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Research Article

In Vitro Screening of Seed Extracts of Medicinal Plants for Protease Inhibitory Activity

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ABSTRACT

Protease inhibitors (PIs) are deployed in the plant kingdom as storage proteins or peptides, regulators of endogenous proteases, and plant protection agents against insect pests and pathogen attack. In humans, they are identified as chemopreventive agents against a range of cancers and have potential as drug to treat an array of disease associated with aberrant activity of proteases. The present investigation reports PIs activity data from 30 medicinal plants. The screening for PIs activity was done by dot blot assay using X-ray film coated with gelatin. Among screened seed extracts, *Albizia lebbeck*, *Raphanus sativus*, *Mucuna pruriens, Achyranthes aspera*, and *Coffea arabica* showed high inhibitory activities with trypsin protease. Most of seed extracts exhibited moderate activity, whereas *Ocimum sanctum* showed moderate to low activity against trypsin. The presence of varied protein content is reported from all seed extracts with highest in *A. lebbeck* (50.0 ± 3.4 mg/ml). The data produced in the present investigation could be helpful for further exploration of PIs as therapeutic agent.

Keywords: Dot blot, protease inhibitors, proteases, protein, trypsin

INTRODUCTION

Proteases or peptidases (E.C. 3.4) are one of the largest and the most diverse families of enzymes which selectively catalyze the hydrolysis of peptide bonds in proteins.[1,2] Proteases are physiologically vital in all living organisms for cell growth, cell differentiation and death (apoptosis), cell migration, and invasion and are abundant in a wide variety of sources starting from viruses, bacteria, protozoa, metazoan, or fungi ending with plants and animals.[3,4] They perform a variety of functions during the vital processes in organism such as food digestion, blood clotting, embryogenesis, tissue reorganization (e.g., wound healing, regeneration, molting, metamorphosis, etc.), defense mechanisms, and immune responses.[5,6] From the analysis of several genomes, it is estimated and found that about 2–4% of all gene products are proteases with more than 560 members.[7,8] Proteases are intricately involved in the protein catabolism and their action can be divided into two different categories: Limited proteolysis and unlimited proteolysis. In limited proteolysis, proteases cleave specific peptide bonds in immature proteins or remove the target signals in pre-proteins, whereas the unlimited proteolysis (bulk hydrolysis) of dietary proteins is essential for providing cells with simple metabolites essential for growth and development. On the basis of catalytic types, they can be classified into serine, cysteine, aspartic, threonine, glutamic acid, and metalloproteases.[9]

Serine proteases are numerous and widespread among the mammals, viruses, insects, bacteria, and eukaryotes, suggesting that they are vital to the organisms. Serine proteinases are classically categorized by their substrate specificity, particularly by whether the residue at P1: trypsin-like (Lys/Arg preferred at P1), chymotrypsin-like (large hydrophobic residues such as Phe/Tyr/Leu at P1), or elastase-like (small hydrophobic residues such as Ala/Val at P1).[10] They are found to be involved in tissue degradation, blood coagulation, digestion, development, and immune defense.[11] The aberrant activity of these proteases can lead to various diseases and thus has been the focus of intense investigation as potential therapeutic targets.

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Protease inhibitors (PIs) are molecules that resist the proteolytic actions of proteases and play a key role in regulation of protein catabolism in the body, the aberrant activity of proteases can lead to various diseases and must be regulated.[2] The diseases which could be attributed are metastasis, angiogenesis, Alzheimer's disease, Parkinson's disease, AIDS, and ischemia.[12,13] In general, regulation could be achieved by differential transcription, post-translational processing, subcellular compartmentalization, and the presence and activity of PIs.[14,15] The existence of PIs in nature was first reported by Fermi and Pernossi.[16] They are small proteins which are quite common in nature and also present in all life forms.[17] In plants, PIs are generally concentrated (about 10%) in the seeds as seed storage proteins.[18] In general, PIs are grouped into five groups as serine PIs, cysteine PIs, threonine PIs, aspartic PIs, and metalloproteases.[19] Among these, serine PIs is predominant followed by cysteine PIs and metalloproteases, while aspartic PIs and aspartic PIs are rare and dispersed in different families. Serine PIs are widespread, well-characterized, and most-studied class of PIs in plants.[20,21] Historically, serine PIs were first characterized from soybean plant.[22]

Proteases are proven therapeutic targets and screening of potent PIs against aberrant proteolytic activity could lead to the development of the drugs. Earlier plant-based PIs are found to be effective against cardiovascular diseases, osteoporosis, inflammatory diseases, and neurological disorders. Several plant PIs are under evaluation in *in vitro* clinical trials for the treatment of many diseases. The PIs have widespread applications in different sectors and are one of the prime candidates in biotechnology and medicine. The present investigation reports the detection and screening of PIs from 30 medicinal plants.

MATERIALS AND METHODS

Procurement of Seeds

Dry seeds of medicinal plants were purchased from the local market of Aurangabad (MS), India.

Procurement of Chemicals

Trypsin (bovine pancreas, E.C. 3.4.21.4), bovine serum albumin (BSA), hexane, acetone, and polyvinylpyrrolidone (PVP) were obtained from Sisco Research Laboratories, Mumbai, India. X-ray films were obtained from Fuji film, USA. All other chemicals used in this study were of the highest purity available.

Extractions of PIs from Medicinal Plants

The dried seeds were pulverized to a fine powder in a mixer grinder. The fat was removed from the powder by hexane and acetone washes. Defatted powder was suspended in Milli-Q water (1:6 w/v) containing 1% PVP and incubated overnight at 15°C. The suspension was then centrifuged at ×12,000 g for 20 min at 4°C, and the supernatant was termed as crude PIs.[23]

Detection of PIs by Dot Blot/Spot Test

The dot blot/spot test was carried out to determine the potency of PIs against protease trypsin, using X-ray film.[23] Three different concentrations of the trypsin and PIs were prepared: 1 (1:3), 2 (1:1), and 3 (3:1) v/v. The volume of the reaction mixture was adjusted by 0.1 M Tris-HCl (pH 7.8). The total volume was made up to 20 µl using buffer and loaded onto X-ray film. The film with spots was incubated for 20 min at 37°C, then washed with running tap water and dried in air. Different ratios of enzyme and inhibitor produced different patterns of gelatin hydrolysis on the X-ray film depending on the efficiency of inhibitor, which may be observed visually and scanned at 300 dpi using an HP digital scanner.

Protein Determinations

Protein content was estimated by following the method of Lowry *et al.*[24] using BSA as the standard.

Statistical Analysis

All experiments were conducted and analyzed in triplicate. The means and standard deviations were calculated and compared using Microsoft Excel 2010.

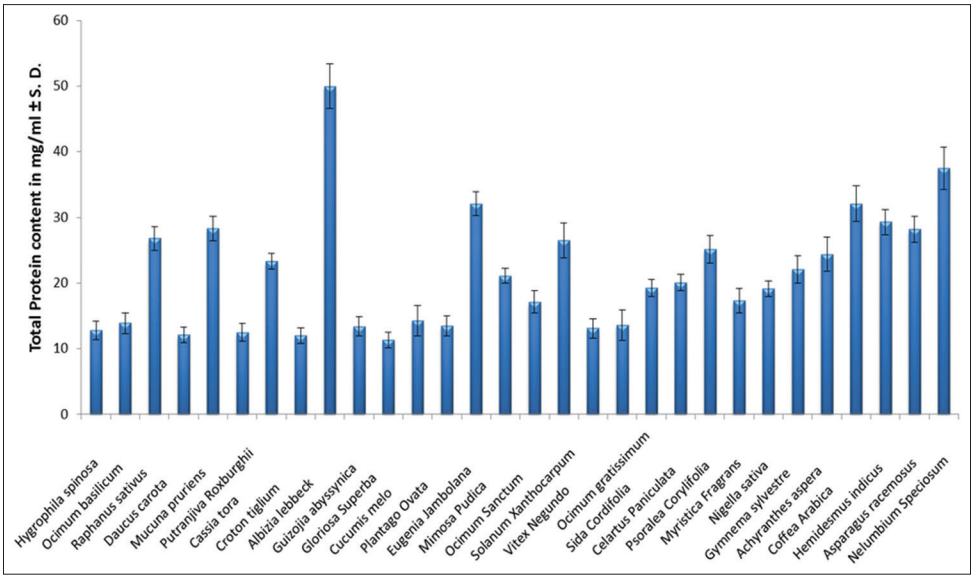

Figure 1: Protein content of selected medicinal plants. Values are mean±standard deviation for at least three replicates

RESULTS AND DISCUSSION

The past few decades have seen a growing interest in the identification, purification, and characterization of novel PIs. They are prime candidates who have numerous applications in agricultural and medicinal biotechnology. Plants are good sources of PIs which protect them against diseases, insects, pests, and herbivores.[25] They also explored as natural drugs for the treatment of an array of diseases including cancers.[13,26] The current study was undertaken to detect the presence of PIs activity in seeds of selected medicinal plants. Here, we screened about 30 medicinal plants for PIs activity.

The initial experiments were designed to explore total protein content from hexane defatted seed powder of these plants. The total protein content was estimated by protein assay developed by Lowry *et al*.[24] in terms of BSA equivalence [Figure 1 and Table 1]. The protein content was found to be higher in medicinal plants belonging to Fabaceae family with highest in *Albizia lebbeck* (50.0 ± 3.4 mg/ml). The higher protein content is attributing to the additional nitrogen that legumes receive through the process of nitrogen fixation.[27] The seed extracts of other medicinal plants were reported to have varied protein content as reported in Table 1.

The crude seed extracts of these plants were further investigated for PIs activity. The presence of PIs activity was confirmed by spot test (dot blot) analysis.[23] Detection of PIs was observed by mixing various concentrations of protease and inhibitor and spotted on the X-ray film, then the clearing zone formed due to gelatin hydrolysis by trypsin and a reduction in the clearing zone by trypsin incubated with an inhibitor was compared. Dot blot method was used to screen a large number of PIs. The results of detection of PIs activity by dot blot are shown in Figure 2 and results for screening of seed sample of 30 plants for inhibition of proteases are given in Table 2.

Table 1: Classification and protein content of selected medicinal plants

Botanical name	Family	Common name	Total protein content (mg/ml±SD)
Hygrophila spinosa	Acanthaceae	Talimkhana	12.8±1.4
Ocimum basilicum	Lamiaceae	Sweet basil	13.9±1.6
Raphanus sativus	Brassicaceae	Radish	26.8±1.8
Daucus carota	Apiaceae	Wild carrot	12.1±1.2
Mucuna pruriens	Fabaceae	Velvet bean	28.3±1.9
Putranjiva roxburghii	Putranjivaceae	Putranjiva	12.5±1.4
Cassia tora	Leguminosae	Sickle Senna	23.3±1.2
Croton tiglium	Euphorbiaceae	Jamaal gota	12.0±1.2
Albizia lebbeck	Fabaceae	Siris tree	50.0±3.4
Guizojia abyssynica	Asteraceae	Black seed	13.4±1.5
Gloriosa superba	Colchicaceae	Flame lily	11.3±1.2
Cucumis melo	Cucurbitaceae	Muskmelon	14.3±2.3
Plantago ovata	Plantaginaceae	Ispaghul	13.5±1.5
Eugenia jambolana	Myrtaceae	Jambolan	32.1±1.8
Mimosa pudica	Fabaceae	Sensitive Plant	21.1±1.1
Ocimum sanctum	Lamiaceae	Tulasi	17.1±1.7
Solanum xanthocarpum	Solanaceae	Kantakari	26.5±2.7
Vitex negundo	Lamiaceae	Nirgundi	13.1±1.5
Ocimum gratissimum	Lamiaceae	Ram Tulsi	13.6±2.3
Sida cordifolia	Malvaceae	Kharinta	19.3±1.3
Celastrus paniculata	Celastraceae	Malkagani	20.1±1.2
Psoralea corylifolia	Fabaceae	Bavachi	25.1±2.1
Myristica fragrans	Myristicaceae	Jatiphala	17.3±1.9
Nigella sativa	Ranunculaceae	Kalonji	19.1±1.2
Gymnema sylvestre	Apocynaceae	Gurmar	22.1±2.1
Achyranthes aspera	Amaranthaceae	Chaff-flower	24.4±2.6
Coffea arabica	Rubiaceae	Coffee	32.1±2.7
Hemidesmus indicus	Apocynaceae	Nannari	29.3±1.9
Asparagus racemosus	Asparagaceae	Shatavari	28.2±2.0
Nelumbium speciosum	Nelumbonaceae	Lotus/Kamal	37.5±3.2

SD: Standard deviation

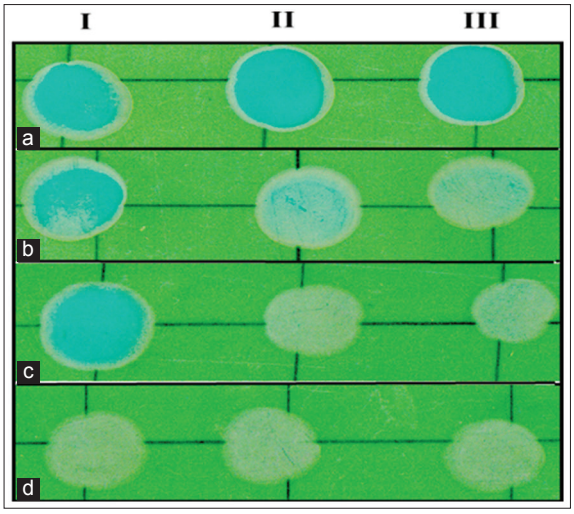

Figure 2: Detection of protease inhibitor (PI) of trypsin by the dot blot method. Three different concentrations of enzymes and inhibitors (I [3:1], II [1:1], and III [1:3]) were used for screening of PIs. The faint green spot (gelatin) indicates total inhibition of enzymes, while dark blue spot (gelatin hydrolysis) indicates no inhibition. TI profile of *Hygrophila spinosa* (a), *Ocimum sanctum* (b), *Cucumis melo* (c), and *Albizia lebbeck* (d)

Table 2: Screening of PIs from seeds extract of medicinal plants by dot blot method

Botanical name	Trypsin		
	3:1	1:1	1:3
Hygrophila spinosa	N	N	N
Ocimum basilicum	N	N	N
Raphanus sativus	P	Y	Y
Daucus carota	N	N	N
Mucuna pruriens	Y	Y	Y
Putranjiva roxburghii	N	N	N
Cassia tora	N	N	P
Croton tiglium	N	N	N
Albizia lebbeck	Y	Y	Y
Guizojia abyssynica	N	P	P
Gloriosa superba	N	N	P
Cucumis melo	P	Y	Y
Plantago ovata	N	N	N
Eugenia jambolana	P	P	Y
Mimosa pudica	N	N	N
Ocimum sanctum	N	N	P
Solanum xanthocarpum	N	N	P
Vitex negundo	N	N	P
Ocimum gratissimum	N	N	Y
Sida cordifolia	N	P	Y
Celastrus paniculata	N	N	N
Psoralea corylifolia	N	N	N
Myristica fragrans	P	Y	Y
Nigella sativa	P	P	Y
Gymnema sylvestre	P	P	Y
Achyranthes aspera	Y	Y	Y
Coffea arabica	Y	Y	Y
Hemidesmus indicus	P	P	Y
Asparagus racemosus	P	Y	Y
Nelumbium speciosum	P	Y	Y

(contd...)

Table 2: *(Continued)*

N: No inhibition, P: Partial inhibition, Y: Total inhibition, PIs: Protease inhibitors

Among screened samples, *A. lebbeck, Raphanus sativus, Mucuna pruriens, Achyranthes aspera,* and *Coffea arabica* showed high inhibitory activities against trypsin. At all selected ratio (1:3, 1:1, and 3:1) of trypsin and the inhibitor, all these samples showed inhibition. Most of the screened seed samples such as *Cucumis melo, Eugenia jambolana, Sida cordifolia, Myristica fragrans, Nigella sativa, Gymnema sylvestre, Hemidesmus indicus, Asparagus racemosus,* and *Nelumbium speciosum* exhibited moderate to high trypsin inhibitory activity, while *Ocimum sanctum* showed moderate to low activity against trypsin. The present study revealed that seed extracts of *A. lebbeck, R. sativus, M. pruriens, A. aspera,* and *C. arabica* were effective in inhibiting the trypsin and could be explored as PIs source.

Proteases are enzymes involved in protein digestion. These enzymes are omnipresent in plants, animals, and also most microorganisms. They are importantly involved in health and well-being by performing a pivotal role in survival and maintenance of the organism. They constitute about 2% of the human genome. The conduct of proteases in spite of many advantages has to be closely regulated and controlled to avoid the excess activity of these enzymes, as it may possibly damage its host organism. This task of controlling is carried out by PIs. The PIs have coevolved with proteases, to control their destructive nature.[28] Cancer is a collection of over 100 devastating diseases that share a number of characteristics, a primary hallmark of which is out-of-control growth. There is a positive correlation between the aggressiveness of a tumor and the secretion of various proteases.[29] A number of reviews on various aspects of the use of PIs as a mean to combat cancer have been published recently. Therefore, the aim of this study was to screen Indian medicinal plants for PIs and *A. lebbeck, R. sativus, M. pruriens, A. aspera,* and *C. arabica* were found to exhibit PIs activity. This study could be useful for the identification of PIs, designed for therapeutic applications.

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AUTHORS' CONTRIBUTIONS

Faiyaz Shaikh designed, performed, and coordinated the main study. Sarwan Hamad, Saber Hamad, and Ashok Shinde helped in the manuscript preparation and editing.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

REFERENCES

1. A. J. Barrett, N. D. Rawlings and J. J. F. Woessner. "*Hand Book of Proteolytic Enzymes*". London: Acadamy Press, 1998.
2. S. G. Fan and G. J. Wu. "Characteristics of plant proteinase inhibitors and their applications in combating phytophagous insects". *Botanical bulletin of Academia Sinica*, Vol. 46, pp. 273-292, 2005.
3. A. N. Flynn and A. G. Buret. "Proteinase-activated receptor 1 (PAR-1) and cell apoptosis". *Apoptosis*, Vo. 9, pp. 729-737, 2004.
4. G. A. Joanitti, S. M. Freitas and L. P. Silva. "Proteinaceous protease inhibitors: Structural features and multiple functional faces". *Current Enzyme Inhibition*, Vol. 2, no. 3, pp. 199-217, 2006.
5. P. K. Lawrence and K. R. Koundal. "Plant protease inhibitors in control of polyphagous insects". *Electronic Journal of Biotechnology*. Vol. 1, pp. 93-109, 2002.
6. D. Ivanov, C. Emonet, F. Foata, M. Affolter, M. Delly, M. Fisseha, S. Blum-Sperisen, S Kochhar and F. Arigoni. "A serpin from the gut bacterium *Bifidobacterium longum* inhibits eukaryotic elastase like serine proteases". *Journal of Biological Chemistry*, vol. 281, no. 25, pp. 17246-17252, 2006.
7. A. J. Barrett, N. D. Rawlings and E. A. O'Brien. "The MEROPS database as a protease information system". *Journal of Structural Biology*, vol. 134, pp. 95-102, 2001.
8. X. S. Puente, L. M. Sanchez, C. M. Overall and C. Lopez-Otin. "Human and mouse proteases: A comparative genomic approach." *Nature Reviews Genetics*, vol. 4, pp. 544-558, 2003.
9. D. N. Rawlings, P. D. Tol1e and A. J. Barrett. "Evolutionary families of peptidase inhibitors". *Biochemical Journal*, vol. 378, pp. 705-716, 2004.
10. J. D. Tyndall, T. Nall and D. P. Fairlie. "Proteases universally recognize beta strands in their active sites." *Chemical Reviews*, vol. 105, pp. 973-999, 2005.
11. A. J. Molehin, G. N. and D. P. Gobert. "McManus: Serine protease inhibitors of parasitic helminthes." *Parasitology*, vol. 139, no. 6, pp. 681-695, 2012.
12. M. L. J. Korsinczky, H. J. Schirra and D. J. Craik. "Sunflower trypsin inhibitor-1". *Current Protein and Peptide Science*, vol. 5, p. 351, 2004.
13. M. H. Lingaraju and L. R. Gowda. "A Kunitz trypsin inhibitor of *Entada scandens* seeds: Another member with single disulfide bridge." *Biochimica et Biophysica Acta*, vol. 1784, pp. 850-855, 2008.
14. P. R. Shewry. "Tuber storage proteins". *Annals of Botany*, vol. 91, pp. 755-769, 2003.
15. N. D. Rawlings, A. J, Barrett and A. Bateman. "MEROPS: The database of proteolytic enzymes, their substrates and inhibitors". *Nucleic Acids Research*, vol. 40, pp. 343-350, 2012.
16. C. Fermi and L. Pernossi. "Untersuchungen uber die enzyme, ver-gleichende studie". Zeitschrift für Hygiene und Infektionskrankheiten, vol. 18, pp. 83-89, 1894.
17. H. Fritz. Foreword. In: K. von der Helm, B. D. Korant and J. C. Cheronis, editors. "*Proteases as Targets for Therapy*". Berlin: Springer-Verlag, pp. 5-6, 2000.
18. F. De Leo, M. Volpicella, F. Licciulli, S. Liuni, R. Gallerani and L. R. Ceci. "PLANT-PIs: A database for plant protease inhibitors and their genes". *Nucleic Acids Research*, vol. 30, no. 1, pp. 347-348, 2002.
19. G. Fear, S. Komarnytsky and I. Raskin. "Protease inhibitors and their peptidomimetic derivatives as potential drugs." *Pharmacology and Therapeutics*, vol. 113, pp. 354-368, 2007.
20. G. C. Mello, M. L. V. Oliva, J. T. Sumikawa, O. L. T. Machado, S. Marangoni, J. C. Novello and M. L. R. Macedo. "Purification and characterization of a new trypsin inhibitor from *Dimorphandra mollis* seeds". *Journal of Protein Chemistry*, vol. 20, pp. 625-632, 2001.
21. S. Haq, S. Atif and R. Khan. "Protein proteinase inhibitor genes in combat against insects, pests, and pathogens: Natural and engineered phytoprotection". *Archives of Biochemistry and Biophysics*, vol. 431, pp. 145-159, 2004.
22. J. W. Read and L. W. Haas. "Studies on the baking quality of flour as affected by certain enzyme actions. V. Further studies concerning potassium bromate and enzyme activity". *Cereal Chemistry*, vol. 45, pp. 59-68, 1938.
23. M. V. Padul, R. D. Tak and M. S. Kachole. "Protease inhibitor (PI) mediated defense in leaves and flowers of pigeonpea (protease inhibitor mediated defense in pigeonpea)". *Plant Physiology and Biochemistry*, vol. 52, pp. 77-82, 2012.
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall. "Protein measurement with folin phenol reagent". *Journal of Biological Chemistry*, vol. 193, pp. 265-275, 1951.
25. C. A. Ryan. "Protease inhibitors in plants: Genes for improving defenses against insects and pathogens". *Annual Review of Phytopathology*, vol. 28, pp. 425-449, 1990.
26. P. J. Magee, R. Owusu-Apenten, M. J. McCann, C. I. Gill and I. R. Rowland. "Chickpea (*Cicer arietinum*) and other plant-derived protease inhibitor concentrates inhibit breast and prostate cancer cell proliferation *in vitro*". *Nutrition and Cancer*, vol. 64, pp. 741-748, 2012.
27. J. I. Sprent. "Legume Nodulation: A Global Perspective". Ames, Iowa: Wiley-Blackwell, pp. 12, 2009.
28. N. Vergnolle. "Protease inhibition as new therapeutic strategy for GI diseases". *Gut*, vol. 65 pp. 1215-1224, 2016.
29. C. Fields, P. Mallee, J. Muzard and G. U. Lee. "Isolation of Bowman-Birk inhibitor from soybean extracts using novel peptide probes and high gradient magnetic separation". *Food Chemistry*, vol. 134, pp. 1831-1838, 2012.