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Heat Stable Protease Inhibitors from *Sesbania grandiflora* and *Terminalia catappa*

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Authors' contributions

This work was carried out in collaboration between all authors. Author HKIP was concept and design of the study, literature search, analysis and interpretation, manuscript preparation and revision of the manuscript. Author BDSJ performed the experiments and collected data. Author SR collaborated with the trypsin inhibitory assay. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Protease inhibitors play a vital role in the regulation of protease activity and display promising therapeutic effects against diseases in humans. The aim of this study was to investigate protease inhibitory activities and their heat stabilities from parts of five medicinal plants.
Study Design: Experimental.
Place and Duration of Study: Department of Biochemistry, Faculty of Medicine, University of Peradeniya, Sri Lanka between March 2012 and February 2013.
Methodology: Water extracts were prepared using fresh plant parts of *Mangifera zeylanica* (MZ) bark, *Sesbania grandiflora* (SG) bark, flower, leaves and seeds, *Terminalia bellerica* bark and seed, *Terminalia catappa* (TC) bark, fruit and leaves and *Terminalia chebula* bark and seed. Percentage inhibitory activities of the extracts against pepsin and trypsin were measured. Final concentrations of the extracts used for pepsin and trypsin inhibitory assays were 0.2 and 0.13% respectively. Remaining inhibitory activities were measured after heating the extract at 60, 80 and 100°C up to

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1 h. All the experiments were conducted in triplicate for three times.
Results: Maximum pepsin inhibitory activity was detected in TC bark (98%) followed by SG bark (67%) and MZ bark (31%). Rest of the extracts showed 10 to 16% pepsin inhibition or no inhibition. Maximum trypsin inhibitory activity was detected in SG bark (95%) followed by TC bark (86%) and MZ bark (39%). Rest of the extracts showed 6 to 18% trypsin inhibition or no inhibition. Inhibitory activities of SG and TC barks remained when heated for 1 h at 60, 80 and 100℃. The maximum loss recorded was with the trypsin inhibitory activity (27% loss) when SG bark was heated at 100℃ for 1 h.
Conclusion: *S. grandiflora* and *T. catappa* barks demonstrated strong protease inhibitory activities which were heat stable. Further studies are necessary to isolate, characterize and elucidate the structures of these protease inhibitors.

Keywords: Protease inhibitors; heat stable; Sesbania grandiflora; Terminalia catappa.

1. INTRODUCTION

Proteases constitute one of the largest and diverse families of enzymes which are involved in regulating all aspects of physiological functions including cell growth, differentiation, migration and protein turnover. Almost 2% of the genes in higher organisms are expressed into proteases [1] and some of them such as angiotensin-converting enzyme catalyze highly specific proteolytic processing events. Proteases are classified into six classes based on the mechanism of action, which include aspartic proteases and serine proteases. Even though protease function is indispensable, unregulated protease-catalyzed reactions underlie multiple pathological processes in humans. Furthermore, parasitic proteases are involved in invasion and migration in to host tissues [2]. Proteases are known to mediate various pathological conditions such as hypertension, cancer metastasis, malaria, acquired immune deficiency syndrome (AIDS) and Alzheimer's disease [3]. Therefore proteases have become a major focus in pharmaceutical industry as potential drug targets. Aspartic proteases have drawn much attention due to the involvement in many human diseases such as cancer and AIDS, even though they belong to a relatively smaller group [3].

Strict regulation of the activity of proteases is vital in all forms of life in order to prevent unrestrained cleavage of proteins [4]. Protease inhibitors (PIs) produced by organisms represent an important strategy in the regulation of protease activity. Number of synthetic PIs has displayed promising therapeutic activities in humans for cancer, degenerative disorders, inflammatory diseases, immunological conditions, cardiovascular conditions, respiratory conditions and infections [3,5]. For instance there are several synthetic inhibitors produced against aspartic proteases such as renin, cathepsin D, plasmepsin, human immunodeficiency virus (HIV) protease and β-secretase which mediate pathological conditions [3]. However, the drug resistance and side effects are significant problems associated with these drugs. Protease inhibitors from plants may serve as an important avenue of therapy because of their lesser side effects and low cost as remedies.

Several families of PIs including the most studied Bowman-Birk and Kunitz inhibitors are present in plants [6]. These two inhibitors are proteins and are isolated mainly from leguminous plants [7]. PIs provide defense in the plants by acting against insect and microbial attack [8]. Non protein inhibitors are also identified from plants [9]. Pepstatin is a low molecular weight aspartic protease inhibitor isolated from Streptomyces species [10]. In general protease inhibitors predominantly act against serine proteases while aspartic protease inhibitors are relatively rare [11]. Proteinaceous plant PIs are generally known to have a high content of cysteine residues [12]. This feature leads to the formation of disulfide bridges [13] and confers the stability of PIs [12].

Terminalia catappa L. (TC) known as tropical almond is a large tree native to Southeast Asia. It is widely used for shade due to its shape with long horizontal branches and large leaves [14]. TC is also used for a wide range of medicinal effects, ornamental purposes and for edible nuts [15]. Anticancer, antimetastatic, antioxidant, antiinflammatory, antifungal, antiparasitic, antidiabetic and hepatoprotective effects of TC leaves are reported [15]. *Sesbania grandiflora* (L.) (SG) is a fast growing, short lived small tree. Leaves and flowers are consumed as vegetables in the diet. Different parts of SG are used for medicinal purposes. Anticancer, antiurolithiatic

and hepatoprotective effects of SG leaf and wound healing, antiinflammatory and antiarthritic activities of SG bark were reported [16].

The aim of this study was to investigate aspartic protease and serine protease inhibitory activities and their heat stabilities using pepsin and trypsin respectively from parts of five Sri Lankan medicinal plants.

2. MATERIALS AND METHODS

Plant parts were collected from Kurunegala and Colombo Districts, Sri Lanka. Plant species used were *Mangifera zeylanica* (Blume) (MZ) Hook.f., *Sesbania grandiflora* (L.) Poir., *Terminalia bellirica* (Gaertn.) Roxb., *Terminalia catappa* L. and *Terminalia chebula* Retz (Table 1). Plants were authenticated and voucher specimens were deposited at Bandaranayake Memorial Ayurvedic Research Institute, Maharagama, Sri Lanka.

2.1 Extract Preparation

Fresh plant materials were washed thoroughly in water. Ten grams of plant material was mixed in 50 ml of distilled water and prepared 20% plant extracts using an electrical blender. Extracts were centrifuged at 8000 g for 15 min at 4°C and filtered to remove debris. Appropriate dilutions of the filtered supernatants were used for the protease inhibitory assay and extracts were stored at -40°C until used.

2.2 Measurement of Pepsin Inhibitory Activity

The assay procedure described by Athauda et al. [17] was used with modifications. Briefly, 10 µl of 0.2 mg/ ml porcine pepsin and 50 µl of 1 M phosphate buffer (pH 2) were pre-incubated with 50 µl of 2% plant extract (final concentration 0.2%) for 15 min at 37°C. There after 2% denatured hemoglobin (400 µl) was added and continued the incubation at 37°C for 30 min. Pepstatin A (1 µM) was used as the standard inhibitor. The reaction was terminated by adding 800 µl of 5% trichloroacetic acid. After 10 min, reaction mixture was centrifuged at 10,000 g for 10 min. Absorbance of the acid soluble peptide products present in the supernatant was recorded at 280 nm in a UV-Visible spectrophotometer (Shimadzu, Japan). A control representing 100% pepsin activity was conducted in an identical form without the plant extract. Appropriate blanks were used. Percentage inhibitory activity of each extract was calculated using following equation.

$$\% \text{ inhibition} = (A_{280}\text{Control} - A_{280}\text{Test}) \times 100 / A_{280}\text{Control}$$

A_{280}Control = Absorbance of the Control - Absorbance of the Control blank

A_{280}Test = Absorbance of the Test- Absorbance of the Test blank

2.3 Measurement of Trypsin Inhibitory Activity

The assay procedure described by Tripathi et al., [18] was used with modifications. Briefly, 20 µl of 0.5% trypsin and 280 µl of 0.05 M phosphate buffer (pH 7.6) were pre-incubated with 50 µl of 2% plant extract (final concentration 0.13%) for 15 min at 37°C. There after 400 µl of 1% casein was added and incubated at 37°C, for another 30 min. Phenylmethylsulfonyl fluoride (PMSF) (0.5 mM) was used as the standard inhibitor. Rest of the procedure was similar to that of pepsin inhibitory assay. Percentage inhibitory activity of each extract was calculated using the equation used for to measure pepsin inhibitory activity.

2.4 Determination of the Thermal Stability of the Inhibitors from SG and TC

Plant extracts with more than 60% inhibitory activity towards pepsin and trypsin were selected. Thermal stability of the inhibitors from of SG and TC bark extracts were evaluated at different temperatures by incubating the crude extract at 60°C, 80°C and 100°C for different time intervals (30 and 60 min). Volumes of the extracts were adjusted to the original volume. A volume of 50 µl of 1% plant extract was used for the assays. Remaining percentage inhibitory activities against pepsin and trypsin were measured in comparison to those of unheated extracts.

2.5 Statistical Analysis

All experiments were performed three times and duplicate measurements were taken. Data are presented as mean ± standard deviation. Analysis of the data was performed using ANOVA [18]. Values of $p < 0.05$ were considered as significant.

Table 1. Plants and plant parts used for the study

Plant name	Family	Common name	Part/s used
Mangifera zeylanica	Anacardiaceae	*Etamba*	Bark
Sesbania grandiflora	Fabaceae	*Kathurumurunga*	Bark, leaf, flower, seed
Terminalia bellirica	Combretaceae	*Bulu*	Bark, Seed
Terminalia catappa	Combretaceae	*Kottamba*	Bark, leaf, fruit
Terminalia chebula	Combretaceae	*Aralu*	Bark, Seed

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Pepsin and trypsin inhibitory activities of plant extracts

Maximum pepsin inhibitory activity was observed with the bark extracts of TC (98%) followed by SG (67%). Maximum trypsin inhibitory activity was observed with the bark extracts of SG (95%) followed by TC (86%). Other parts collected from these two plants except for TC leaf (12% pepsin inhibitory activity) did not show any inhibitory activity. MZ barkextract showed 30-39% protease inhibition where as the other plant extracts used showed minor inhibitory activities (Fig. 1).

3.1.2 Thermal stability of the inhibitory compounds of SG and TC barks

Incubation of the SG bark extract for 1 hour at 60°C and 80°C did not result any loss of pepsin inhibitory activity. There was a marginal loss of approximately 2% of pepsin inhibitory activity when heated for 1 hour at 100°C (Fig. 2A).

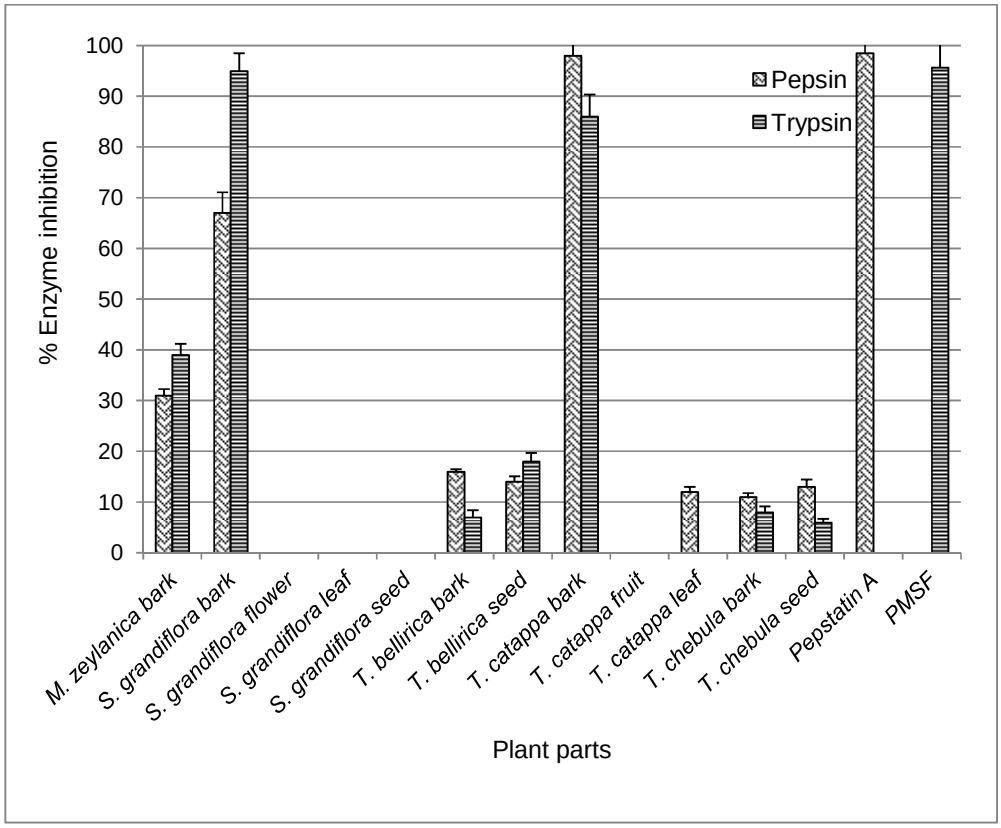

Fig. 1. Pepsin and trypsin inhibitory activities of the plant extracts

Protease inhibitory activities are expressed as mean ± standard deviation. Final concentration of the extracts used for pepsin and trypsin inhibitory assays were 0.2% and 0.13% respectively

With regard to trypsin, 100% inhibition was remaining after incubation of the SG bark extract for 1 hour at 60°C. However some decline in the remaining trypsin inhibitory activity was observed when heated at 80°C and 100°C for 1 hour with a loss of 15% and 27% activity respectively (Fig. 2B).

The percentages of pepsin and trypsin inhibitory activities remained in TC bark extract after 30 min were remained 100%. Even after 1 hour incubation of TC extract at 100°C, 89% pepsin inhibitory activity and 96% trypsin inhibitory activity were remained (Fig. 3).

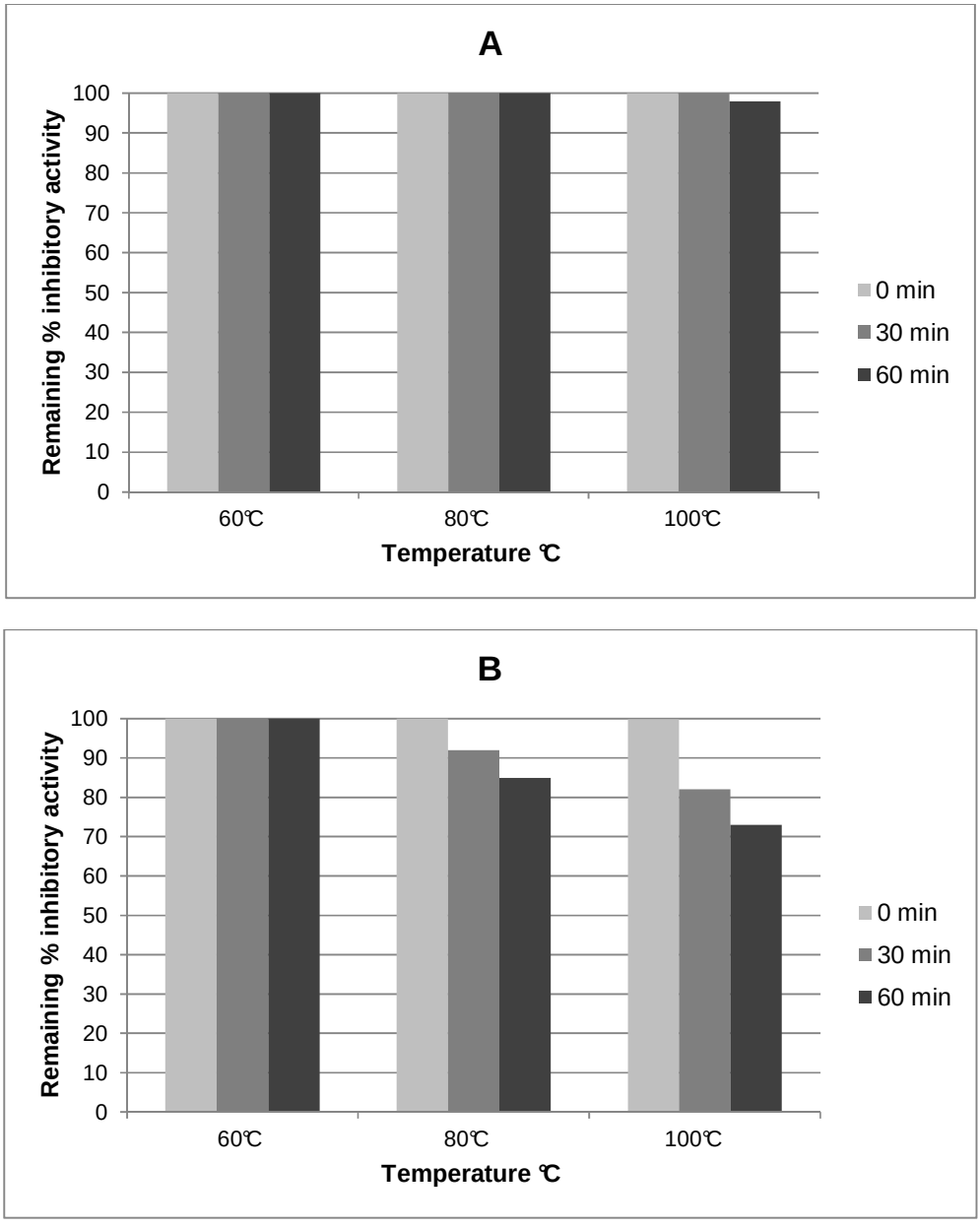

Fig. 2. Remaining proteases inhibitory activities of SG bark

*SG bark extract was heated at 60, 80 and 100℃ for 30 and 60 min and measured the remaining inhibitory activities against pepsin and trypsin in comparison to those of unheated extract (0 min) **A:** Remaining percentage inhibitory activity against pepsin, **B:** Remaining percentage inhibitory activity against trypsin*

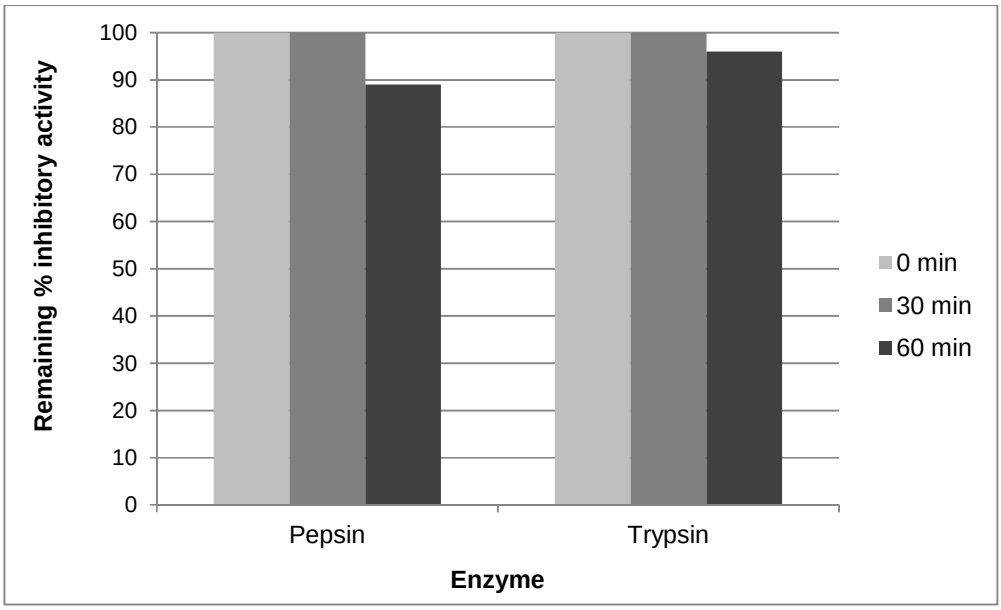

Fig. 3. Remaining proteases inhibitory activities of TC bark

TC bark extract was heated at 100℃ for 30 and 60 m in and measured the remaining inhibitory activities against pepsin and trypsin in comparison to those of unheated extract (0 min)

3.2 Discussion

In the present study, pepsin and trypsin were used as model enzymes to investigate aspartic and serine protease inhibitory activities respectively. Among the tested extracts, barks of SG and TC showed strong PI activities against both pepsin and trypsin. Other parts tested from these two plants did not show considerable protease inhibition. The pepsin inhibitory effects of SG and TC barks and trypsin inhibitory effect of TC bark showed 100% remaining activity after heating at 100℃ for 30 min indicating their stability. The maximum loss of activity was observed with the trypsin inhibitory activity of SG which amounted to 27% when heated for 1 h at 100℃. The current study could have been improved with a proper quantification of the extract concentration if dry extracts were used.

Plant PIs are recognized as compounds with a great potential in therapeutic applications [19,20] and in agriculture [21]. A role of plant PIs in preventing cancers was suggested when epidemiological studies demonstrated a decline in the occurrence of cancers in vegetarians [22]. This observation led to study the value of plant PIs as cancer chemopreventive agents [23]. Some studies have investigated for HIV protease inhibitors from plant sources using pepsin as an alternative for HIV protease [24]. PI genes are proposed as a tool to design an eco-friendly method to produce insect resistant plants [25] with an aim of blocking the digestion process of insects to follow an eventual death. Several transgenic plants expressing PIs have been developed [26].

Various parts of SG and TC such as bark, flowers, fruits, leaves and roots are used in traditional medicine. Antibacterial [27,28] and antiinflammatory effects [29,30] of SG and TC barks were reported. Furthermore, antimetastatic effects of TC mediated by the inhibition of a matrix metalloproteinase was demonstrated [31].

Number of studies has investigated the HIV-protease inhibitory effects of plant extracts. The structural and catalytic resemblance of the two aspartic proteinases HIV-1 protease and pepsin has been discussed [32]. HIV-1 protease has become a main target in the treatment of HIV, as its inhibition leads to the release of non infectious immature virion particles [24]. Water extract of SG leaves and flowers have demonstrated mild HIV-protease inhibitory activities (8.9% and 9.8% respectively) at 100 µg/ ml [33]. Same study found absence of inhibitory activity in the ethanol extracts of SG leaves [33]. These findings are in agreement with the findings of the current study. A potent anti HIV-1 protease activity of *Adhatodavasica* was detected using pepsin [24].

When four plants of Combretaceae family were investigated, *Terminalia arjuna* (fruit and bark), *Terminalia chebula* (arial parts and fruit) and *Terminalia horrida* (resin), were found to be active against HIV-1 protease while *Terminalia bellirica* fruit was inactive [34]. However, in the current study, seed extracts of both T. *chebula* and *T. bellirica* showed mild pepsin inhibitory effects (13% and 14% respectively).

Venkatalakshmi et al. [30] demonstrated high trypsin inhibitory effects of TC bark and fruit in a recent study when the effects were used as a tool to monitor the antiinflammatory activity. Even though the results of TC bark in the current study matched with that of Venkatalakshmi et al. [30] current study did not identify any inhibitory activity in TC fruit. In another study, highest inhibitory activity against trypsin was reported in the seeds of *Garcinia xanthochymus* (96.7%) among 19 plants tested. *Datura stramonium* and *Ricinus communis* seeds and *Phyllanthus amarus* fruits exhibited 86.1, 74.1 and 72.1% trypsin inhibition [35].

Most plant PIs are known to be active up to 50℃ [36]. Even proteinacious PIs have shown stability at high temperatures. This stability may be attributed to the presence of number of disulfide bonds which stabilize a tight conformation [37]. A trypsin inhibitor isolated from the seeds of *Abelmoschus moschatus* was found to be heat stable with 100% remaining activity at 80℃ for 10 min [38]. However, this inhibitor lost the inhibitory activity completely when heated at 100℃ for 30 min. A soybean proteinacious PI isolated was stable at temperatures below 60℃ and lost 45% of activity at 80℃ and a complete loss of activity at 100℃ [39]. Inhibitory effects seen in the current study with SG and TC were stable than the ones investigated in many studies as only the trypsin inhibitory effect of SG showed a decline of 18% at 100℃ for 30 min while others did not show any loss of effect.

4. CONCLUSION

Strong *in vitro* pepsin and trypsin inhibitory activities (67-98% when 0.2% and 0.13% extracts respectively were used) were detected in the water extracts of *Sesbania grandiflora* and *Terminalia catappa* barks. To the best of our knowledge this is the first time reporting pepsin and trypsin inhibitory activities from *S. grandiflora* bark. Furthermore, the study revealed that the inhibitory activities observed in both extracts are heat stable. Current findings suggest the potential of these extracts to be used in therapeutic applications. Further studies are necessary to isolate, characterize and elucidate the structures of these inhibitors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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