J. Res. Technol. Eng. 5 (2), 2024, 115-125

Journal Of Research Technology & Engineering
www.jrte.org

Occurrence and partial characterization of serine protease inhibitors in the bark extract of some medicinal plants in Sri Lanka

* T.S.G.S.B. Wijayarathna, R. G. S. C. Rajapakse

Postgraduate Institute of Science, University of Peradeniya, Sri Lanka
*gayanjali90@gmail.com

Received:08 Feb 2024; Revised: 06 Mar 2024; Accepted: 16 Mar 2024; Available online: 10 Apr 2024

Abstract— Protease inhibitors play a vital role concerning proteases. Serine proteases are responsible for a variety of physiological processes and pathological conditions. Therefore, recently interest in protease inhibitors has increased, especially from natural sources such as plants.

This study aimed at the identification of a potential candidate plant, the development of an assay procedure to detect the maximum serine protease inhibitory activity, and the characterization and partial purification of serine protease inhibitors from plant bark extracts.

Preliminary assays were conducted on aqueous extracts made with ten medicinal plant species in Sri Lanka, revealing that *Garcinia quaesita* exhibited the highest inhibitory activity against trypsin.

The crude bark extract of *Garcinia quaesita* displayed significant inhibition of trypsin (62%) at 1.25% concentration. The crude extract retained more than 58% inhibitory activity over a broad temperature range from 37 ºC – 95 ºC for 30 minutes. The crude bark extract retained over 95% and 85% inhibition at 4 ºC and room temperature, respectively, over one month. However, incubation at 37 ºC for a month resulted in a slight decrease to 54% inhibition. According to the results obtained from dialysis, it can be suggested that the serine protease inhibitors present were likely proteinaceous molecules with molecular weights higher than 8 kDa. However, the significant thermal stability suggested the presence of a combination of proteinaceous and non-proteinaceous inhibitors.

The study reveals that the mature bark extract of *Garcinia quaesita* harbors potent serine protease inhibitors, specifically targeting trypsin activity against casein. Further research on purified inhibitors is recommended for a comprehensive understanding of their structure and properties. This assay provides a quantitative measurement of inhibitory activity in the crude bark extract, laying the foundation for future investigations.

Index Terms — *Garcinia*, Medicinal Plants, Protease Inhibitors, Serine, Sri Lanka.

1 INTRODUCTION

Protease inhibitors are molecules that can alter or inhibit the catalytic activity of proteolytic enzymes. Those can eventually slow down or stop the catalysis and exert dramatic biological effects. These changes can be permanent or transient [1], [2]. Sometimes drugs and poisons serve as enzyme inhibitors [3]. Many act as substrates by binding directly to the active site of the proteases. Others bind to surface sites adjacent to the actual active sites [2], [4]. Protease inhibitors can be either proteinaceous or non-proteinaceous. At the end of the reaction, the inhibitor can be modified or non-modified. Often, the formation of an enzyme-inhibitor complex brings out conformational changes in the inhibitor. Protease inhibitors have a broad spectrum of sources and have been isolated from a large number of plants, animals, and microorganisms.

Protease inhibitors are important in regulating biological processes such as protein functions, growth factor activities, receptor clearance signals, and carcinogenesis activities [5].
Plants are a common source of protease inhibitors that are widely used in research, therapeutics, and biotechnological applications [6], [7], [8].
Serene protease inhibitors or serpins are specific for serine proteases. Ser protease inhibitors are the widely distributed in nature and the well-studied class of inhibitors. Serpins are considered irreversible suicide inhibitors. The soybean trypsin inhibitor crystallized by Kunitz in 1947 was the first serpin identified. Serpins have been categorized into certain groups and Bowman-Birk (BBI) and Soybean in which soya bean Kunitz type inhibitors are the most predominant. [9], [10].

In Sri Lanka, the rich biodiversity offers a great opportunity to explore and harness the potential of plant-derived serine protease inhibitors for pharmaceutical and therapeutic purposes. Understanding the presence and characteristics of serine protease inhibitors in medicinal plants is a crucial step toward unlocking their full therapeutic potential.

Clusiaceae plants have received attention for their significant therapeutic applications in cancer, inflammation, and viral infections [11]. Kostermans in 1976, in the Revised Flora of Ceylon, mentioned a total of ten *Garcinia* species that have been identified in Sri Lanka based on morphological characteristics which include five endemic species named, *Garcinia quaesita*, *G. zeylanica*, *G. hermonii*, *G. terpnophylla* and *G. thwaitesii* [12]. Three indo-Sri Lankan species named *G. morela*, *G. echinocarpa* and *G. spicata*, one species cultivated more than 80 years ago, *G. mangostana,* and one introduced species, *G. xanthochymus* which is considered a semi-naturalized species. Now *G. thwaitesii* and *G. zeylanica* species have been listed as globally endangered, while *Garcinia quaesita* has been listed as globally vulnerable [13].

The present study aimed at screening several potential plant species for the presence of serine protease inhibitory activity, selecting a potential candidate plant, developing an assay procedure to detect the maximum serine protease inhibitory activity, and characterizing serine protease inhibitor/s from plant bark extracts.

By investigating the inhibitory activities against serine proteases, such as trypsin, and performing partial characterization, we seek to contribute valuable insights into the bioactive components of these plants, paving the way for their further utilization in medical and biotechnological applications.

3 Research Methodology

An assay procedure was developed to determine the serine protease activity. A solution of 0.2 mg/ml Trypsin and 1% casein was used as the candidate enzyme and substrate. 800µl of 5% TCA was used to terminate the reaction. The optimization of enzyme volume, pre-incubation time, and incubation time was determined experimentally (Fig.1).

Serine inhibitory activities of bark extracts were determined by the spectrophotometric stop rate determination method. A 0.2 mg/ml Trypsin solution and 1% casein substrate used with plant extracts (inhibitor). After incubating for 1hour. the reaction was terminated by adding 5% TCA solution. The final volumes of the reaction mixtures were maintained at 1.5ml. Each test and control procedure were

conducted in duplicate three times. Finally, the absorbance of the soluble peptides was measured at 280nm against the blank (phosphate buffer, 0.1 M, pH 7.6), 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, the 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 º C - 95 º C for 30 min and an optimized protease inhibitory assay was carried out. Another set of samples were pre-incubated at 4 º C, room temperature, and 37 º 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 serine protease inhibitors was carried out by ion exchange chromatography and ammonium sulfate precipitation methods. (Fig. 2)

Preliminary serine protease inhibitory assays were conducted for aqueous extracts of ten different plant species belonging to five different families using trypsin and casein as the serine protease and substrate, respectively.

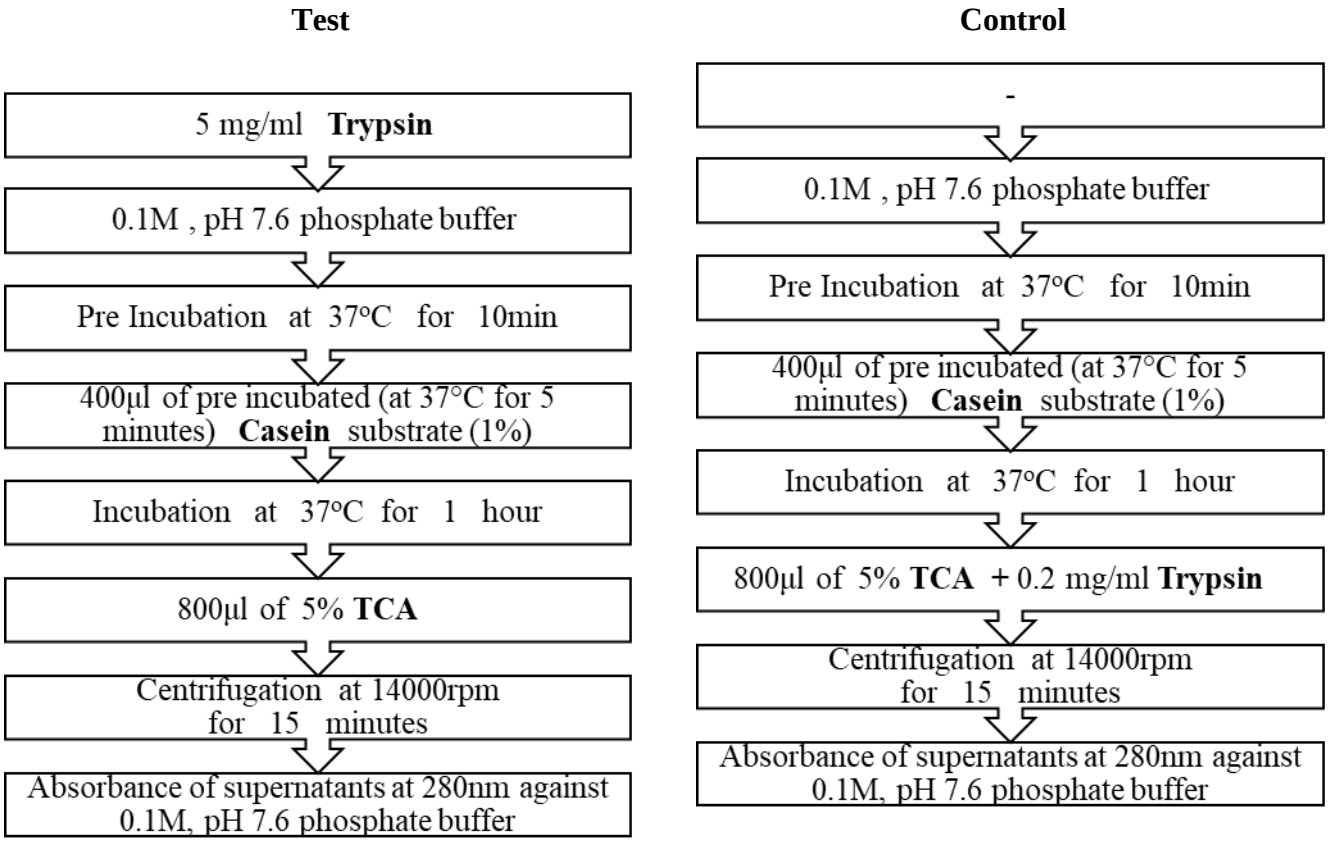

Fig. 1 Experimental procedure for determining the enzyme activity

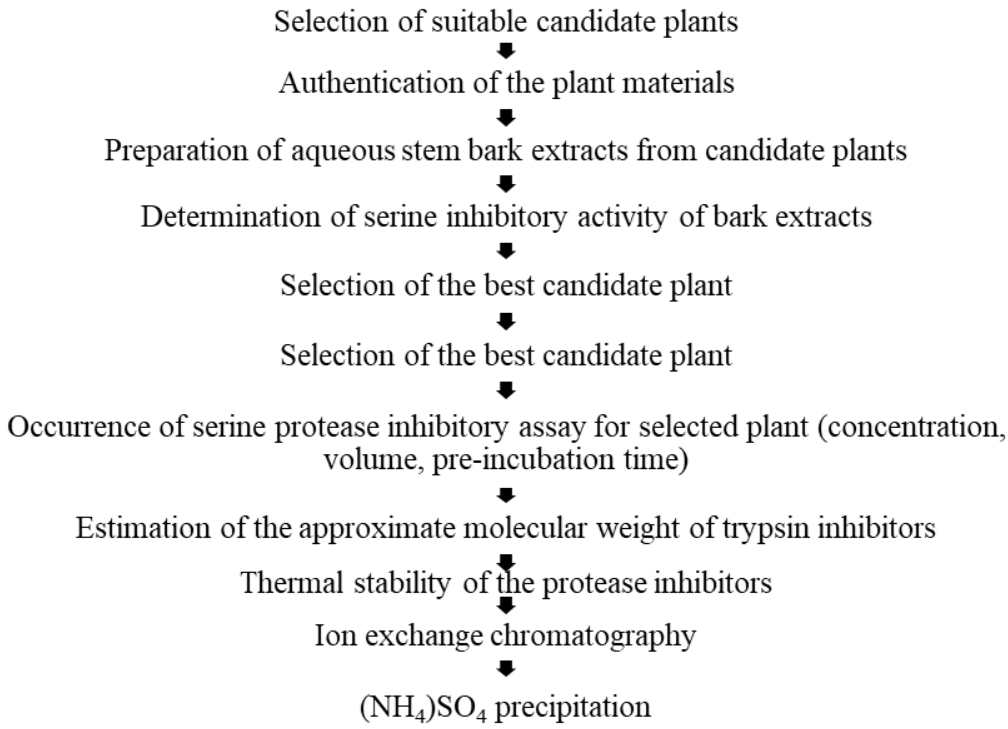

Fig. 2 Flow chart of experimental procedure carried out during the experiment

Table 1. Candidate plant species used for selection

Family	Plant species	Common names	
		Sinhala	English
Annonaceae	*Annona muricata* L.	Katu Anoda	Prickly Custard Apple
Clusiaceae	*Garcinia quaesita* Pierre	Rath Goraka	Red Mango
Fabaceae	*Bauhinia tomentosa* L.	Kaha Pethan	Yellow Bell Orchid Tree
	Caesalpinia bonduc	Kumburu	Gray Nicker bean
	Crotalaria laburnifolia	Yakberiya	Old land Rattlepod
	Crotalaria micans	Kaha Andanahiriya	Caracas Rattlebox
	Entada zeylanica kosterm	Pus Wel	Elephant creepers
	Erythrina suberosa	Katu Erabadu	Corky Coral Tree
Meliaceae	*Azadirachta indica* A.juss	Kohomba	Neem
Rutaceae	*Limonia acidissima* L.	Divul	Wood apple

4 Results and Discussion

For assaying enzymes, initially, standardization of parameters such as temperature, and enzyme concentration is important to determine the optimal values and to ensure that the enzyme and substrate function properly. In the determination of enzyme activity, 60 µl (Fig. 3) was selected as the optimum trypsin volume for further assays. Fig. 4 shows the graph obtained for optimizing the incubation time, the longer the enzyme was incubated with its substrate the greater the products formed. Hence, for convenience, 60min of the incubation time was selected. Enzyme assay was conducted and 15 min of pre-incubation time was selected as the optimum value.

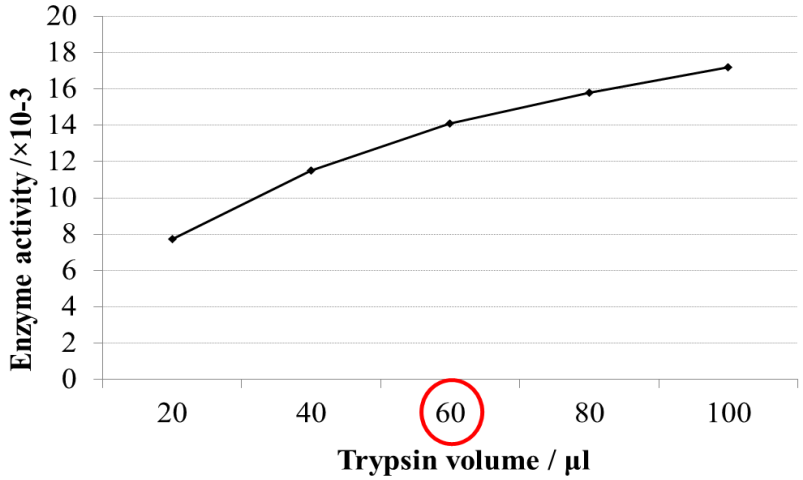

Fig 3. Optimization of the volume of trypsin for the assay procedure

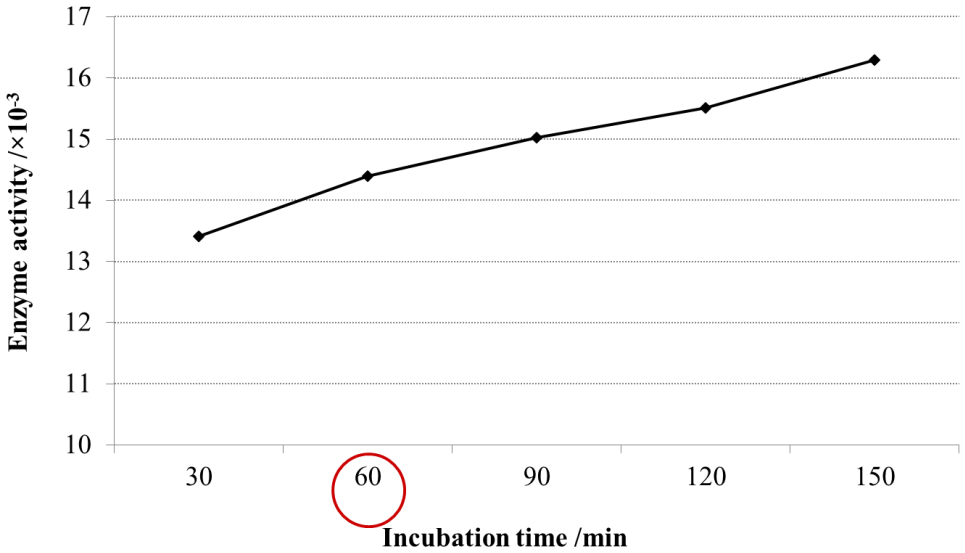

Fig. 4 Optimization of the incubation time for the assay procedure

According to the table 2, maximum inhibition was shown by *Garcinia quesita* Pierre while other species haven't shown any significant value. Hence, *Garcinia quaesita* with maximum inhibitory activity was selected for characterization of inhibitors.

Table 2. Selection of suitable candidate plant

Plant family	Species	Bark concentration		
		5%	10%	20%
		% inhibitory activity		
Annonaceae	*Annona muricata* L.	4%	7%	10%
Clusiaceae	***Garcinia quaesita* Pierre**	**55%**	**47%**	**37%**
Fabaceae	*Bauhinia tomentosa* L.	48%	37%	24%
	Caesalpinia bonduc	5%	27%	24%
	Crotalaria laburnifolia L	13%	6%	2%
	Crotalaria micans Link	11%	4%	2%
	Entada zeylanica kosterm	13%	27%	34%
	Erythrina suberosa	11%	6%	19%
Meliaceae	*Azadirachta indica* A.juss	21%	35%	40%
Rutaceae	*Limonia acidissima* L.	32%	16%	10%

Then optimum bark concentration, volume and pre-incubation time for inhibitory assay were determined. According to the Fig. 5, 1.25% of bark extract showed maximum inhibition. With the increase of the concentration the inhibition has decreased. This may be due to the presence of molecules that hinder or interfere with the activity of serine protease inhibitors. Considering the results, 20 µl (Fig. 6) of 1.25% *Garcinia* bark extract was used for further assays.

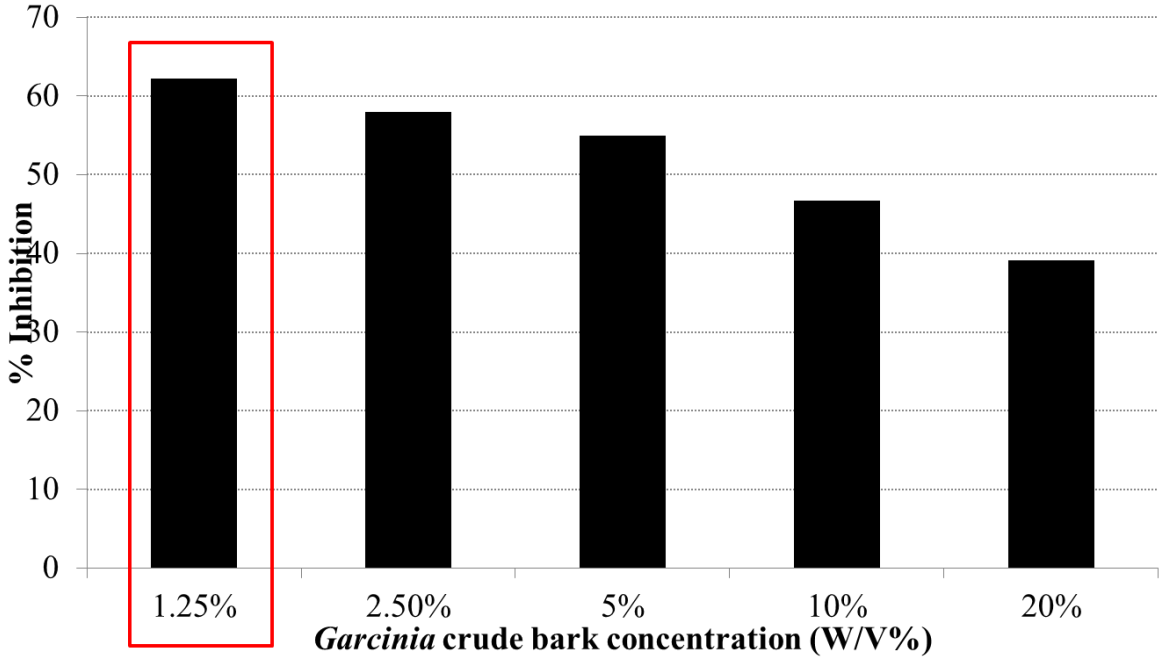

Fig. 5 Optimization of the concentration of the *Garcinia* bark extract

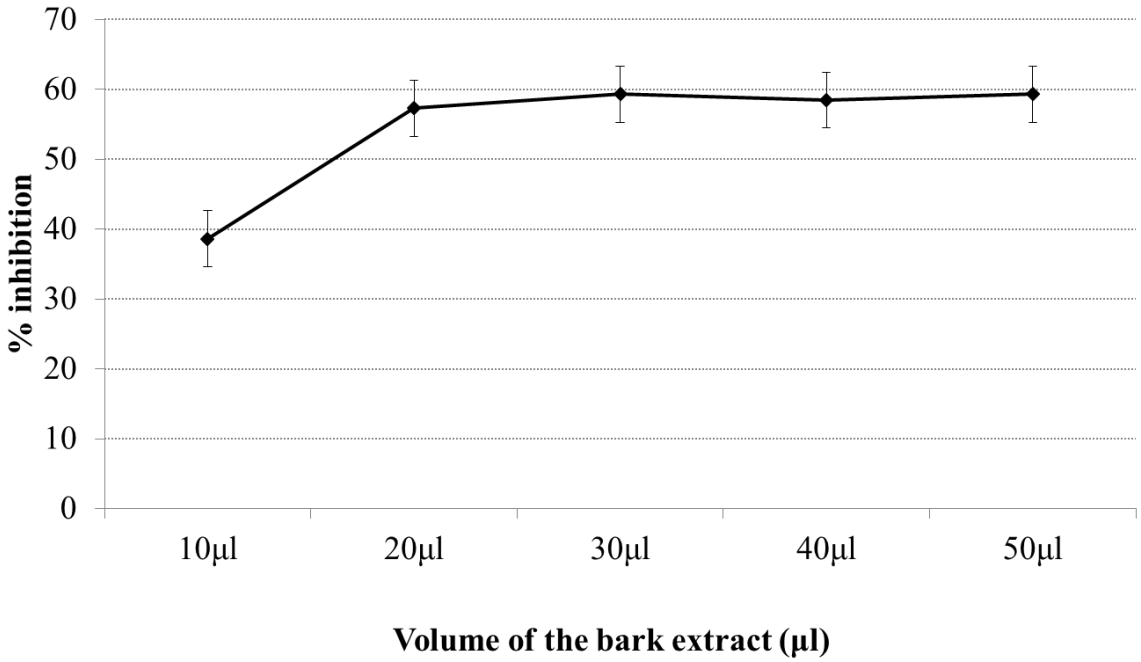

Fig. 6 Optimization of the volume of bark extract

Inhibitors require time to bind to the enzyme and equilibrate which is provided through pre-incubation time with the enzyme. In most of the previous studies recommended pre incubation time lie between 5-20 min. for this study, 10 min of pre-incubation time was selected as there is no much increase after 10 minutes as depicted in graph shown in Fg. 7.

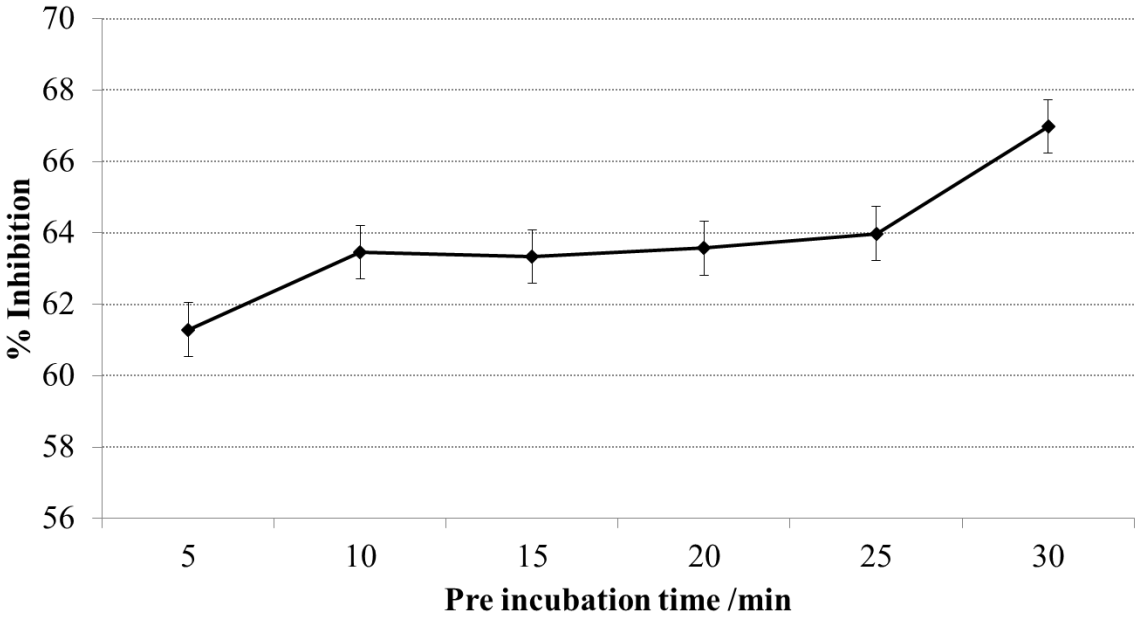

Fig. 7 Optimization of the pre-incubation time of the inhibitory assay

The thermal stability of the bark extracts was determined by incubating the samples at 4 ^{o}C, room temperature, and 37 ^{o}C separately over one month and the remaining protease inhibitory activity was calculated at different time intervals. According to Fig. 8, inhibitors have retained about 62% of inhibitory activity over a wide range of temperatures from 26^{o}C to 95^{o}C suggesting the highest thermal stability of the inhibitor/s. This type of thermal stability implies the presence of non-proteinaceous serine protease inhibitors.

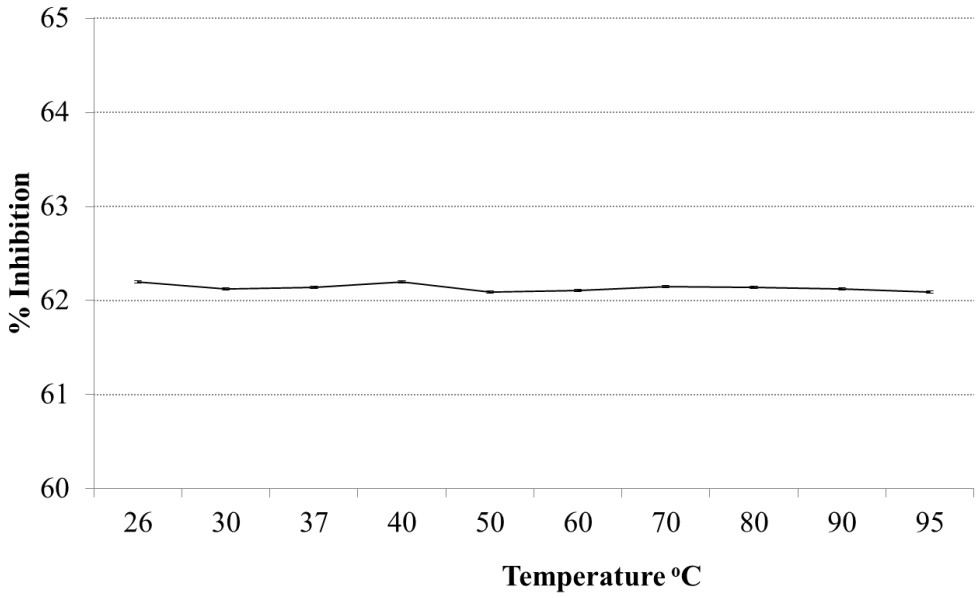

Fig. 8 Thermal stability of the inhibitors in crude bark extract
(after pre-incubating 30 min)

The crude bark extract retained over 95% and 85% inhibition at 4 ^{o}C and room temperature, respectively, over one month. However, incubation at 37 ^{o}C for a month resulted in a slight decrease to 54% inhibition during one month period (Fig. 9).

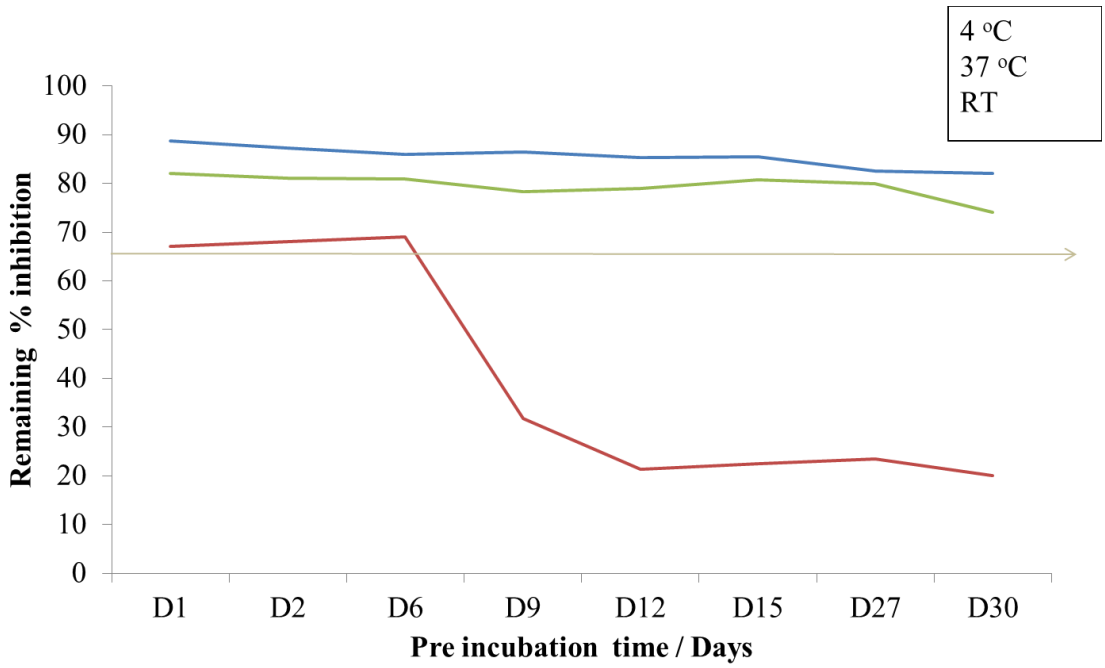

Fig. 9 Thermal stability of the dialyzed sample over a month at three different temperatures

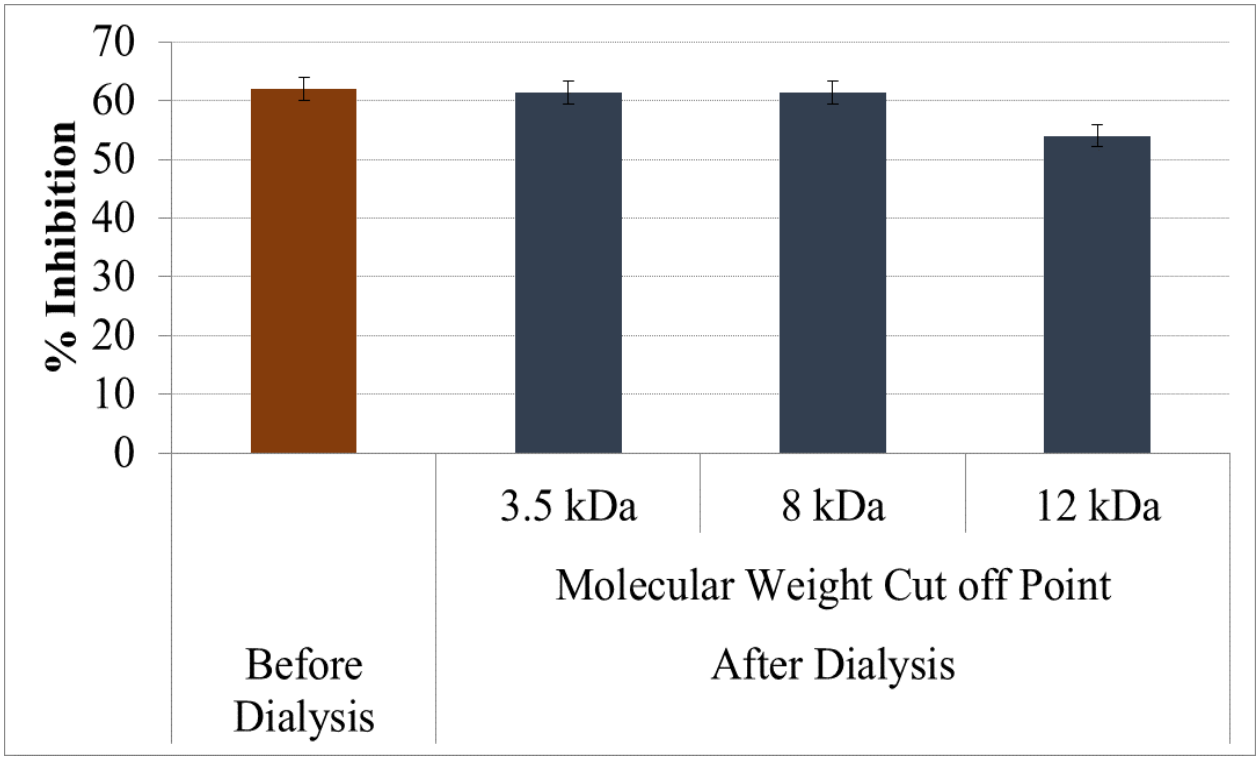

Fig. 10 Approximate molecular weight of the serine protease inhibitor/s

The approximate molecular weights of the inhibitors were estimated at pH 7.6 using dialysis membranes with different molecular weight cut-off points.

According to Fig. 10, protease inhibitors are larger molecules with a molecular weight greater than 8 kDa. It implied that these serine inhibitors are most likely to be proteinaceous molecules. The relatively high thermal stability exhibited by the inhibitory activity could be due to thermal stable proteinaceous or non-proteinaceous molecules. Thus inhibitors may contain a cocktail of proteinaceous and nonproteinaceous inhibitors.

Partial purification of serine protease inhibitors was attempted using ion exchange chromatography and ammonium sulfate precipitation methods. Results are not shown here, as the ammonium-desalted fractions and fractions obtained from the chromatography column did not give significant inhibition of trypsin. As purification methods were not successful, we can assume that there are less amount of proteinaceous protease inhibitors in the inhibitor cocktail extracted from the inner fleshy later of the mature stem bark of *Garcinia quaesita*. However, there are certain cases reported where protein inhibitors didn't bind to the chromatography column due to the zero net charge of the protein. Hence, to confirm the presence of proteinaceous inhibitors further studies have to be conducted.

5 Conclusion

The mature bark extract of *Garcinia quaesita* has significant serine protease inhibitory activity that specifically inhibits trypsin activity against the casein. Inhibitors contain macromolecules that are relatively thermally stable. This assay procedure provided the quantitative measurement of the inhibitory activity for the inhibitor/s present in the crude bark extract. Further studies on purified inhibitors are necessary to characterize and elucidate the structure/s of the inhibitors.

6 References

[1] M. Hartl, A.P. Giri, H.Kaur and I.T. Baldwin, the multiple functions of plant serine protease inhibitors, Plant signalling and behaviors, 6(7), 1009-1011, 2011. doi: 10.4161/psb.6.7.15504.

[2] D.J. Holme, and H. Peck, Enzymes. In Analytical Biochemistry, 3rd edn. Longman, Harlow, Essex, U.K, Chapter 8,258-290, 1998.

[3] V.Menon and M.Rao, Protease inhibitors: Emphasizing the functional aspects of aspartic protease inhbitors, fuctional plant science and biotechnology, 6(2), 1-67, 2012. https://api.semanticscholar.org/CorpusID:40170457

[4] K. Hibbetts, B. Hines and D Williams, An overview of proteinaceous inhibitors, Journal of Veterinary International Medicine, 13(4), 302-308, 1999. doi: 10.1892/0891-6640(1999)013<0302:aoopi>2.3.co;2

[5] H. Habib and K.M.Fazil, Plant protease inhibitors: A defense strategy in plants, Biotechnology and Molecular Biology Review, 2(3), 68-85, 2007.

[6] R.A.Copeland, Assay consideration for compound library screening. In Evaluation of enzyme inhibitors in drug discovery, 2nd edn. John Wiley & Sons, New Jersey, USA, Chapter 4, 300, 2013.

[7] H. Bisswanger, Enzyme assays. Perspectives in Science 1 (1-6), 41-55, 2014.

[8] S.G.Fan, and G.J. Wu, Characteristics of plant proteinase inhibitors and their applications in combating phytophagous insects. Botanical Bulletin of Academia Sinica 46 (4), 273-292, 2005. Id: 2005310749.

[9] P.Lison, I. Rodrigo and V. Conejero, A novel function for the Cathepsin D inhibitor in tomato, Plant Physiology, 142(3), 1329-1339. 2006. doi: 10.1104/pp.106.086587

[10] K.V. Rachel, G.V. Sirisha, A Review of protease inhibitor from different sources. International Journal of Applied, Physical and Bio-Chemistry Research, 4(2), 1-18, 2014.

[11] G.P. Marasinghe, Chemistry and antiviral/ anti-HIV activity studies of family Clusiaceae, university of Peradeniya, 2000.

[12] P.S. Pathirana and T.R. Herath, Comparative vegetative anatomical study of the genus *Garcinia* in Sri Lanka, Ceylon Journal of Science (Biological Sciences), 32, 39-66, 2004. http://repository.ou.ac.lk/handle/94ousl/1072

[13] W.J.Nimanthika and H.S.Kaththriarachchi, Systematics of genus *Garcinia* (Clusiaceae) in Sri Lanka: New insight from vegetative morphology, Journal of the National Science Foundation of Sri Lanka, 38(1), 29-44, 2010. doi: 10.4038/jnsfsr.v38i1.1723