BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, March 2023. Vol. **20**(1), p. 85-95

Isolation and Characterization of Protease Inhibitors with Antimicrobial Activity from Sirisa (*Albizia lebbeck*) Seed Protein Extract

Rutwika S. Thete[1], Shahriar Roushani[2], Faiyaz K. Shaikh[1]*, Jyoti Kulkarni[1] and Raghavendra H.L. [1]

[1]Centre for Biotechnology, Pravara Institute of Medical Sciences (Deemed to be University), Rahata Taluk, Ahmednagar District, Loni-413736, Maharashtra, India.
[2]Department of Microbiology, Dr. Balasaheb Vikhe Patil Rural Medical College, Pravara Institute of Medical Sciences (Deemed to be University), Rahata Taluk, Ahmednagar District, Loni-413736, Maharashtra, India.

http://dx.doi.org/10.13005/bbra/3071

(Received: 27 November 2022; accepted: 09 February 2023)

The present study aimed to identify protease inhibitors (PIs) with antimicrobial activity from sirisa (Albizia lebbeck) seed protein extracts that may be a natural alternative to overcome multi-drug resistance, toxicity, and side effects of existing antimicrobial drugs. The crude PIs were extracted from seeds of A. lebbeck in 1% PVP and further partially purified by ammonium sulphate (NH4)2SO4 fractionation. The total protein content was found to be high in 0-30 % (NH4)2SO4 saturated protein fraction F1 (7.3 ± 0.17 mg/ml). Reasonably high PI activity towards trypsin was observed in 60–90 % (NH4)2SO4 saturated fraction F3 assessed by the agar well diffusion method and in vitro solution assay. Electrophoretic profiling of proteins from the F3 fraction showed nine bands on the gel with differential mobility. The presence of a zone of inhibition (ZOI) for different concentrations of F3- 60–90 % (NH4)2SO4 saturated PIs on agar plate demonstrated antimicrobial activity against E.coli, S. aureus, and P. aeruginosa with MIC values of 100 ± 5 μg/ml, 100 ± 4 μg/ml, and 90 ± 3 μg/ml respectively. Our results indicate that PIs from seeds of sirisa display potent antimicrobial activity against the tested microorganisms and could be investigated further in the future use in designing or formulating natural antimicrobial drugs to treat microbial infection-related diseases.

Keywords: Albizia lebbeck; Ammonium sulphate fractionation; Antimicrobial agent; PIs; Sirisa.

Proteases are the enzymes (hydrolases) that cause the hydrolysis of proteins into small polypeptides or oligopeptides, or amino acids.[1] They are intricate in many biological processes, such as the digestion of ingested proteins, protein catabolism (breakdown of old proteins), cell signaling, cell growth, and cell death.[2-3] Proteases could be grouped into exopeptidases and endopeptidases according to the site of enzyme action. Endoproteases are found to break peptide bonds within proteins, whereas exopeptidases hydrolyze peptide bonds close to the N- or C-terminal ends of the proteins.[4]

*Corresponding author E-mail: faizbiochem@gmail.com

Proteases are biologically important enzymes that play a vital role in almost all metabolic pathways and networks. The un-regulated expression of these proteases is reported by researchers in diseases like coagulopathies, bleeding disorders, necrotic skin infections, destruction of hemostasis, neurodegeneration, inflammation, bacterial diseases, viral diseases, hypertension, and cancer.[5-10] All serine proteases like trypsin, chymotrypsin, elastase, etc. are known to be predominant and regulate vital biological events, which makes them attractive targets in therapy. Indeed, in recent years, scientific communities have worked to uncover the physiology, biochemistry, and genetics of these enzymes in order to establish them as potential drug targets. A literature review suggests that drug discovery approaches that target proteases are useful for the management of human diseases.[11] The general therapeutic strategy to regulate proteases and manage of diseases is to identify specific inhibitors of these proteases. These are known as protease inhibitors (PIs) and are pervasive.[12] Within an organism, these PIs govern and regulate the activity of proteases, as an imbalance can cause severe damage and diseases in the host body.[13]

The PIs are small molecules that are omnipresent in the plant kingdom and are mostly located in aerial parts, tissues, tubers, and seeds.[14] The plant PIs are known for their strong defence mechanisms against insects and phytopathogens.[14] Structurally, PIs are classified into various subfamilies. Of these, serine proteases i.e. Kunitz and Bowman-Birk have been comprehensively studied and are considered candidates of therapeutic interest.[15-16] In recent times, PIs have been known as the principal candidates who have applications in medicine and biotechnology.[17] These molecules are explored to study protein-protein interactions and as drug candidates to treat various human complications or diseases related to the uncontrolled actions of proteases.

Due to the indiscriminate use of broad-spectrum antibiotics, multi-drug-resistant microbial strains and the emergence of strains with less susceptibility to antibiotics have recently increased and are causing pathological complications in humans. Therefore, there is an urgent need to look into novel potent anti-microbial molecules to control microbial infections. Recently, many investigations have concentrated on the antimicrobial activity of plant PIs.[18-20] In this regard, the current investigation was designed to explore the antimicrobial property of PIs from sirisa (*A. lebbeck*), a medicinal plant with a wider occurrence in the Indian subcontinent.

MATERIALS AND METHODS

Chemicals and reagents

HiMedia and Sigma-Aldrich, India provided all the chemicals and reagents utilised in the mentioned experiments. All chemicals and reagents used in the current investigation were of analytical grade.

Microorganisms

The microorganisms (*E. coli, S. aureus* and *P. aeruginosa*) selected for the current investigation were procured from the Department of Microbiology, Dr. Balasaheb Vikhe Patil Rural Medical College, Pravara Institute of Medical Sciences (DU), Loni (MS) India.

Sample collection

The dry seeds of sirisa (*A. lebbeck*) were collected from the Pravara Institute of Medical Sciences, Loni Tal. Rahata Dist. Ahmednagar (MS), India with latitude and longitude of 18.182°N 74.36455°E. The plant and seeds were authenticated by Dr. A.S. Wabale, Vice Principal, and Research Guide, Department of Botany, Padmashri Vikhe Patil College of Arts, Science, and Commerce, Loni (Ref: /PVPC/Bot/2022-23/36 Date: 22-08-2022).

Defatting and depigmentation

The sirisa (*A. lebbeck*) seeds were minced to powder by a mixer grinder. The fine powder was subjected to the defatting and depigmentation procedure to remove fats and pigments. Defatting was carried out in a 1:6 (w/v) ratio of powder to n-hexane for 2 hours.[21] At regular 20 minute intervals, the mixture was slightly stirred, and the sample was filtered through filter paper. The residue was dried at room temperature (27-28 ºC). The dried powder was defatted once more to ensure that all fats were removed. The defatted powder was further subjected to depigmentation by acetone in a ratio of 1:10 (w/v), and the resulting dry powder was used for the extraction of crude proteins.

Crude protein extraction

The crude proteins of the defatted and

depigmented powder of the seeds of *A. lebbeck* were extracted in 1% PVP with a 1:6 (w/v) ratio of powder to PVP overnight at 4 ºC.[21] The sample was centrifuged at 10000 rotations per minute (rpm) at 4ºC for 15 minutes. The resulting supernatant was further used as a crude protein extract for the study.

Ammonium sulphate fractionation

Crude protein extract (40 ml) was subjected to ammonium sulphate $(NH_4)_2SO_4$ fractionation to get three different salt precipitated fractions of proteins i.e. F_1-0-30%, F_2-30-60%, and F_3-60-90%. All these fractions were dialyzed against a 0.1M phosphate buffer, pH 6.8 to remove the $(NH_4)_2SO_4$.

Profiling of *A. lebbeck* proteins by 10% native PAGE

Native PAGE is employed to separate proteins in their native conformations.[22] The proteins with biological activities viz. enzymes, generally retain their activity during the run in electrophoresis. By maintaining the "native" condition, the proteins migrate based on the charge density that depends on the primary amino acid sequence of proteins and pH during electrophoresis. A 10% polyacrylamide gel was cast in the gel chamber of the native PAGE assembly. The assembly was filled with the electrode buffer, and the gel chamber was inserted into the assembly. The assembly was connected to the power pack and started on 100 Volt. The samples (approx. 50 µg; crude and (NH4)2SO4 fractions) were loaded into the well separately along with the sample buffer. After the electrophoretic run, the gel was removed from the chamber and placed in a protein stain (0.1% Coomassie brilliant blue R-250) overnight. The next day, the gel was put in a destaining solution until bands of protein were visualized.

Detection of PIs by the agar well diffusion method

The agar well diffusion method was used to detect PI activity from crude and $(NH_4)_2SO_4$ fractions (F_1, F_2 and F_3). One gram of agar was heated and allowed to cool to about 55 ºC before adding 0.1 gram of gelatin.. The gelatin-containing agar plates were prepared and allowed to solidify. The wells were made by using a well borer. Three variable concentrations of enzymes and PIs viz. 3:1, 1:1, and 1:3 v/v were made. The final volume of the reaction mixture was adjusted with 0.1 M Tris-HCl buffer at pH 7.8. The wells were loaded with these mixtures (100 µl) separately with trypsin as the positive control and buffer as a negative control. The plates were incubated at 37 °C overnight, and the next day, 10% TCA was added to the wells and kept for 30 minutes. The gelatin hydrolysis was visually assessed around the well as hydrolysis zones. The well with the minimum hydrolysis zone diameter in comparison to control trypsin specifies inhibition and also indicates the presence of PIs.

Protease inhibitors (PIs) assay

The inhibitory action of PIs against proteases was assessed by an *in vitro* solution assay by the procedure described earlier with minor modification.[23] This method allows measurement of the TCA (trichloro acetic acid) soluble fractions of peptides formed by the action of trypsin (protease used) on the protein substrate casein. The residual proteolytic or caseinolytic activity in the presence of PIs is considered a measure of protease inhibitory activity. During the assay, suitable blanks for enzymes, substrate, and inhibitors were prepared along with the tests.

The procedure of PI assay is summarised as follows: 100 µl aliquot of trypsin (prepared in 0.1 N Tris HCl buffer, pH 7.2) was preincubated with different aliquots of PIs (crude and $(NH_4)_2SO_4$ fractions) i.e. 20 ìl, 40 ìl, and 60 ìl and appropriate volume of 0.1 N Tris HCl buffer, pH 7.2. In the reaction mixture, 1%, casein was mixed and incubated at 37 °C for 30 min. The reaction was terminated by adding 1 ml of 10% TCA and the tubes were then incubated for 10 minutes. The reaction mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was harvested. To 0.5 ml of supernatant, 1 ml of 20% Na_2CO_3 was added. The mixture was incubated for 20 minutes. Then, to this mixture, 0.5 ml of Folin-Ciocalteu (FC) reagent in a 1:3 dilution was added and allowed to be incubated for 10 minutes. The absorbance was read at 660 nm. The TCA soluble peptide fractions obtained during the assay were quantified by comparing them with tyrosine on the tyrosine standard curve. The resulting PIs activity was articulated in terms of the percent inhibition of trypsin (protease) activity.

Antimicrobial activity of PIs of *A. lebbeck*

Antimicrobial activity of PIs of *A. lebbeck* against *E.coli, S. aureus,* and *P. aeruginosa* was carried out by agar well diffusion method. About

50,100,150 and 200 µg proteins from $(NH_4)_2SO_4$ fractions (F_1, F_2 and F_3) were loaded in wells prepared on nutrient agar plates (swabbed with the mentioned microorganisms) with positive controls (Ampicillin for *E. coli*) and chloramphenicol for *P. aeruginosa* and *S. aureus* separately). The resulting plates were incubated at 37ºC for 24 hours, and minimum inhibitory concentration (MIC) was documented.

Total protein quantification

The total protein present was estimated by the Lowry method (1951) using bovine serum albumin as a standard.[24]

Statistical analysis

Experimental values were taken in duplicate or triplicate. The mean values and standard deviations (S.D.) were calculated using Microsoft Excel 2010. The results are presented in the form of bar digram using mean value ± S.D.

RESULTS

Total protein content

Here, crude proteins and proteins from $(NH_4)_2SO_4$ fractions (F_1, F_2 and F_3) were quantified by Lowry method.[24] The total protein content was found to be high in fraction F_1 (7.3 ± 0.17 mg/ml) in comparison to fractions F_2 and F_3 where it was 7.0 ± 0.12 and 6.5 ± 0.15 mg/ml, respectively (Table 1 and Figure 1).

Protein profiling by 10% native PAGE

On the gel, nine protein bands with different mobility were identified based on their colour intensity, such as thin and distinct, diffuse and thick (Figure 2**)**. When compared to F1 and F2, F3 had a more uniform and clear banding pattern, whereas the crude seed extract of *A. lebbeck* had fewer bands. Among all, 02 bands showed very slow migration on the gel, 02 were moderately moving, and the remaining 05 bands showed relatively faster migration on the gel finding a place at the bottom of the gel. The findings indicate that *A. lebbeck* seeds contain a diverse range of molecular weight proteins. Our results also demonstrated partial purifications of these proteins, as seen in the F_3 fraction containing lane.

Table 1. Total protein contents in seeds of *A. lebbeck*.F_1- 0–30 % $(NH_4)_2SO_4$ saturated fraction, F_2- 30–60 % $(NH_4)_2SO_4$ saturated fraction, and F_3- 60–90 % $(NH_4)_2SO_4$ saturated fraction

Sr. No.	Sample	Protein mg/ml ± SD
1	Crude	9.3 ± 0.34
2	F_1	7.3 ± 0.17
3	F_2	7.0 ± 0.12
4	F_3	6.5 ± 0.15

Detection of PI activity by agar well diffusion method

The agar well diffusion method was used to test the PI activity of crude extract and F3, 60-90% (NH4)2SO4 saturated fraction of *A. lebbeck* seeds. When the E:I ratio is 1:3, the PI activity is considered low, moderate when the ratio is 1:1, and high when the ratio is 3:1. Figure 3 presents a photograph of the agar-well diffusion method showing the potency of crude extract and F_3- 60–90 % $(NH_4)_2SO_4$ saturated fraction against trypsin. Crude extract and F_3- 60–90 % $(NH_4)_2SO_4$ saturated fraction showed moderate to higher activity against trypsin in all tested ratio, suggesting the presence of potent PI activity.

Protease inhibition solution assay

In our study, it was noted that all PIs could be precipitated at 0 - 90% (w/v) saturation of $(NH_4)_2SO_4$). Although, 60 - 90% (w/v) saturation of $(NH_4)_2SO_4$) was found to be effective for precipitating the PIs equated to other fractions. An in vitro caseinolytic assay against the protease trypsin was used to determine PI activity. The TCA soluble peptide fractions obtained during the assay (in the presence or absence of PIs) were quantified by comparing them with tyrosine on the tyrosine standard curve, as shown in Figure 4.

Results from Figure 5 and Table 2 show that F_3- 60–90 % $(NH_4)_2SO_4$ saturated fraction possesses potent PI activity. According to the findings, PI at a concentration of 150 µg/ml is effective and inhibits approximately 74.81±4.03 percent of trypsin activity. With higher

Table 2. Percent (%) inhibition of trypsin by F_3- 60–90 % $(NH_4)_2SO_4$ saturated fraction

	F_3 protein in µg/ml	% trypsin inhibition
1	150	74.81 ± 4.03
2	300	68.69 ± 4.30
3	450	70.12 ± 3.57

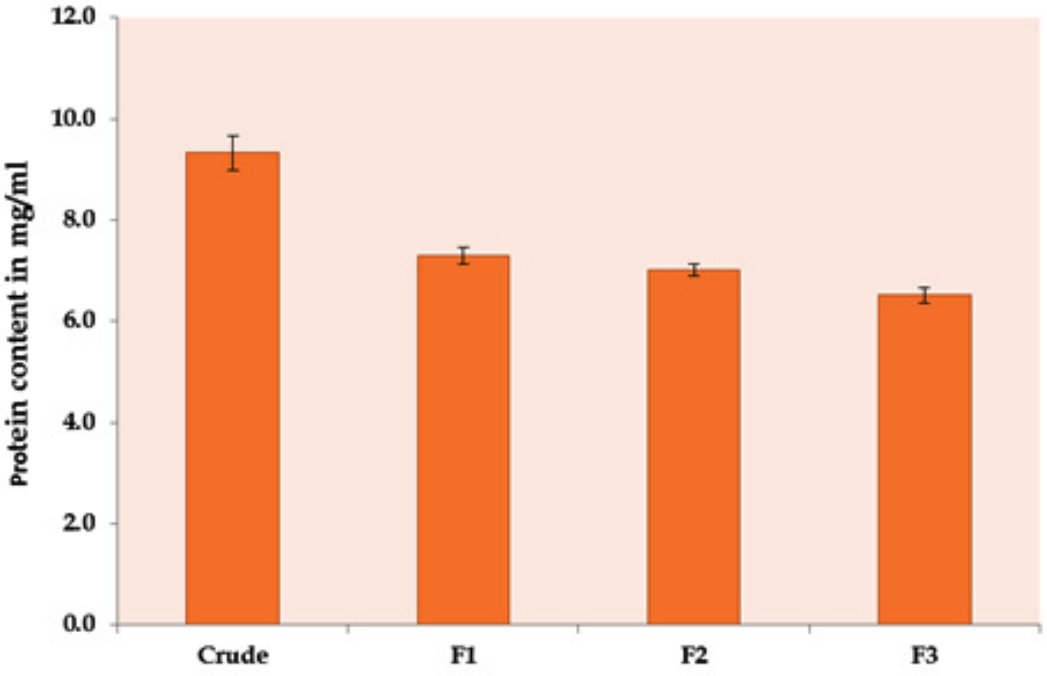

Fig. 1. Total protein contents in crude extract and three $(NH_4)_2SO_4$ saturated fractions of seeds of *A. lebbeck*. Results are mentioned as mean ± SD, n = 3. F_1- 0–30 % $(NH_4)_2SO_4$ saturated fraction, F_2- 30–60 % $(NH_4)_2SO_4$ saturated fraction, and F_3- 60–90 % $(NH_4)_2SO_4$ saturated fraction

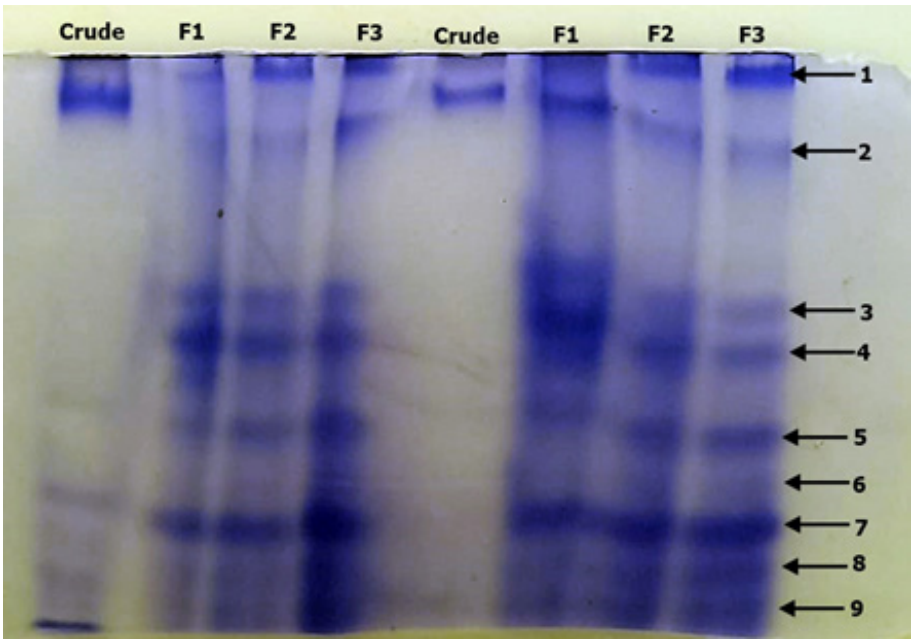

Fig. 2. 10% Native PAGE- Protein banding patterns in crude extract and three $(NH_4)_2SO_4$ saturated fractions of seeds of *A. lebbeck* (from right to left 10 µg & 20 µg sample of each loaded in different lanes). F_1- 0–30 % $(NH_4)_2SO_4$ saturated fraction, F_2- 30–60 % $(NH_4)_2SO_4$ saturated fraction, and F_3- 60–90 % $(NH_4)_2SO_4$ saturated fraction

concentrations, i.e. 300 µg/ml and 450 µg/ml, no significant change was observed.

Antimicrobial activity of PIs of *A. lebbeck*

The agar-well diffusion method is found to be an effective method to check the antimicrobial efficacy of compounds and hence used to test the antibacterial efficacy of F_3- 60–90 % $(NH_4)_2SO_4$ saturated protein fraction. The wells of 9 mm diameter were made in pre-inoculated nutrient agar plates with the test organisms, i.e. *E.coli, P. aeruginosa,* and *S. aureus.*

The presence of a zone of inhibition (ZOI) for different concentrations of F3- 60–90 % $(NH_4)_2SO_4$ saturated PIs revealed antimicrobial activity against *E.coli, P. aeruginosa,* and *S. aureus* (Figure 6). The antimicrobial activity of

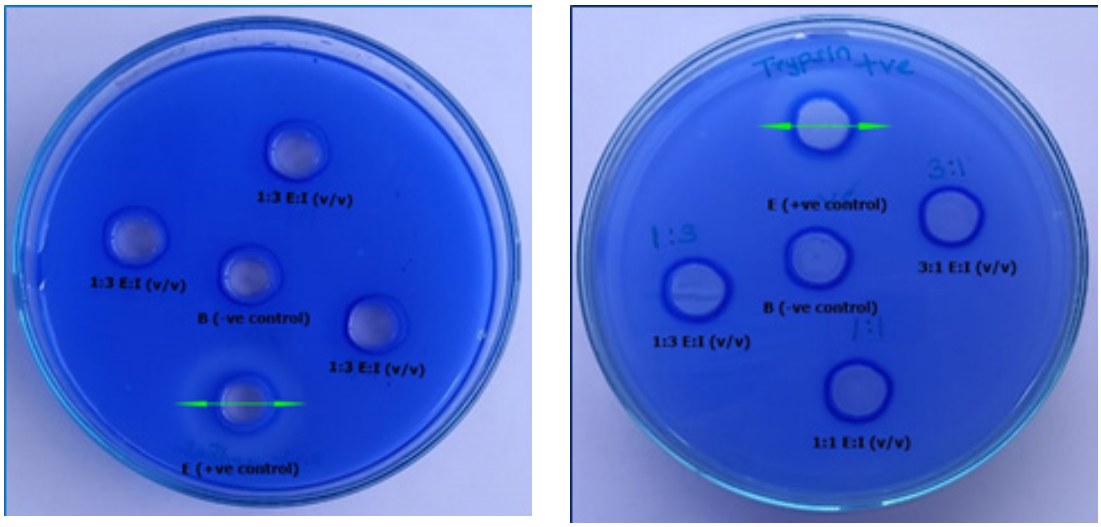

Fig. 3. Detection of PI activity by agar well diffusion method. Different trypsin (E) and I (crude extract and F_3-60–90 % $(NH_4)_2SO_4$ saturated fraction separately) ratios were incubated and loaded in wells on gelatin-containing agar. The detailed procedure is mentioned in the material and method section

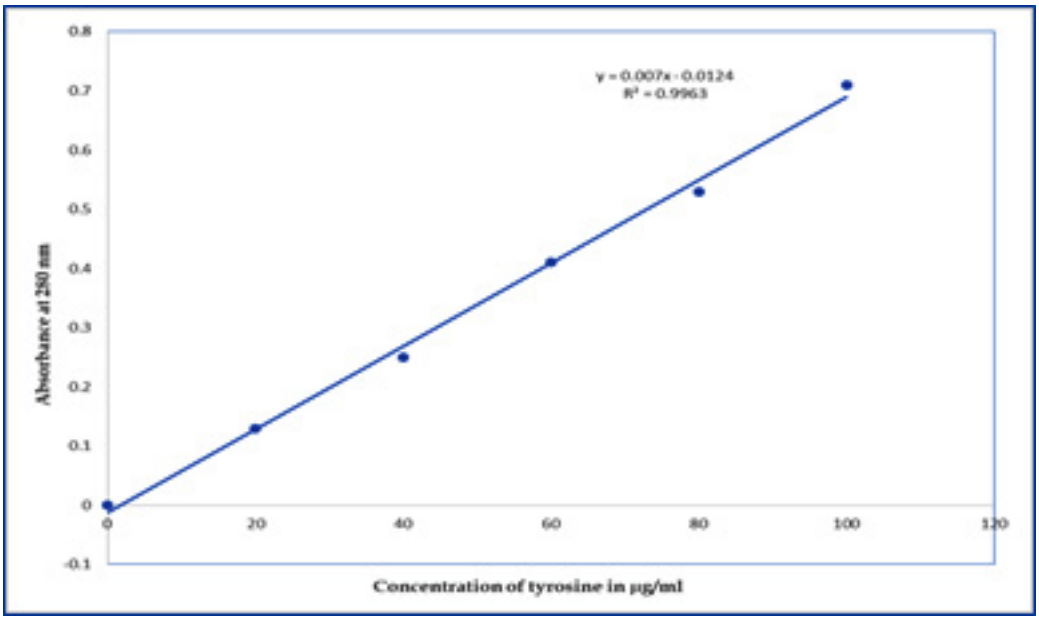

Fig. 4. Standard curve for tyrosine at 280 nm

different concentrations of F3- 60–90 % $(NH_4)_2SO_4$ saturated PIs was compared with ampicillin in the case of *E.coli* and chloramphenicol in the case of *P. aeruginosa* and *S. aureus* (Figure 7). Standard antibiotics (ampicillin and chloramphenicol) had the largest inhibition zone, but F3- 60-90% $(NH_4)_2SO_4$-saturated PIs inhibited all bacterial strains with MIC values of 100 ±5 µg/ml, 90 ±3 µg/ml, and 100 ±4 µg/ml, respectively (Figure 8). The results indicate that F3- 60–90 % $(NH_4)_2SO_4$ saturated PIs are potent antimicrobial agents against the tested microorganisms i.e. *E.coli, P. aeruginosa,* and *S. aureus*.

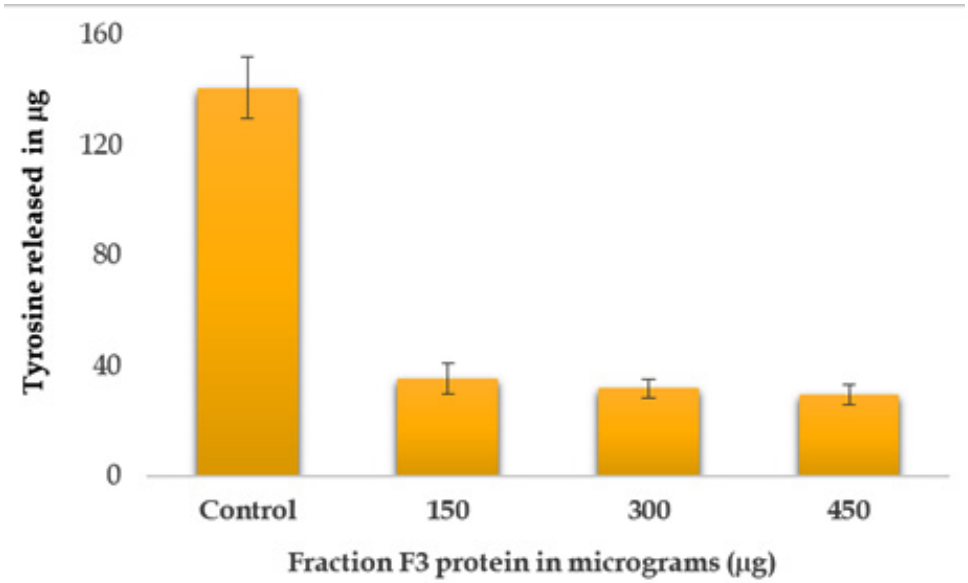

Fig. 5. PIs activity of F_3- 60–90 % $(NH_4)_2SO_4$ saturated fraction of *A. lebbeck* against trypsin by caseinolytic assay

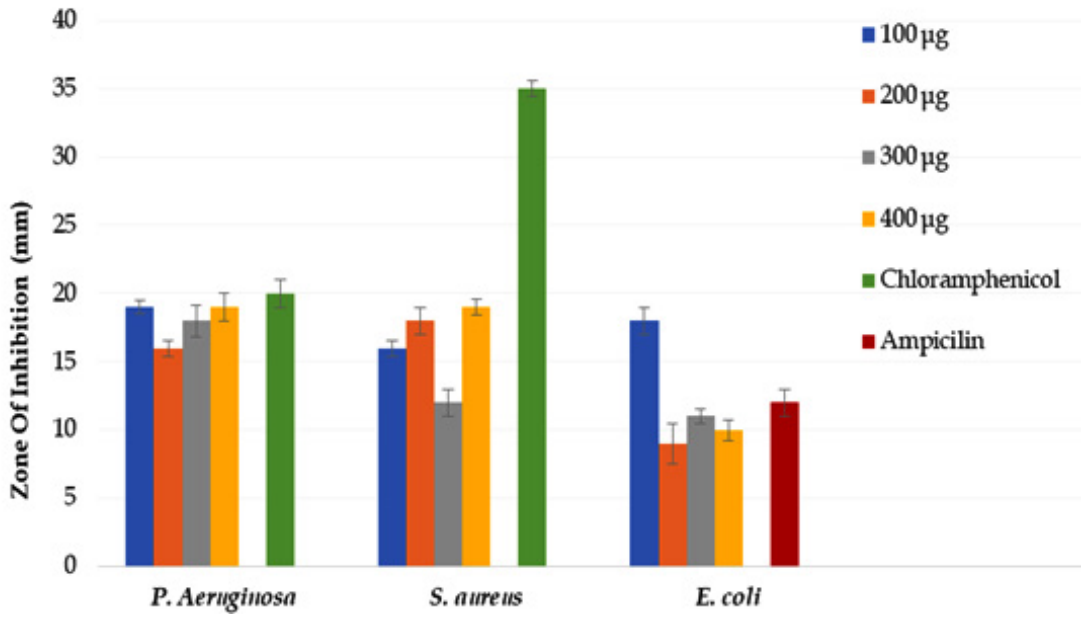

Fig. 6. Antimicrobial activity by different concentrations of F3- 60–90 % $(NH_4)_2SO_4$ saturated PIs

DISCUSSION

In the past few years, renewed interest has been gained in the identification, purification, and characterization of novel PIs worldwide. Having several applications in agricultural and medicinal biotechnology, they are widely studied. Plants are the major source of PIs. Plant PIs play an important role in their defense mechanisms such as protecting them against insects, pesticides, and diseases.[14] Previously, PIs were isolated from Thai legume seeds.[25] Godbole et al., found PIs in pigeon pea.[26] These PIs have a keen interest in research for the treatment of HIV and cancer.[27,28] We were interested in understanding the antimicrobial activity of these PIs which may be therapeutic agents to treat microbial diseases in humans. In this context, the study aimed to determine the antimicrobial potential of the proteinaceous seed extract of Sirisa (*A. lebbeck*), a well-known source of PIs as previously identified by Shaikh et al.[21] and Sharma et al.[29]

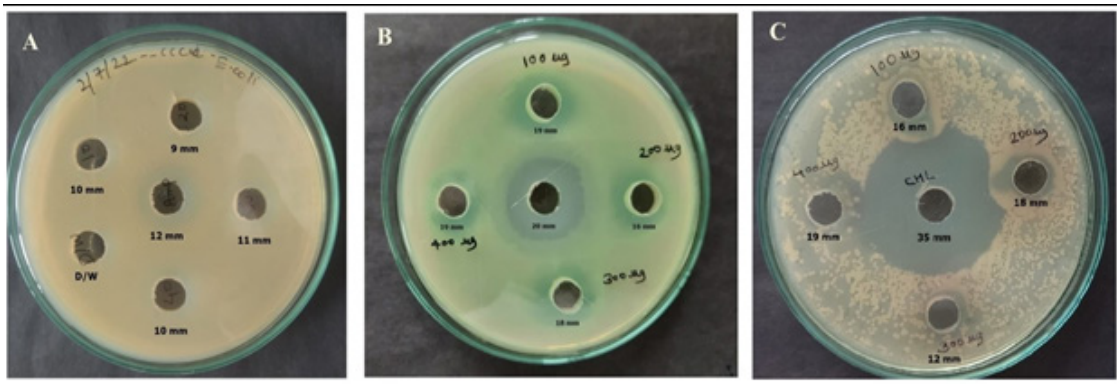

Fig. 7. Antimicrobial activity by different concentrations of F3- 60–90 % $(NH_4)_2SO_4$ saturated PIs determined using agar well diffusion method. Photograph of A) *E.coli* with ampicillin as control antibiotic in the middle well B) *P. aeruginosa* with chloramphenicol as control antibiotic in the middle well and C) *S. aureus* with chloramphenicol as control antibiotic in middle well

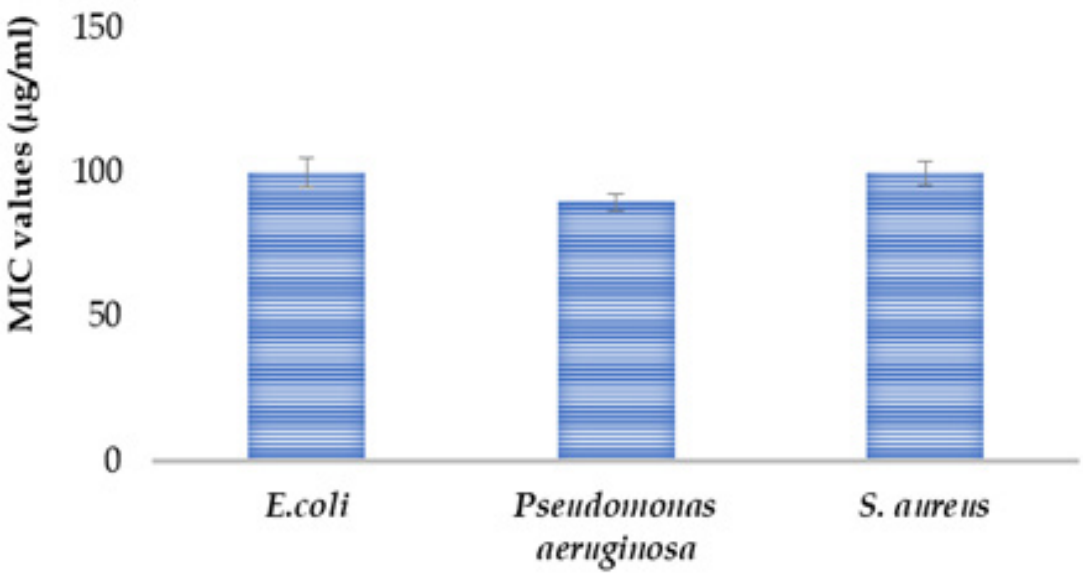

Fig. 8. Minimum inhibitory concentration (MIC) of F3- 60–90 % $(NH_4)_2SO_4$ saturated PIs against *E.coli, P. aeruginosa,* and *S. aureus*

The toxicity and side effects of synthetic or chemical antimicrobial drugs incline researchers toward natural sources for antibiotics.[30] Here, the experiments were designed to explore the total protein content of hexane defatted and acetone depigmented seed powder from *A. lebbeck*. The total protein content was estimated by a protein assay described by Lowry et al., in 1951 in terms of BSA equivalence.[24] The protein content was found to greater in seed powder of *A. lebbeck* in agreement with earlier findings.[21] Initial detection of PIs by the gelatin plate agar-well diffusion method demonstrated potent inhibitory activity against trypsin in crude protein extract and F_3- 60–90 % $(NH_4)_2SO_4$ saturated protein fraction as compared to other $(NH_4)_2SO_4$ saturated protein fractions, i.e. F_1 0-30 % and F_2 30-60 %. These findings indicate the presence of a significant amount of PIs in the proteinacous seed extract of *A. lebbeck*, as reported earlier.[21,29] The PIs potency of F_3- 60–90 % $(NH_4)_2SO_4$ saturated protein fraction was assessed by casienolytic assay using casein as substrate. More than 70% inhibition of trypsin was observed with 150 µg/ml of F_3- 60–90 % $(NH_4)_2SO_4$ saturated protein fraction. These results corroborated our earlier findings with PIs in the seed extract of *A. lebbeck*. [21,31]

The current study suggests that the protein extracts of *A. lebbeck* possess antimicrobial activity against *E. coli. P. aeruginosa and S. aureus* at altered concentrations. This will help to prevent the antimicrobial effects caused by these harmful pathogens on humans. Similarly, the antimicrobial potential of PIs with different concentrations was reported for *Lawsonia inermis*.[32] The microorganisms tested were *S. aureus* and *P. aeruginosa*. Indiscriminate use of commercially available antimicrobial drugs leads to multiple drug resistance, which is a great threat to public health. In this situation, plant-based antimicrobials could be an alternative to synthetic/chemical drugs that could avoid drug resistance.[33,34] In this perspective, our investigation may be helpful in designing plant-based antimicrobial and proteinaceous seed extracts of *A. lebbeck* with PIs that could be possible antimicrobial drug candidates.

CONCLUSION

This research aimed to identify protease inhibitors (PIs) with antimicrobial activity from the seeds of *Albizia lebbeck*. The PIs were partially purified by ammonium sulphate $(NH_4)_2SO_4$ fractionation. The salt precipitated fraction F_3 was a prominent source of PI activity as assessed by the agar well diffusion method and *in vitro* solution assay using casein. This fraction was further assessed for antimicrobial potential against *E. coli. P. aeruginosa and S. aureus*. Our results indicate that *A. lebbeck* PIs is a potent antimicrobial agent against the above-tested microorganisms. This study could be useful for designing plant-based antimicrobials that could be an alternative to antimicrobial drugs containing synthetic chemicals. The exhaustive analysis of PIs from seed extract of *A. lebbeck* with regard to their purification, structural identification, and functional characterization as antimicrobial agents is the future perspective of this study.

ACKNOWLEDGEMENT

The authors are thankful to Centre for Biotechnology, Pravara Institutes of Medical Sciences (Deemed to be University), Loni, Ahemadnagar (MS) India, for providing the related support required for execution of this research work. The authors are also thankful to Department of Microbiology, Dr. Balasaheb Vikhe Patil Rural Medical College, Pravara Institute of Medical Sciences (DU), Loni (MS) India, for providing microorganisms (*E. coli. P. aeruginosa and S. aureus*) for this study.

Conflict of Interest

The authors declare that there is no conflict of interest.

Funding Sources

There is no Funding Sources.

REFERENCES

1. López-Otín, Carlos., Bond J.S. Proteases: Multifunctional Enzymes in Life and Disease. J. Biol. Chem. 2008; 283 (45): 30433–30437.
2. King J. V., Liang W. G., Scherpelz K. P., Schilling A. B., Meredith S. C., Tang W. J. Molecular basis of substrate recognition and degradation by human presequence protease. Structure 2014; 22 (7): 996–1007.
3. Shen Y., Joachimiak A., Rosner M. R., Tang W. J. Structures of human insulin-degrading enzyme

reveal a new substrate recognition mechanism. Nature 2006; 443 (7113): 870–874._

4. Atkinson J. M., Siller C. S., Gill J. H. Tumour endoproteases: the cutting edge of cancer drug delivery?. British J Pharmacol. 2008; 153(7): 1344–1352.
5. Hamza T.A. Bacterial Protease Enzyme: Safe and Good Alternative for Industrial and Commercial Use. Int. J. Chem. Sci. 2017; 3(1): 1-10.
6. Dusing R., Sellers F. ACE inhibitors, angiotensin receptor blockers and direct renin inhibitors in combination: a review of their role after the ONTARGET trial. Curr. Med. Res. Opin. 2009; 25: 2287–2301.
7. Rijken D. C., Lijnen H. R. New insights into the molecular mechanisms of the fibrinolytic system. Journal of thrombosis and haemostasis: JTH. 2009; 7(1): 4–13.
8. Krishnaswamy S. Exosite-driven substrate specificity and function in coagulation. J. Thromb. Haemost. 2005; 3: 54–67.
9. Hu J., Van den. Steen PE., Sang Q.X. et al. Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. Nat. Rev. Drug Discov. 2007; 6: 480–498.
10. Adams J., Kauffman M. Development of the proteasome inhibitor Velcade (Bortezomib). Cancer Investig. 2004; 22 (1): 304–311.
11. Drag M., Salvesen G.S. Emerging principles in protease-based drug discovery. Nat. Rev. Drug Discov. 2010; 9(9): 690-701.
12. Koiwa H., Bressan R. A., Hasegawa P. M. Regulation of protease inhibitors and plant defense, Trends Plant Sci. 1997; 2(10): 379-384.
13. Rawlings N.D., Tolle D.P., Barrette A.J. MEROPS: the peptidase database. Nucleic Acids Resources. 2008; 32: 160–164.
14. Ryan C.A. Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annu. Rev. Phytopathol. 1990; 28: 425-449.
15. Mosolov V.V., Valueva T. A. Proteinase inhibitors and their function in plants. A review. Appl. Biochem. Microbiol. 2005; 41: 227-246.
16. Migliolo L., Oliveira A.S., Santos E.A. et al. Structural and mechanistic insights into a novel non-competitive Kunitz trypsin inhibitor from *Adenanthera pavonina* L. seeds with double activity toward serine and cysteine-proteinases. J. Mol. Graph. Model 2010; 29: 148-156.
17. Srikanth S., Chen, Z. Plant Protease Inhibitors in Therapeutics-Focus on Cancer Therapy. Front. Pharmacol. 2016; 7: 470.
18. de Souza Cândido E., Pinto M.F.S., Pelegrini P.B. et al. Plant storage proteins with antimicrobial activity: novel insights into plant defense mechanisms. The FASEB J. 2011; 25: 3290-3305.
19. Costa H.P.S., Oliveira J.T.A., Sousa D.O.B., et al. JcTI-I: a novel trypsin inhibitor from *Jatropha curcas* seed cake with potential for bacterial infection treatment. Front. Microbiol. 2014; 5 (1): 1-12.
20. Kim J.Y., Park S.C., Hwang, I. et al. Protease inhibitors from plants with antimicrobial activity. Int. J. Mol. Sci. 2009; 10: 2860-2872.
21. Shaikh F.K., Gadge P.P., Shinde A.A., et al. Characterization of the AlTI13 protein from Indian Siris (*Albizia lebbeck*) that inhibit the growth of cotton bollworm (*Helicoverpa armigera*). Asia Pac. Entomol. 2014; 17: 319–325._ _
22. Davis B.J. Disc Electrophoresis. 2, Method and application to human serum proteins. Ann. New York Acad. Sci. 1964; 121: 404-427.
23. Kunitz M.J. Crystalline soyabean trypsin inhibitor II General properties. J. Gen. Physiol. 1947; 30: 291-310.
24. Lowry O.H., Rosebrough N.J., Farr A.L. et al. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951; 193: 265-275.
25. Benjakul S., Visessanguan W., Thummaratwasik P. Isolation and characterization of trypsin inhibitors from some thai legume seeds. J. Food Biochem. 2000; 24 (2): 107-127.
26. Godbole S. A., Thirumalai G. Krishna et al. Purification and Characterisation of Protease Inhibitors from Pigeon Pea (*Cajanus cajan* (L) Millsp) Seeds. J. Sci. Food Agric. 1994; 64: 87-93.
27. Lingaraju M. H., Gowda L. R. A Kunitz trypsin inhibitor of *Entada scandens* seeds: Another member with single disulfide bridge. Biochim. Biophys. Acta 2003; 91: 755-769.
28. Magee P. J., Owusu-Apenten R., McCann M. J. et al. Chickpea (*Cicer arietinum*) and other plant derived protease inhibitor concentrates inhibits breast and protease cancer cell proliferation *in vitro*. Nutr. Cancer 2012; 64: 741-748.
29. Sharma P., Nath A.K., Bhardwaj S.V. Purification, characterization and evaluation of insecticidal activity of trypsin inhibitor from *A. lebbeck* seeds. J. For. Res. 2012; 23 (1): 131-137.
30. Alam M.K., Jamal M. A. H. M., Ajam M., et al. Antifungal and antibacterial activity of the different parts of mature *Benincasa hispida* against various microbial infectious agents. Afr. J. Microbiol. Res. 2021; 15(7): 349-359.
31. Shaikh F., Bradosty S., Hamad S. et al. *In Vitro* Screening of Seed Extracts of Medicinal Plants for Protease Inhibitory Activity. CUESJ. 2019: 3(1): 61-65.

32. Dabhade P. A., Patel P., Patil U. Proteinaceous protease inhibitor from *Lawsonia inermis*: purification, characterization and antibacterial activity. Nat. Prod. Commun. 2013; 8 (1): 1467-1470.
33. Zhang Y.J., Gan R.Y, Li S., et al. Antioxidant Phytochemicals for the Prevention and Treatment of Chronic Diseases. Molecules 2015; 20(12): 21138-56.
34. Karuppiah P., Rajaram S. Antibacterial effect of *Allium sativum* cloves and *Zingiber officinale* rhizomes against multiple-drug resistant clinical pathogens. Asian Pac. J. Trop. Biomed. 2012; 2(8): 597-601.