar plates. The organism was identified by carrying out internal transcribed spacer/5.8S ribosomal DNA (rDNA) sequencing. The sequence so obtained was searched for homology in the NCBI nucleotide database. Phylogenetic tree of *Penicillium sp.* SDBF1 constructed with MEGA 4 software using the neighbor joining method.

2.2. Isolation of genomic DNA

The fungal culture was grown in 100 ml of MGYP broth (pH 7.0) at 28^{o}C ± 0.5 for 7 days with continuous shaking at 160 rpm. Genomic DNA of the fungal culture was extracted by using the salting out method [10]. 5g fungal mycelia were ground into a powder using liquid nitrogen and suspended in 5 ml SET buffer (75mM NaCl, 25mM EDTA (pH 8.0) and 20 mM Tris (pH 7.5). 100 µl lysozyme (final conc. 1mg/ml) was added to the above solution and incubated at 37^{o}C for 60 min. 140 µl of proteinase K (final conc. 0.5 mg/ml) and 600 µl of 10% SDS were added, and incubated for 2 h at 55^{o}C with occasional mixing. 2 ml of 5M NaCl (final conc. 1.25M) was added and the mixture was cooled to 37^{o}C. 5ml chloroform was added and mixed for about 30 min at room temperature and then the mixture was centrifuged for 20 min, at 4500 x g. The supernatant was transferred into a fresh tube and 0.6 vol of isopropanol was added to precipitate the DNA. The DNA was pelleted and washed twice with 70% ethanol, air dried and dissolved in 1-2 ml of TE buffer (10 mM Tris, 1mM EDTA, pH 8.0) at 28ºC. DNA was quantified by taking absorbance at 260 nm.

2.3. PCR Amplification of rDNA

The primers used for the identification of the fungal species were universal primers for fungal amplification: ITS1 (5'–TCC GTA GGT GAA CCT GCG G-3'), which hybridizes at the end and ITS4 (5'–TCC TCC GCT TAT TGA TAT GC-3') which hybridizes at the beginning. The 25µl Polymerase chain reaction (PCR) was set using the fungal genomic DNA as template. The reaction mixture typically contained genomic DNA 30 ng 1µl, 10X PCR Buffer containing 15mM $MgCl_2$ 2.5µl, 0.2mM deoxyribonucleoside

triphosphates (dNTPs) 2.5µl, forward and reverse primers 10 pmole (1.0µl each), distilled water 16.0µl, and 1µl of *Taq* DNA polymerase, 1U/ µl (Bangalore Genei). All the additions were done on ice and the PCR reaction was performed on C1000 Thermal Cycler of Bio-Rad Inc. Limited, USA. The PCR conditions for gene amplification were: Initial denaturation 95°C for 3min, followed by 35cycles of 94°C for 1min, 55°C for 1min, 72°C for 1min and final extension at 72°C for 10 min. 5µl of the above PCR amplified product was used for electrophoresis using 1.0% agarose gel in 0.5X TBE buffer (Working solution: 0.5X; Stock: 5X, 54 g Tris base, 27.5 g boric acid, 20 ml 0.5M EDTA [pH 8]). The gel was run at 80V for 90min, using 0.5X TBE as running buffer. The gel was stained in 1% ethidium bromide for 45 min and was observed under UV illumination.

2.4. Purification of PCR amplified product

10 µl of the above PCR amplified product was used for electrophoresis using 1.0% agarose gel in 0.5X TBE buffer (Working solution: 0.5X; Stock: 5X, 54g Tris base, 27.5g boric acid, 20ml 0.5M EDTA [pH 8]). The gel was run at 80V for 90min, using 1X TBE as running buffer. The gel was observed under UV illumination and the DNA band ~550 bp was cut and purified with AxyPrep DNA gel extraction kit by Axygen Biosciences, USA. Purity of DNA was checked again on gel.

2.5. Sequencing of the purified PCR product

The sequencing reactions were carried out using Taq DNA polymerase dye terminator cycle sequencing using the 'ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit' (Perkin Elmer Applied Biosystems Division, Foster City, CA) according to the manufacturer's protocol. This Kit contains the four dNTPs with different fluorescence labels termed as BigDye Terminators. 2 µl PCR product and 3 pmol of the sequencing primer were used in a 20 µl sequencing reaction. The sequencing reaction mixes were subjected to 25 cycles in a Perkin Elmer thermal cycler 9700. Each cycle consisted of 95°C for 10min, 50°C for 5min and 60°C for 4min. DNA sequencing was carried out on ABI 3700 DNA sequencer. The analysis of nucleotide sequence was done at NCBI server using BLAST-n.(www.ncbi.ncm.gov/blast)

2.6. Production of the cysteine protease inhibitor

The media composition for the production of cysteine protease inhibitor (CPI) was malt extract (0.3%), glucose (0 .1%), yeast extract (0.3%), peptone (0.5%) and ultra marine salt (1.1%). The pH of the medium was adjusted to 7.0 before autoclaving. Production of the CPI was carried out by inoculating 1% of freshly grown culture broth into 500 ml Erlenmeyer flasks containing 100 ml of the above-mentioned broth medium and was incubated under shaking condition (160 rpm) at 28^{o}C for 7 days.

2.7. Assay for inhibitory activity of CPI towards papain

The inhibitory activity of CPI against papain was determined by assaying the proteolytic activity of 50 µl of papain (1mg/ml) in Tris-HCL buffer, pH 6.5 in the presence of 10 mM DDT and 10 mM EDTA, using BAPNA (1.5 mM) as the substrate in the presence and absence of CPI.

2.8. Time course for the production of CPI

The production of inhibitor at various time intervals was checked by removing samples at 24 h time intervals and assayed for its anti-papain activity. The time course of production of CPI was estimated in terms of percent inhibition against papain activity.

2.9. Purification of cysteine protease inhibitor

The extracellular culture filtrate of the isolate was obtained by centrifugation of growth medium constituents at 5000 rpm, at 4^{o}C for 20 minutes. Further CPI was purified by ultra-filtration (UM-30 and UM-3) and gel filtration (Sephadex LH-20) to remove high molecular weight impurities. The fractions were eluted on isocratic mode with H_2O at a flow rate of 0.5 ml/min and monitored at a wavelength of 280 nm. Then CPI was purified

by RP-FPLC, by using rp-column prepared by polystyrene/divinyl benzene monodisperse beads (15µm diameter). The fractions were eluted on a linear gradient of 5-95% acetonitrile with H_2O containing 0.05% TFA at a flow rate of 0.5 ml/min and monitored at wavelength 220 nm. The active fractions obtained from RP-FPLC were pooled, lyophilized and loaded on RP-HPLC using prepacked µBondaPak column. The fractions were eluted on a linear gradient of 0-95% acetonitrile with H_2O containing 0.05% TFA at a flow rate of 0.5 ml/min and monitored on dual wavelengths 220 nm and 280 nm. The eluted sample was lyophilized and dissolved in distilled water to check the anti-papain activity.

2.10. Biochemical characterization of CPI

The molecular mass of purified CPI was determined by qudrupole electrospray ionization mass spectrometer. Methanol–water (1:1) system was used as mobile phase. The purity of inhibitor was also checked on UPLC-MS (ultra pressure liquid chromatography) system coupled with mass spectrophotometer system (WATERS). Acquity UPLC BEh C18 column (2.1 x 50 mm) was used for analysis. The run was for 8 min in isocratic mode and CPI showed a retention time of 3.2 min.

2.11. Assay for inhibitory activity of CPI towards trypsin and Chymotrypsin

The inhibitory activity of CPI against Chymotrypsin and trypsin was determined by assaying the proteolytic activity of 25 µl each of Chymotrypsin and trypsin (1mg/ml) in phosphate buffer, pH 7.0, in the presence of 10 mM EDTA and using BAPNA (1.5 mM) as the substrate. A blank reaction was performed in the absence of CPI.

2.12. Temperature and pH stability of CPI

The temperature stability of CPI was determined by incubating CPI (50 µl) at temperatures from 30-100^oC up to 6 hours and by estimating anti-papain activity at different time intervals. The pH stability of CPI was determined by incubation of CPI at different range of pH values (2-11) in appropriate buffers up to 2 hours and its inhibitory activity against papain was estimated.

2.13. Antimicrobial activity

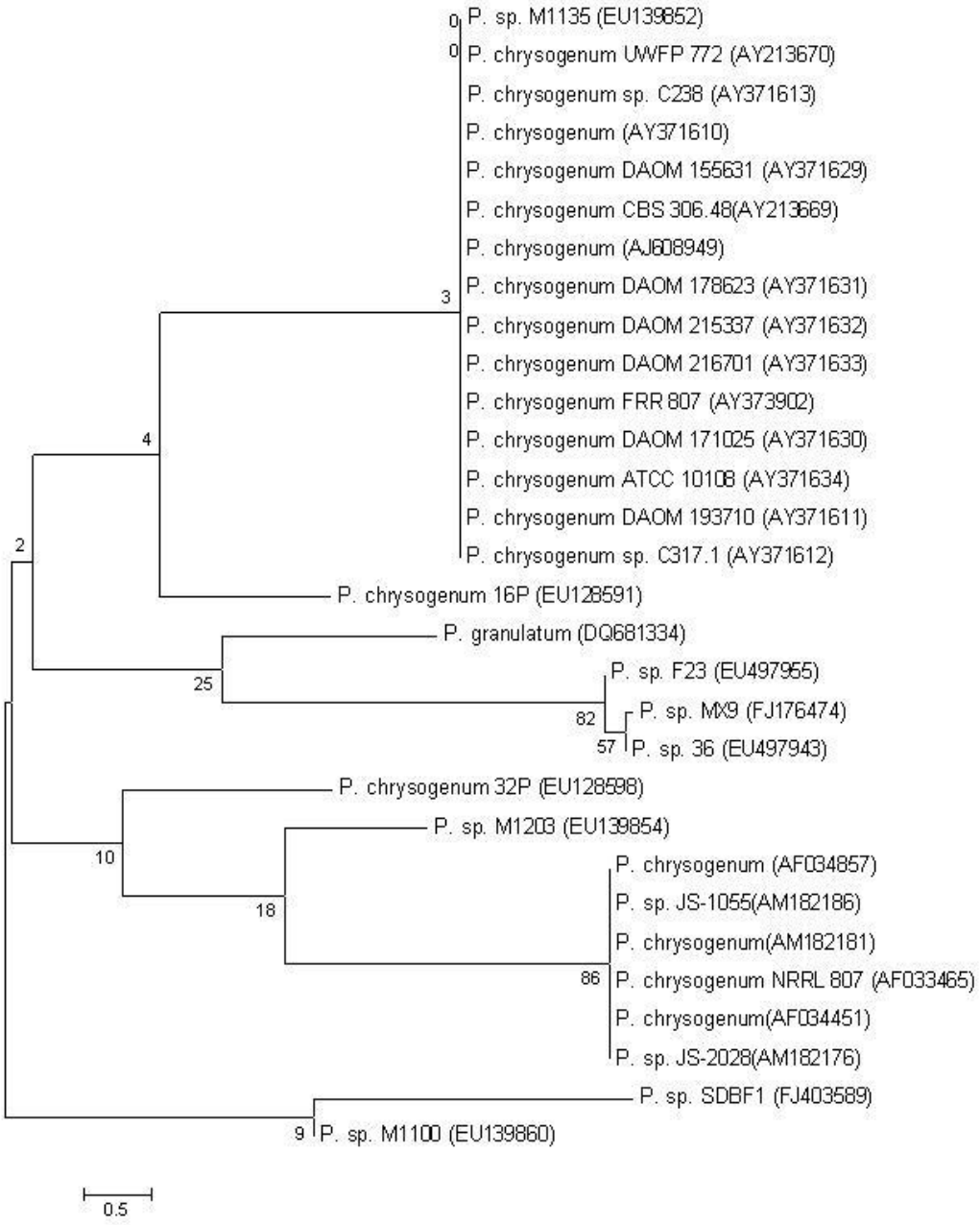
Fungal mycelia were washed twice with sterile distilled water, to remove the extracellular impurities. They were frozen in liquid nitrogen and then crushed using a mortar and pestle. Water extract was centrifuged at 5000 rpm for 10 min and supernatant was used for disk diffusion assay.

Microbes *Serratia marcescens* (NCIM No. 2078); *Bacillus circulans* (NCIM No. 2160) were obtained from National Collection of Industrial Microorganisms (NCIM), NCL, Pune, India.

[III] RESULTS AND DISCUSSION

After extensive screening, a salt tolerant fungus was isolated, which produces a cysteine protease inhibitor (CPI). The organism produced CPI in the peptone ultra marine salt complex media at 28^oC and pH 7. The *Penicillium sp.* was isolated from leaves of the mangrove plant *Avicennia marina*, where it resides as a plant pathogen. This *Penicillium sp.* is an aerobic, spore forming fungus and its mycelia were large, irregular, sticky and white colored. The plant leaves were collected from Mandovi estuaries, Goa, India. The genomic DNA was isolated and checked on agarose gel, was found to be of high molecular weight and intact. The spectro-photometeric analysis of the DNA showed that the DNA had an A260/A280 ratio of 1.84, which indicated the purity of the DNA preparation. The sequence was submitted to NCBI database under accession number FJ403589 and the fungus identified as *Penicillium sp. SDBF1*. The sequence on NCBI Blast showed closest homology (99%) to *Penicillium chrysogenum*. Phylogenetic tree was constructed with MEGA 4 software using neighbor joining method. In **[Figure-1]**

Fig: 1. Phylogenic tree constructed with MEGA 4 software using neighbor joining method.



Optimum fermentation condition for *Penicillium sp.* SDBF1 was found to be 28°C±0.5 and pH 7.0 for 7 days. Growth of the organism in fermentation media was measured by taking O.D. at 600 nm. Maximum production of CPI was observed from 4 to 7 days of growth using the fermentation medium containing malt extract (0.3%), glucose (1%), yeast extract (0.3%), peptone (0.5%) and ultra marine salt (1.1%). Ultra marine salt (1.1%) is essential for production of CPI from the *Penicillium* species. The time profile for the production of the CPI by the organism was showing a sharp decrease in the inhibitor production after 7 days. In **[Figure-2]**

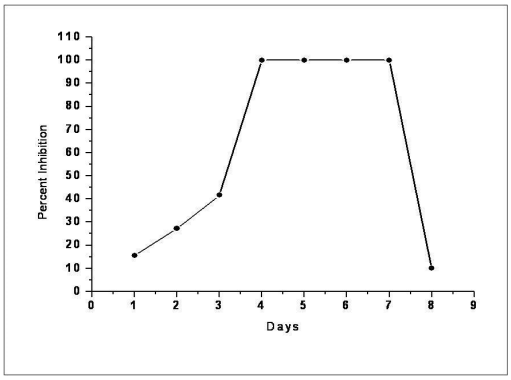


Fig: 2. Time profile for the production of CPI

The production of CPI was also achieved even in the stationary growth condition as CPI level remained same in the flasks.

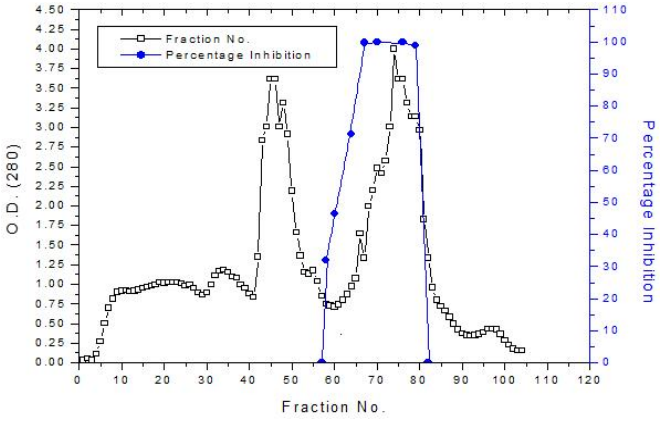


Fig: 3. Gel chromatographic profile of inhibitor on Sephadex LH-20 matrix column

The extracellular culture filtrate of *Penicillium sp.* was subjected to centrifugation and ultra filtration (30 KDa cut off and 3 KDa cut off). The filtrate was concentrated and loaded on gel filtration column (Sephadex LH-20). In **[Figure-3]**

The gel filtration fractions 58-80 showing papain inhibitory activity was pooled, concentrated and loaded on RP-FPLC column (prepared by polystyrene /divinyl benzene monodisperse beads of 15 µm diameter). The gradient was made of 5% -95% acetonitrile and pre equilibrated with 5% acetonitrile and 0.05% TFA. The fractions 15 to 16 showed a papain inhibitory activity. In **[Figure-4]**

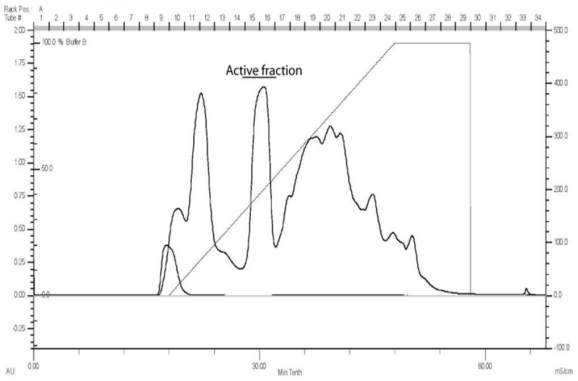


Fig: 4. Reverse phase-FPLC purification of CPI

These fractions further concentrated and loaded in µBondaPak prepacked column for RP-HPLC. The anti papain activity was associated with the peak (Active Fraction) indicated as arrow, having a retention time of 14 min **[Figure-5A]** and some other eluted peak with differing retention time. The fractions showing the inhibitory activity were pooled and lyophilized. Homogeneity of the active fractions containing peak A was indicated by a single peak as analyzed on RP-HPLC with retention time 14 min. In **[Figure-5B]**

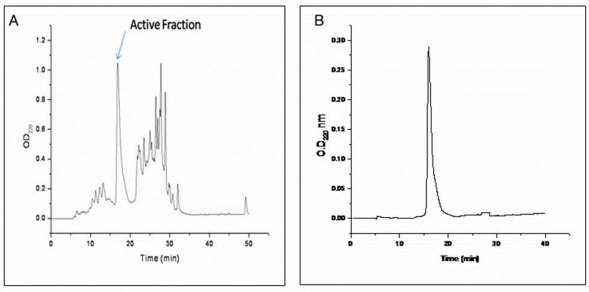


Fig: 5. Reverse phase-HPLC purification of CPI.

A. 40µl of the lyophilized CPI sample was loaded on prepacked µBondaPak column (waters RP-C18) pre equilibrated with acetonitrile (CH3CN) and trifluoroacetate (TFA). The fractions containing the peaks A were collected manually and assayed for the anti-papain activity.

B. 50µl of the pooled fractions containing the peak A (associated with the anti papain activity) was reloaded onto the reverse-phase HPLC system under similar experimental

conditions. The peak detected showed a retention time of 15.76 min

The chromatographic profile of UPLC and HPLC shows that the CPI is a purified compound with almost no impurity. We had used diode array detector for scanning the compounds present in sample, only single peak of CPI was detected In **[Figure-6]**. The molecular mass of the inhibitor was found to be 437.312 Da. The mass spectra also shows fragmentation pattern of CPI. The compound was fragmented into small peaks of 139.084, 150.949, 151.101, 183.196 and 197.114. In **[Figure-6]**

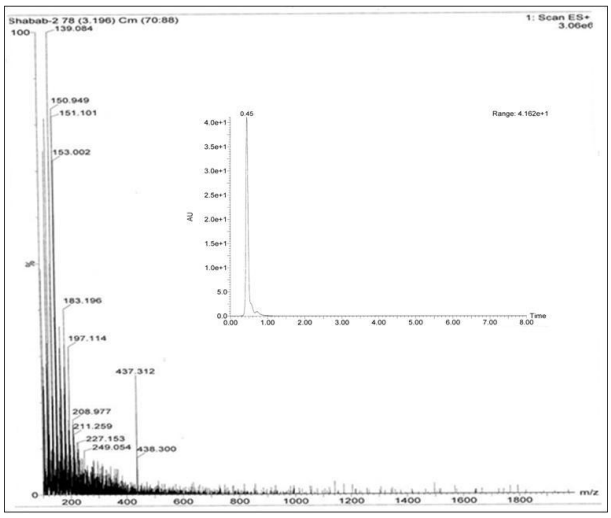


Fig: 6. Molecular mass of CPI. The purified CPI was analyzed for the determination of the Mr by electrospray ionization spectra (437.312 Da). (Inset)- Reverse phase-UPLC profile of CPI. 0.5 ml of the lyophilized CPI sample was loaded on rp-UPLC C18 column. The inhibitor was monitored on diode array detector.

In general, the pH stability of CPI was found to stable at pH ranging from 2-11 for two hours, indicating the stability of CPI over a wide range of ionic environments. CPI was stable at 30-100^{o}C for about 6 hours. A very high inhibitory activity of CPI was recorded against papain. The CPI was also tested for inhibition against other classes of enzymes like aspartic acid protease (pepsin) and serine proteases (trypsin and chymotrypsin). However, it did not show any inhibition activity for these enzymes.

Fungi are prolific producers of structurally diverse and biologically active secondary metabolites. High levels of environmental stress and intense and frequent interactions with other organisms promote the production of metabolically diverse compounds [11]. The salt tolerant plant pathogenic fungi are an underexploited group of significant taxonomic diversity and represent one of the largest untapped pools of novel fungi. There are few reports of biologically active metabolites from this class of fungi, like cyclo-(N-MeVal-N-MeAla) from endogenous fungus of *Avicennia marina* [12]. Intracellular fraction of *Penicillium sp.* SDBF1 was found to contain potent antibacterial activity against human pathogen *Serratia marcescens* and *Bacillus circulans*. *S. marcescens* is a species of gram-negative rod shaped bacteria in the family enterobacteriaceae. It is involved in nosocomial infections; particularly catheter associated bacterimia, urinary tract infections and wound infections [13]. Most *S. marcescens* strains are resistant to several antibiotics because of the presence of R factors which is a type of plasmid that carry one or more genes that encode resistance proteins; all are considered intrinsically resistant to ampicillin, macrolides and first generation cephalosporins [13]. *B. circulans* are gram-positive, aerobic, spore-forming rods. *B. circulans* is used for producing L-glutamic acid. Although not a major pathogen, *B. circulans* has been reported to cause infections [14]. This may be an important data for further studies.

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