

Exploring plant seed extracts as potential sources of trypsin inhibitors for insect pest management: screening and characterization of inhibitory activity

S. K. Sreejina and V. M. Kannan*

Department of Zoology, University of Calicut, Thenhippalam 673635, Malappuram, Kerala, India.

Email: kannanvm@yahoo.com

ABSTRACT: This study aimed to identify seeds containing trypsin inhibitors by screening 17 plant species. Plant seed extracts were prepared by homogenizing soaked seeds in a bicarbonate buffer (pH 9.0), followed by centrifugation. The resulting supernatants, rich in soluble proteins, were subjected to a protease inhibition assay. Among the seventeen seed extracts examined, ten exhibited significant trypsin inhibition, with notable examples including *Pterocarpus marsupium* ($98.20 \pm 0.5\%$), *Barleria repens* ($97.75 \pm 0.25\%$), *Ipomoea sepiaria* ($93.80 \pm 0.15\%$) and *Distimake vitifolius* ($91.79 \pm 0.71\%$) have not been previously reported as sources of trypsin inhibitors. © 2025 Association for Advancement of Entomology

KEY WORDS: Inhibitor, protease inhibition assay, serine protease, pest management

Insect pests intensively feed on host plants to obtain sufficient nutrients for their growth and development. This process involves a complex interplay of proteases, including trypsin, chymotrypsin, aminopeptidase, elastase, and carboxypeptidase, which aid in the digestion of proteins within the caterpillar's digestive system. These proteases are categorized into exopeptidases, which cleave proteins from the N or C-terminus, and endopeptidases, which cleave internal peptide bonds (Fan and Wu, 2005; Rawlings *et al.*, 2010). Lepidopteran insects are some of the most significant pests threatening various economically valuable crops. During their larval stage, these insects predominantly utilize serine proteases for digestion, with trypsin-like enzymes constituting

around 90% of their digestive protease profile, while chymotrypsin-like enzymes represent about 5% (Srinivasan *et al.*, 2006).

Plant protease inhibitors (PIs) are natural, biodegradable defense molecules that can be utilized as biochemical markers to identify crop plants with varying resistance levels against insect pests (Swathi *et al.*, 2021). Seeds harbour relatively high levels of proteinase inhibitors, most of which exhibit activity against serine endopeptidases (Chougule *et al.*, 2008). PIs, particularly serine group inhibitors like Bowman-Birk inhibitors (BBIs) and Kunitz inhibitors (KIs), have shown promise in managing lepidopteran pest populations. These plant PIs often bind to the active site of enzymes, forming enzyme-

* Author for correspondence

inhibitor complexes that block their activity (Norton, 1991; Nanasahe *et al.*, 2008). The inhibition of protease enzymes leads to a depletion of essential amino acids crucial for insect growth and development (Leo *et al.*, 2002). Specifically, the unavailability of essential amino acids in the insect body due to the inhibition of trypsin-like enzymes can result in insect pest mortality (Samiksha *et al.*, 2019). As evidenced by reports from diverse plant species, plants containing serine protease inhibitors can resist various insects and pathogens (Leung *et al.*, 2000; Oliva and Sampaio, 2009; Ramos *et al.*, 2009). Leguminous plant seeds, in particular, are abundant in PIs, which have been shown to inhibit insect gut proteolytic activity both in vivo and in vitro (Richardson, 1991). Therefore, employing PIs in resistance breeding programs could facilitate the development of innovative defense strategies in domestic crop varieties. Additionally, a gene stacking approach with different mechanistic classes of PIs can effectively target the dynamic expression of digestive proteases in highly polyphagous pests, such as *Helicoverpa* and *Spodoptera* (Swathi *et al.*, 2021). Hence, the study aimed to screen plant seed extracts to identify seeds containing the protease enzyme (trypsin) inhibitors, thereby contributing to our understanding of plant defenses against insect herbivory.

Reagents: N- α -Benzoyl-DL Arginine-P-NitroAnilide (BAPNA) was purchased from Sigma-Aldrich Chemicals, and trypsin was purchased from HIMEDIA, India. All other chemicals were of analytical grade. The reagents used were 200 mM Tris Buffer with 20 mM Calcium Chloride, pH 7.8, 0.1 per cent (w/v) N α -Benzoyl-DLArginine-P-NitroAnilide (BAPNA). Hydrochloric Acid Solution, 1 mM. Trypsin solution (Dissolved 2.5mg of Bovine trypsin in 20ml of cold Reagent C (7mM Na-K tartrate, 0.81M sodium carbonate, 0.5N NaOH final concentration)), 0.9 per cent (w/v) Sodium chloride solution.

Protease assay: Protease assay was carried out in a total volume of 1ml containing 536 μ l Tris Buffer (200 mM Tris; 20 mM Calcium chloride, pH 7.8), 67 μ l bovine trypsin (0.125 mg/ml; dissolved in 1mM HCl), 67 μ l NaCl (0.9%) and 330 μ l N α -

Benzoyl-DLArginine-P-NitroAnilide (BAPNA) (1mg/1ml) as substrate. A blank was also done along with it by adding 67 μ l 1mM HCl, instead of trypsin (Divya *et al.*, 2014).

Protease inhibition assay: Seventeen plant species were selected for this study based on the availability of the seeds and traditional knowledge. Fully matured dry seeds of *Albizia lebbbeck*, *Barleria repens*, *Bixa orellana*, *Calliandra haematocephala*, *Cleome viscosa*, *Crotalaria pallida*, *Cucumis sativus*, *Fioria vitifolia*, *Hibiscus surattensis*, *Ipomoea sepiaria*, *Leucaena leucocephala*, *Distimake vitifolius*, *Passiflora foetida*, *Pterocarpus marsupium*, *Swietenia mahagoni*, *Trichosanthes cucumerina*, and *Vigna trilobata* were collected from Calicut University Botanical Garden (11°8'1.57"N; 75°53'21.74"E), Thodeekkalam (11°51'12.51"N; 75°39'18.96"E), and Srirampura (13°3'51.15"N; 77°37'12.76"E). These were harvested at their peak ripeness and then dried under shade to ensure consistency in our extraction process. The seeds were washed and soaked in bicarbonate buffer (pH 9.0; 1 ml/g tissue) overnight, then homogenized. The homogenates were centrifuged at 10,000 $\times$ g in a Rota 4R-V/FA, Plasto Crafts centrifuge for 10 minutes at 4°C, and the supernatant containing soluble proteins was used for the protease inhibition assay.

In protease inhibition assay the extract was pre-incubated with the enzyme (25 μ l of plant extract with 67 μ l of trypsin (0.125 mg/ml⁻¹) for 10 minutes, followed by addition of 67 μ l NaCl (0.9%) and 511 μ l Tris buffer. The reaction was started by adding 330 μ l BAPNA (1mg ml⁻¹). Proteolytic activity was measured by continuous spectrophotometric rate determination method using UV Spectrophotometer, by recording the increase in absorbance at 405nm for 5minutes. All assays were done three times in duplicate and percentage of inhibition calculated by taking the activity of the enzyme alone as 100% (Divya *et al.*, 2014). We took meticulous care to ensure that the pH of the assay medium was consistently maintained at 7.8 across all experiments.

Calculations: The absorbance of the control was subtracted from the absorbance of the inhibitor alone to quantify the protease activity attributed to the plant extract. This calculated value was then subtracted from the absorbance measured in the presence of both the inhibitor and the enzyme, allowing for the determination of the actual absorbance devoid of protease activity from the plant extract. The absorbance of the enzyme alone was designated as representing 100 per cent enzyme activity. Control Activity ($\Delta A_{405nm}/\text{min}$) represents the enzyme's absorbance rate without any plant extract, and taken as 100 per cent activity. With this reference point, the absorbance recorded in the presence of the inhibitor was translated into percentage activity. Finally, this percentage was subtracted from 100 to derive the percentage inhibition.

Percentage inhibition is calculated as follows:

$$= (\Delta A_{405nm}/\text{minute uninhibited} - \Delta A_{405nm}/\text{minute inhibited}) / (\Delta A_{405nm}/\text{minute uninhibited} - \Delta A_{405nm}/\text{minute blank}) \times 100$$

Seventeen plant seeds were screened to identify protease inhibitors targeting trypsin. Among these, ten seed extracts exhibited significant trypsin inhibition, with inhibition levels exceeding 40 per cent (Table 1). In this study, *Pterocarpus marsupium* showed the highest trypsin inhibition at 98.2 per cent $\pm$ 0.5 per cent, followed closely by *Barleria repens* (97.75% $\pm$ 0.25%), *Ipomoea sepiaria* (93.80 per cent $\pm$ 0.15%), *Distimake vitifolius* (91.79% $\pm$ 0.71%), and *Calliandra haematocephala* (88.74 per cent $\pm$ 0.26%). There are no prior reports of trypsin inhibitors from these seeds, highlighting their potential as novel sources. In contrast, previous research has documented trypsin inhibitors in other species of the *Calliandra*. For example, *C. selloi* Macbride demonstrated a trypsin inhibitor with a molecular weight of approximately 20,000 Da (Yoshizaki *et al.*, 2007), supporting the relevance of *Calliandra* species as potential sources of protease inhibitors.

In agreement with earlier studies, *Albizia lebbbeck*

exhibited substantial trypsin inhibition, showing 80.8 per cent inhibition in our study. Prior study identified a Bowman-Birk inhibitor from *A. lebbbeck* that was effective against gut proteases of *Helicoverpa armigera*, leading to larval mortality and growth suppression in *Pieris brassicae* and *Spodoptera littoralis* (Hivrale *et al.*, 2013). This consistency across studies reinforces the potential of *A. lebbbeck* for pest management.

Similarly, *Vigna trilobata* demonstrated 76.7 per cent trypsin inhibition, despite a lack of prior reports documenting protease inhibitors from this species. Nonetheless, study on *Vigna unguiculata* has identified inhibitors belonging to the Bowman-Birk family (Parveen *et al.*, 2018), indicating a possible commonality within the genus. *Fioria vitifolia* and *Cleome viscosa* showed trypsin inhibition at 49.43 and 40.15 per cent, respectively. The observed inhibition might be linked to phytochemicals known for anti-proteinase activity (Pillai and Nair, 2014). *Crotalaria pallida* demonstrated 46.33 per cent inhibition, which aligns with previous findings of proteinaceous trypsin inhibitors effective against various pests, including *Callosobruchus maculatus* and *Spodoptera mauritia* (Gomes *et al.*, 2005; Remya and Kannan, 2019). For the first time, this study reports seven plant seeds such as *P. marsupium*, *B. repens*, *I. sepiaria*, *D. vitifolius*, *C. haematocephala*, *V. trilobata* and *F. vitifolia* as sources of PIs targeting trypsin. These findings suggest broader applications in pest management, particularly in targeting lepidopteran pests.

Plant-derived protease inhibitors (PIs) play a key role in plant defense, both in natural ecosystems and agricultural systems. The effectiveness of PIs in various plants is well-documented. In this study, among the 17 plant species screened, five species from the Fabaceae family demonstrated significant trypsin inhibition, each exhibiting more than 40% inhibitory activity. This notable trend highlights the potential of Fabaceae species as promising sources of protease inhibitors, warranting further investigation into their biochemical properties. For instance, soybean-derived inhibitors like the Kunitz soybean trypsin inhibitor (STI) and the Bowman-Birk inhibitor (BBI) exhibit significant anti-

Table 1 List of plant seed extracts exhibiting trypsin inhibition, with inhibition values presented as the mean of six experiments along with standard error (SE)

No.	Plant species	Family	Common name	Mean $\pm$ SE % inhibition
1	<i>Pterocarpus marsupium</i> Roxb.	Fabaceae	Indian kino tree	98.20 $\pm$ 0.50
2	<i>Barleria repens</i> Nees.	Acanthaceae	Small bush violet	97.75 $\pm$ 0.25
3	<i>Ipomoea sepiaria</i> Roxb.	Convolvulaceae	Purple heart glory	93.80 $\pm$ 0.15
4	<i>Distimake vitifolius</i> Burm. f.	Convolvulaceae	Grape leaf wood rose	91.79 $\pm$ 0.71
5	<i>Calliandra haematocephala</i> Hassk.	Fabaceae	Red powder puff	88.74 $\pm$ 0.26
6	<i>Albizia lebbeck</i> (L.) Benth.	Fabaceae	Indian siris tree	80.80 $\pm$ 0.06
7	<i>Vigna trilobata</i> (L.) Verdc.	Fabaceae	Wild gram	76.7 $\pm$ 1.90
8	<i>Fioria vitifolia</i> (L.) Mattei	Malvaceae	Wild okra	49.43 $\pm$ 0.57
9	<i>Crotalaria pallida</i> Ait.	Fabaceae	Streaked rattle pod	46.33 $\pm$ 1.66
10	<i>Cleome viscosa</i> Linn.	Cleomaceae	Asian spider flower	40.15 $\pm$ 0.85
11	<i>Bixa orellana</i> Linn.	Bixaceae	Lipstick plant	36.00 $\pm$ 1.10
12	<i>Cucumis sativus</i> Linn.	Cucurbitaceae	Cucumber	30.50 $\pm$ 0.40
13	<i>Hibiscus surattensis</i> Linn.	Malvaceae	Bush sorrel	28.10 $\pm$ 0.80
14	<i>Leucaena leucocephala</i> (Lam.) de Wit	Fabaceae	Lead tree	24.04 $\pm$ 0.75
15	<i>Passiflora foetida</i> Linn.	Passifloraceae	Stinking passion flower	22.23 $\pm$ 1.00
16	<i>Swietenia mahagoni</i> Linn.	Meliaceae	Mahogany	19.50 $\pm$ 1.50
17	<i>Trichosanthes cucumerina</i> Linn.	Cucurbitaceae	Snake gourd	8.79 $\pm$ 1.50

proteolytic activities and are widely distributed across plant families like *Gramineae* (Poaceae), *Leguminosae* (Fabaceae), and *Solanaceae* (Haq *et al.*, 2004; Bijina *et al.*, 2011; Marathe *et al.*, 2019). Bowman-Birk inhibitors, such as those from *Dolichos biflorus*, have demonstrated effectiveness in deterring herbivores and fungal pathogens, highlighting the multifunctionality of PIs (Kuhar *et al.*, 2013). These inhibitors often exist as zymogens, inactive forms activated under stress, enhancing their defensive capabilities (Graham *et al.*, 1985; Koiwa *et al.*, 1997).

In agricultural contexts, PIs are valuable as natural insecticides. These proteins specifically target digestive enzymes in herbivorous insects' midguts, impairing digestion and leading to slowed growth and mortality (De Leo *et al.*, 2001; Ribeiro *et al.*, 2006). PIs offer a sustainable alternative to chemical pesticides by targeting pests with minimal

impact on beneficial organisms, thereby preserving ecological balance (Gatehouse *et al.*, 1997; Schneider *et al.*, 2017; Marathe *et al.*, 2019). Studies have consistently demonstrated the efficacy of PIs in pest control, making them suitable for integrated pest management (IPM) strategies (Dunse *et al.*, 2010; Pawar *et al.*, 2024). Although transgenic crops expressing insecticidal genes have gained popularity, concerns about pest resistance and consumer acceptance remain, particularly regarding Bt crops. PIs, being naturally occurring, offer a safer alternative for pest management with fewer health risks and greater public acceptance (Kumar, 2024). Hence, this study underscores the significance of plant-derived protease inhibitors in natural pest management and sustainable agriculture. The newly identified inhibitors from plant seeds suggest the possibility of developing effective, eco-friendly strategies for IPM.

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