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DEVELOPMENT OF AN ASSAY PROCEDURE FOR ASPARTIC PROTEASE INHIBITORY ACTIVITY AND SCREENING OF SOME MEDICINAL PLANTS IN SRI LANKA

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Abstract— Aspartic acid proteases participate in many physiological processes and their activities are associated with the spreading of diseases such as Alzheimer's disease, hypertension, cancers, and inflammatory, cardiovascular, viral, and other parasitic diseases. Aspartic proteases have become a widely spoken topic due to human immunodeficiency virus protease and malaria parasite protease that are targeted as key therapeutic intervention points in treating AIDS and malaria, respectively. But, relatively few studies have been reported on natural aspartic inhibitors. Therefore inhibition of proteases is one of the most promising approaches as therapeutic drugs for such conditions. In this case, plants have become a good source of proteinaceous and non-proteinaceous inhibitors. The present study is aimed at the identification, characterization, and partial purification of potent aspartic protease inhibitor/s from several medicinal plants in Sri Lanka.

For this study plant samples were collected from Kandy district, Sri Lanka. Aqueous extracts of equal concentrations were prepared separately using fresh mature bark samples from *Annona muricata*, *Garcinia quaesita* Pierre, *Bauhinia tomentosa*, *Caesalpinia bonduc*, *Crotalaria laburnifoloria*, *Crotalaria micans*, *Entada zeylanica* (kosterm), *Erythrina suberosa*, *Azadirachta indica*, and *Limonia acidissima* L. To our knowledge to recent, there are no aspartate inhibitors identified from these plants. Aspartate inhibitory activity was determined by spectrophotometric stop rate determination method using 0.1mg ml-1 pepsin enzyme, 2.5% bovine hemoglobin substrate with plant extracts (inhibitors). The reaction was terminated by adding 5% TCA solution. The absorbance of the acid-soluble peptides was measured at 280 nm. The inhibitor was purified by ammonium sulfate precipitation and ion exchange chromatography methods. Molecular weight was estimated by dialysis. All the experiments were conducted in duplicate three times.

Maximum inhibition of pepsin was shown by *Garcinia quaesita* Pierre while other species didn't show any significant inhibition of pepsin. Hence for further studies, *Garcinia quaesita* bark extract was selected. The thermal stability of the inhibitor in the crude extract was studied by incubating the extract at different temperatures and determining the remaining activity. The inhibitors were subjected to ammonium Sulphate precipitation and Ion exchange chromatography, in an attempt to purify the inhibitor/s. Molecular weight was estimated by dialysis which implies that the inhibitors comprise small and large molecules with different molecular weights ranging from less than 3.5 kDa to more than 12 kDa. Crude extract of *Garcinia quaesita* retained more than 50% of inhibitory activity over a wide range of temperatures (4-95 ^{o}C) for 30 minutes and also at 4^{o}C for one month. But the remaining inhibitory activities of the crude extract incubated at room temperature and 37 ^{o}C for one month were 20% and 10%, respectively. This suggests that inhibitor molecules are moderately thermostable. This assay procedure provided a quantitative measurement of the inhibitory activity of the inhibitor/s present in the crude bark extract. Fractions obtained from ammonium sulfate precipitation and ion exchange chromatography didn't show inhibition towards pepsin suggesting that inhibitors could be non-proteinaceous.

Further studies on purified inhibitors and necessary to characterize and elucidate the structure of the inhibitors

Index Terms— Aspartic acid protease, *Garcinia quaesita* Pierre, Inhibitors, Pepsin

1 INTRODUCTION

Proteases or proteolytic enzymes are a group of enzymes that break down chain-like protein macromolecules into smaller fragments of peptides and ultimately into their constituent amino acids. Proteases are present in plants, bacteria, viruses, archaea, and certain types of algae and most abundantly in animals [1]. They are encoded by around 2% of the genes in the whole genome set. Proteases involve in protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth, metastasis, zymogen activation, hormones and peptide release and transportation of secretary proteins across membrane.

There are two types of proteases as endopeptidases and exopeptidases. Endopeptidases have further characterized in accordance with the type of peptide bonds they attack. These endopeptidases play an important role in metabolism. This group contains, aspartic endopeptidases, cysteine endopeptidases, metallo endopeptidases, serine endopeptidases and also threonine endopeptidases and glutamic endopeptidases. In this study, aspartic acid proteases were the concerned protease [2].

Aspartic acid proteases are also named as acidic proteases that have aspartic acid residues in the active site. There are 3 families such as pepsin (A1), retropepsin (A2), and pararetroviruses (A3, 13) in which rennin, cathepsin D, yapsins, chymosin, pepsin, gastricsin and Human Immunodeficiency Virus protease are included. Most of the APs exhibit maximum activity at acidic pH and their molecular weights range from 30-45kDa [2].

As mentioned above APs are involved in several biological processes in the human body. The regulation of aspartic proteases are normally put through in the body itself by regulated expression or secretion and /or activation of pro-proteases, hydrolysis of mature proteases, and inhibition of proteases. Even though still proteases can be harmful to cells or organisms when their activity and presence are out of control and eventually they can cause serious diseases [3], [4].

Aspartate proteases are critical in the spreading of diseases such as cancers, Alzheimer's disease, malaria, AIDS (acquired immunodeficiency syndrome), hepatitis, herpes, cardiovascular, inflammatory, neuroendocrine, virus, and parasitic diseases. Therefore inhibition of aspartate proteases is one of the most promising approaches as therapeutic drugs for such conditions, even though there are only a few studies done yet [5], [2].

Most of the existing synthetic drugs are obtained from natural resources. For many of the recent studies natural inhibitors are widely targeted. The majority of these cannot yet be synthesized economically and are still obtained from plants that are important in pharmacological, and biotechnological studies. These natural compounds can lead new directions allowing the designing and planning of new drugs, the development of drug synthesis, and the discovery of new therapeutic methods. Identification of such inhibitor molecules from natural resources is 1 further useful as they are naturally occurring, cheap, and could be less toxic than synthetic compounds [6].

In this study ten candidate plants were used including Annona *muricata* from the family Annonaceae, *Garcinia quaesita* pierre from the family Clusiaceae. *Bauhinia tomentosa, Caesalpinia bonduc, Crotalaria laburnifoloria, Crotalaria micans, Entada zeylanica* (kosterm), and *Erythrina suberosa* from family Fabaceae, *Azadirachta indica* from family Meliaceae, and finally *Limonia acidissima* L. from family Rutaceae. Water extracts of mature plant bark were used as the inhibitor source.

The objectives and aims of the research were to Screen potential plants for aspartic acid protease inhibitory activity from medicinal plants in Sri Lanka, select a candidate plant, develop an assay procedure to detect maximum aspartic protease inhibitory activity and attempt to characterize inhibitor/s from plant materials, and partial purification of the inhibitor.

2 MATERIALS AND METHODS

Aspartate inhibitory activities of bark extracts were determined by spectrophotometric stop rate determination method using 0.1mg ml-1 pepsin enzyme and 2.5% bovine hemoglobin substrate with plant extracts (inhibitor). After incubating for 1.5 hrs. reaction was terminated by adding 5% TCA solution. The final volumes of the reaction mixtures were maintained at 1.5ml. Each test and control procedures were conducted in duplicate three times. Finally, the absorbance of the acid-soluble peptides was measured at 280nm against the blank (phosphate buffer, 1 M, pH 2) followed by the evaluating percentage of inhibition. Considering the results obtained, a suitable candidate plant with maximum inhibitory activity was selected and characterization and partial purification of the inhibitor/s were carried out. In this manner, optimal bark

concentration, volume, and pre-incubation time for inhibitory assay were determined. Bark extract samples were pre-incubated at different temperatures ranging from 4 ° C – to 95 ° C for 30 min and an optimized protease inhibitory assay was carried out. Another set of samples was pre-incubated at 4 ° C, room temperature, and 37 ° C separately over a period of one month and optimized protease inhibitory assay was carried out at different time intervals to determine the thermal stability of the inhibitor. Using the dialysis method approximate molecular weight of the aspartic acid protease inhibitor was estimated. Partial purification of aspartic acid protease inhibitors was carried out by ion exchange chromatography and Ammonium sulfate precipitation methods.

3 RESULTS AND DISCUSSION

In this study we selected ten plant species from five different plant families after reviewing previous studies carried out in this regard. Even though many diseases are occurring on account of aspartic proteases, very few studies have been conducted yet. Protease inhibitors from natural sources are widely accepted and tested to find out suitable drugs for many human diseases. Hence several plant materials are used to identify protease inhibitors. Natural water-soluble inhibitors will have a high demand to use for therapeutic drugs as most of the inhibitors available are not water-soluble and cannot be administrated orally to patients.

For this inhibitory assay pepsin was used as the aspartate protease as it is a well-characterized, cheap, and easily available enzyme. Pepsin solution was stored in a pH 5.5 buffer solution providing its stable pH and the reaction was carried out at an acidic pH bringing out its maximum activity. The final reaction volume was maintained at 1.5ml to reduce the large use of bark extract 2 and other solutions. All the assay procedures were performed using duplicates for test and control samples for three times to reduce errors. The reaction was terminated by adding 5% trichloro acetic acid solution. Aspartate inhibitory activity was determined by spectrophotometric stop rate determination method where the absorbance of the acid-soluble peptides was measured at 280nm against a suitable blank (phosphate buffer, 1 M, pH 2)

According to the results obtained for the en candidate plants, the highest inhibition (42%) was given by *Garcinia quaesita* Pierre bark extract. *Annona muricata* L. and *Limonia acidissima* L. didn't show any inhibition against pepsin while the remaining plant extracts showed very less inhibitory activities. Hence *Garcinia quaesita* Pierre was selected for further studies. *Garcinia* plant is called red mango in English and Rath Goraka in Sinhala. This is an endemic plant to Sri Lanka which is listed under the vulnerable species of IUCN red list. All the plant parts are useful in traditional healing methods for diseases like fever, heart diseases, fractures, rheumatism, hemorrhoids, abdominal pain, nausea, asthma, vomiting, swellings, nervous system diseases, warm infections, and hypertension. Fruit rinds are very popular as a condiment, food preservative, and dietary supplement.

Table 1. Percentage inhibitory activities of 5%, 10%, and 20% bark extracts of ten candidate plants

Plant Family	Species	Bark concentration		
		5%	10%	20%
		Percentage inhibition		
Annonaceae	*Annona muricata* L.	-	-	-
Clusiaceae	*Garcinia quaesita* Pierre	**42%**	**42%**	**42%**
Fabaceae	*Bauhinia tomentosa*	-	2%	4%
	Caesalpinia bonduc	6%	21%	23%
	Crotalaria laburnifoloria	20%	18%	17%
	Crotalaria micans	26%	21%	16%
	Entada zeylanica (kosterm)	6%	8%	10%
	Erythrina suberosa	9%	10%	11%
Meliaceae	*Azadirachta indica*	18%	17%	17%
Rutaceae	*Limonia acidissima* L	-	-	

An assay was conducted using three different concentrations of *Garcinia* extract (5%, 10%, and 20%) which gave the same inhibitory value towards pepsin. Therefore 5% was selected as the optimum inhibitor concentration to save plant extract.

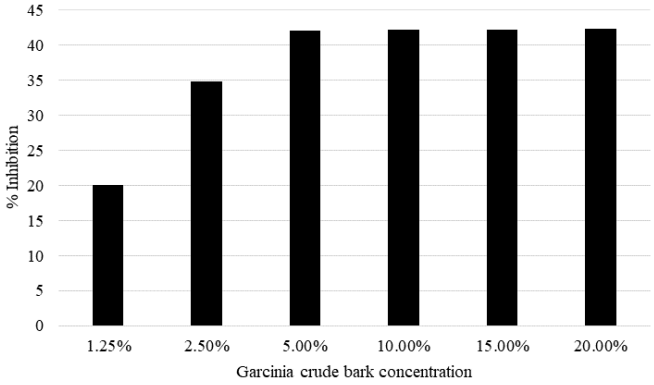

Fig. 1 *Garcinia* crude bark extract concentration

In the same manner, inhibitory assays were conducted to determine optimum inhibitor volume and pre-incubation time. There 20µl showed maximum inhibition. This is because when bark extract volume is less the inhibitor molecule content may also be less in number compared to the enzyme. When bark volume is high, the amount of all the compounds in the water extract increase including inhibitor molecules as well as other molecules that affect or inhibit the aspartate inhibitors. So the inhibition of certain aspartate inhibitors by others could decline the inhibitory activity. With the increasing of the pre-incubation time of the inhibitory assay, the inhibitory activity of the crude bark sample was increased.

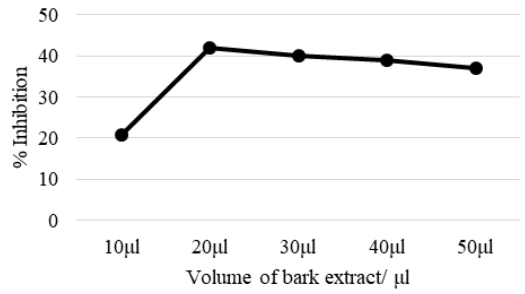

Fig. 2 Optimization of the bark extract of volume for the inhibitory assay

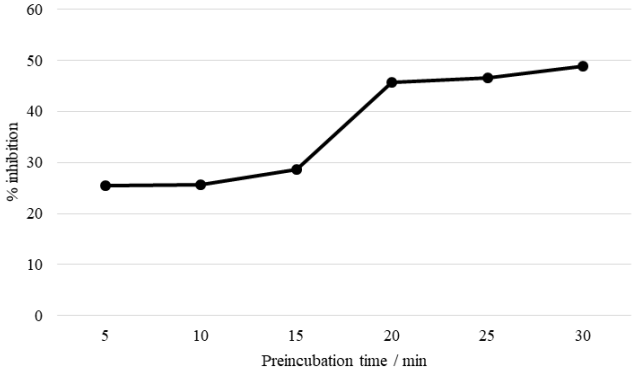

Fig. 3 Optimization of the pre incubation time for the inhibitory assay

Results obtained from dialysis methods indicated that inhibition is carried out by a mixture of inhibitor molecules with different molecular weights ranging from < 3.5kD-12kD.

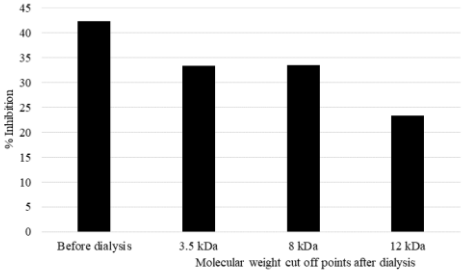

Fig. 4 Inhibitory activity of the dialyzed samples as a comparison to the crude extract shown on the first column

Crude bark samples retained more than 50% inhibitory activity over a wide range of temperatures from 4 ^{o} C – 95 ^{o} C for 30 min and also at 4 ^{o} C for one month could retain inhibitory activity more than 50 %. This suggests that inhibitor molecules are highly thermal stable even in higher temperatures.

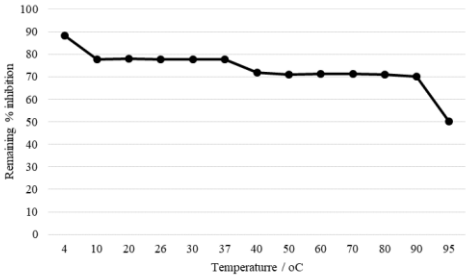

Fig. 5 Thermal stability of the crude bark extract incubated at different temperatures ranging from 4-95^{o}C

Bark extract was partially purified by ion exchange chromatography (Anion exchange chromatography and Cation exchange chromatography) and ammonium sulfate precipitation methods. According to the results inhibitor molecules didn't bind to any of the columns showing no inhibition. Ammonium sulfate precipitated fractions also didn't manifest inhibitory activity against pepsin suggesting that inhibitor molecules could be non- Proteinaceous.

8 Conclusion

In this study we carried out isolation, characterization and partial purification of a new aspartate inhibitor complex from mature bark of *Garcinia quaesita* pierre .It is a useful plant as a traditional medicine, spice and food preservative, but to the best of our knowledge, this is the first time of reporting the potential of this plant as a source of aspartate protease inhibitor. We conclude that *Garcinia quaesita* aspartic acid protease inhibitors mixture could be a valuable source with wide and useful application in biotechnology and medical field. Further studies on purified inhibitors are necessary to characterize and to elucidate the structures of the inhibitors.

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