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Isolation of a new trypsin inhibitor from *Calopogonium Mucunoides* and its toxicity to the larvae of the Dengue vector, *Aedes albopictus*

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Abstract
Plant protease inhibitors are molecules that inhibits proteolytic activity of proteases. They regulate the activity of proteases and also serves as defence proteins. The expression of some plant protease inhibitors is induced by herbivory. These defence proteins can be exploited in pest control. The predominant protease in the gut of mosquito larvae is trypsin-like serine proteases. Thus, serine protease inhibitors find applications in mosquito control. In this investigation, 45 plant extracts were screened for trypsin inhibition activity. Of these, 11 extracts exhibited trypsin inhibition greater than 50%. Six of these protease inhibitors were proteinaceous in nature and among them *Calopogonium mucunoides* had shown the highest trypsin inhibition and showed significant toxicity to *Aedes albopictus* larvae. The *C. mucunoides* trypsin inhibitor was purified by ammonium sulphate fractionation, gel filtration chromatography, ion exchange chromatography and trypsin affinity chromatography. The purified trypsin inhibitor was found to have 22.54 ± 0.27 percentage trypsin inhibition at 0.04 µg/µl protein concentration. When *Aedes albopictus* larvae were treated with fraction eluted from gel filtration column, significant mortality observed with an LC_{50} (48 hours) value of 0.32 ± 0.00 µg/ml. As the trypsin inhibitor from *C. mucunoides* is toxic to *A. albopictus* larvae and it will find applications in mosquito control.

Keywords: trypsin inhibitor, *Calopogonium mucunoides*, protein purification, mosquito larvicide, *Aedes albopictus*

Introduction
Proteases are hydrolytic enzymes that cleaves peptide bonds in target proteins. They are found in a wide range of organisms and catalyse many metabolic processes including digestion for providing essential nutrients, such as amino acids, for the growth of organism. However, they need to be regulated, otherwise continuous protease action evokes some other detrimental effects. Protease inhibitors are molecules that modulate protease activity, thereby regulating its activity. Protease inhibitors are broadly distributed across plants, animals, and microorganisms (Valueva & Mosolov, 2004; Christeller, 2005; Haq *et al*., 2004; Supuran *et al*., 2002; Mosolov & Valueva, 2005; Mosolov *et al*., 2001) [5, 12, 21, 22, 32, 34]. In plants, protease inhibitors are typically found in storage organs or other aerial parts (De Leo *et al*., 2002) [6] and are often induced in response to injury or attacks by insects or pathogens (Ryan, 1990) [27]. These inhibitors are key components of plant defence systems, as they can disrupt the digestive enzymes in the gut of herbivores, reducing herbivory. For example, soybean-derived protease inhibitors have been shown to affect insect pests such as *Tribolium confusum* (Lipke *et al*., 1954) [19]. Over time, numerous studies have reported on protease inhibitors targeting various phytophagous insect pests, and purified the inhibitors (Gupta *et al*., 2000; Lawrence & Koundal, 2002; Garcia *et al*., 2004; Kansal *et al*., 2008; Kaur & Sohal, 2019) [10, 11, 13, 14, 17]. Additionally, plant protease inhibitors have demonstrated antimicrobial properties against bacteria, fungi, and some viruses (Kim *et al*., 2009) [15]. They are also known to possess anti-inflammatory, antioxidant, and anticancer properties, highlighting their potential in therapeutic applications (Li *et al*., 2011; Arbogast *et al*., 2007; Mehdad *et al*., 2016) [1, 20].

Trypsin inhibitors from various plants have been identified for mosquito control, as trypsin is a major gut protease in mosquitoes (Pontual *et al*., 2014; Sasaki *et al*., 2015; Sara Prakash *et al*., 2020; N *et al*., 2021) [26, 29, 30]. A Kunitz type trypsin inhibitor has been purified from the *Cassia leiandra* seeds with an LC_{50} of 2.28 x 10^{-2} M on larvae of *A. aegypti* (Dias *et al*., 2017) [7] and from *Allium sativum* (Shamsi *et al*., 2018) [31] with LC_{50} of 50.83 µg/ml. Another trypsin inhibitor was purified from *Enterolobium contortisiliquum* seeds and caused toxicity to *A. aegypti* on prolonged exposure and had enhanced activity when combined with Bti toxins (Tabosa *et al*., 2020) [33]. When DENV-2 infected *A. aegypti* was treated with soybean trypsin inhibitor, replication rate of DENV-2 in midgut got slower (Molina-Cruz *et al*., 2005) [21]. In this study, we screened plant extracts for proteinaceous trypsin inhibitors and purified a trypsin inhibitor from *Calopogonium mucunoides* with mosquito larvicidal activity.

Materials and Methods
1. Materials
Trypsin and azocasein from SRL, Mumbai, India. Proteinase K was from Himedia Laboratories, Mumbai, India. Cyanogen bromide activated Sepharose 4B was from Sigma, USA and Amicon 3 kDa protein concentrator was purchased from Merck, Germany. All other reagents used were analytical grade.

2. Methods
2.1 Collection of plants and preparation of plant extracts
Plants were collected from Calicut University Botanical Garden, Malappuram, Kerala, India. Leaves, seeds and fruits of plant collected were used. Plant parts were soaked

overnight in 100 mM bicarbonate buffer, pH 9.0 (1g tissue/ml) and homogenized. Homogenates centrifuged at 10000 x g for 10 minutes at 4°C and soluble fraction kept at -20°C until use.

2.2 Screening plant extracts for trypsin inhibitor by trypsin inhibition assay

Plant extracts prepared were screened for the presence of trypsin inhibitor by trypsin inhibition assay. Protease assay was done by modifying Charney and Tomarelli method (Charney & Tomarelli, 1947) [4]. Trypsin was used as the enzyme source and azocasein as the substrate. 5µl trypsin (1 mg/ml) was incubated with 5.2 µl azocasein (3.8 µg/ µl) in a total volume of 20.2 µl at 37°C for 30 minutes. Proteolytic degradation of azocasein by trypsin results in liberation of azo dye. Reaction was stopped by adding 5% TCA and centrifuged at 10000 x g for 10 minutes. The supernatant was mixed with 150 µl of 500mM NaOH and the absorbance was measured at 440nm in a microplate reader. Screening of plant extracts was done by trypsin inhibition assay. In trypsin inhibition assay the plant extract (10ul) was pre-incubated with trypsin at room temperature for 10 minutes and the protease assay continued as described above.

2.3 Proteinase K treatment of plant extracts

Plant extracts having more than 50% inhibition was subjected to proteinase K treatment, to confirm whether the inhibitor is proteinaceous or not. This was done by incubating the plant extract with proteinase K in 9:1 ratio overnight at 56°C. After incubation, the proteinase K was inactivated by keeping the reaction mixture at 96°C water bath for 5 minutes. The mixture centrifuged and supernatant was subjected to trypsin inhibition assay. In the case of proteinaceous inhibitor, proteinase K treatment results in significant reduction in trypsin inhibition. *C. mucunoides* inhibitor was proteinaceous in nature and showed the highest trypsin inhibition was selected for purification of the inhibitor.

2.4 Purification of trypsin inhibitor from *Calopogonium mucunoides*

The crude extract was prepared in 100 mM bicarbonate buffer, pH 9.0 and precipitated by ammonium sulphate (0-30%, 30-50% and 50-70%) (Englard & Seifter, 1990) [9]. The fractions were tested for larvicidal activity on *Aedes albopictus* and fraction that showed highest larvicidal activity subjected to gel filtration chromatography in SEC 650 column. Elution of proteins was done with 100 mM bicarbonate buffer, pH 9.0. The elutes showing highest trypsin inhibition was concentrated and further purified on a 1 ml ion exchange source Q column using FPLC system. The protein was eluted with a gradient of NaCl (0.0-1.0 M) in bicarbonate buffer (100 mM), pH 9.0. The elutes were further purified by trypsin affinity chromatography using batch method. Trypsin affinity resin was prepared by coupling trypsin to cyanogen bromide activated Sepharose 4B. Elution was done with 0.1 M HCl and the elutes neutralized immediately with 0.1 M NaOH.

2.5 Protein estimation

Protein estimation of samples were done by Bradford's dye binding method (Bradford, 1976) [3] using bovine serum albumin as standard.

2.6 SDS-PAGE analysis

Proteins purified in each step of the purification was subjected to SDS-PAGE according to Laemmli's method (Laemmli, 1970) [16] to assess the level of purification and for determining the molecular weight of the inhibitor.

2.7 Toxicity of gel filtration purified fraction to *Aedes albopictus* larvae

The second instar *A. albopictus larvae* were treated with gel filtration purified fraction (GFF) from *C. mucunoides*. For this, 25 *A. albopictus* second instar larvae were taken in 100 ml distilled water and treated with different concentrations of GFF. A control without GFF was also maintained. Mortality and developmental alterations were noted. Probit analysis was used to calculate lethal concentrations.

2.8 Statistical analysis

Statistical analysis was done using SPSS version 23 and Microsoft Excel version 21.

Results

1. Screening of plant extracts for trypsin inhibitor

A total of 45 plant extracts were screened for identifying trypsin inhibitor. The list of plants screened with percentage inhibition is given in table 1.

Table 1: List of plants screened with percentage trypsin inhibition

Sl no.	Plant name	Family	Plant part used	% trypsin inhibition (mean ± SE)
1	*Calopogonium mucunoides* Desv.	Fabaceae	Seeds	87.99 ± 0.38
2	*Lantana camara* L.	Verbanaceae	Seeds	85.35 ± 1.36
3	*Mucuna pruriens* (L.) DC.	Fabaceae	Seeds	70.03 ± 0.46
4	*Coffea arabica* L.	Rubiaceae	Leaves	69.85 ± 1.87
5	*Ocimum tenuiflorum* L.	Lamiaceae	Leaves	62.10 ± 0.76
6	*Globba sessiliflora* Sims	*Zingiberaceae*	Leaves	61.74 ± 2.91
7	*Mussaenda erythrophylla* Schumach and Thonn	Rubiaceae	Leaves	56.05 ± 1.76
8	*Erymgium foetidum* L.	Apiaceae	Leaves	55.09 ± 3.74
9	*Cinnamomum cassia* (L.) J. Presl	Lauraceae	Leaves	52.81 ± 1.45
10	*Plectranthus amboinicus* Lour.	Lamiaceae	Leaves	52.81 ± 1.45
11	*Zingiber officinale* Roscoe	Zingiberaceae	Leaves	51.96 ± 1.70
12	*Mussaenda luteola* Delile	Rubiaceae	Leaves	49.90 ± 6.44
13	*Thunbergia erecta* (Benth.) T. Anderson	Acanthaceae	Leaves	49.90 ± 6.44
14	*Vanilla planifolia* Andrews	Orchidaceae	Leaves	49.02 ± 3.71
15	*Manihot esculenta* Crantz	Euphorbiaceae	Leaves	44.07 ± 0.62
16	*Chassalia curviflora* (Wallich) Thwaites	Rubiaceae	Leaves	44.06 ± 1.13
17	*Grewia nervosa* (Lour.) G. Panigrahi	Malvaceaae	Fruits	41.16 ± 2.70

18	*Piper betle* L.	Piperaceae	Leaves	40.75 ± 0.62
19	*Hemigraphis alternata* (Burm. f.) T. Anderson	Acanthaceae	Leaves	40.04 ± 2.14
20	*Scheicheria oleosa* (Lour.) Oken	Sapindaceae	Leaves	38.37 ± 0.20
21	*Argyreia pomacea* (Roxb.) Choisy	Convolvulaceae	Leaves	33.69 ± 1.34
22	*Murraya koenigii* (L.) Spreng.	Rutaceae	Leaves	33.27 ± 2.28
23	*Justicia wynadensis* (Nees) T. Anderson	Acanthaceae	Leaves	31.84 ± 1.81
24	*Catharanthus roseus* (L.) G. Don	Apocynaceae	Leaves	28.06 ± 3.06
25	*Kopsia fruticose* (Roxb.) A. DC.	Apocynaceae	Leaves	27.69 ± 0.79
26	*Litchi chinensis* Sonn.	Sapindaceae	Seeds	24.74 ± 0.40
27	*Lawsonia inermis* L.	Lythraceae	Leaves	24.32 ± 1.25
28	*Simarouba glauca* DC.	Simaroubaceae	Leaves	21.20 ± 1.87
29	*Carica papaya* L.	Caricaceae	Leaves	21.17 ± 1.83
30	*Pancratium parvum* Dalzell	Amaryllidaceae	Bulb	20.55 ±0.65
31	*Tinospora cordifolia* (Willd.) Miers	Menispermiaceae	Leaves	18.85 ± 0.77
32	*Hibiscus rosasinensis* L.	Malvaceae	Leaves	17.53 ± 0.39
33	*Laggera alata* (D. Don) Sch. Bip. Ex Oliv.	Asteraceae	Leaves	17.48 ±0.28
34	*Clerodendrum viscosum Vent.*	Lamiaceae	Leaves	16.07 ± 0.88
35	*Mimusops elengi* L.	Sapotaceae	Seeds	13.87 ± 1.44
36	*Callisia repens* (Jacq.)	Commelinaceae	Leaves	12.83 ± 0.40
37	*Asparagus racemosus* Willd.	Asperagaceae	Seeds	11.91 ± 2.16
38	*Clitoria ternatea* L.	Fabaceae	Leaves	11.42 ± 2.88
39	*Leea indica* (Burm. f.) Merr.	Vitaceae	Leaves	10.30 ± 0.38
40	*Olea dioica* (Roxb.)	Oleaceae	Leaves	9.89 ± 0.10
41	*Combretum indicum* (L.) DeFilipps	Combretaceae	Leaves	8.26 ± 3.41
42	*Lophopetalum wightianum* Arn.	Celastraceae	Leaves	7.55 ± 1.10
43	*Conyza sps.*	Asteraceae	Leaves	6.79 ± 1.23
44	*Thevetia peruviana* (Pers.) K. Schum.	Apocynaceae	Leaves	4.95 ± 1.56
45	*Pithecellobium dulce (*Roxb.) Benth.	Fabaceae	leaves	0.81 ± 1.17

Values are mean ± standard error.

Out of the 45 plants screened, highest percentage trypsin inhibition was observed for *Calopogonium mucunoides* with 87.99 ± 0.38 %, followed by *Lantana camara* with 85.35 ± 1.36. More than 50% trypsin inhibition obtained in 11 plant extracts. These extracts were subjected to proteinase K digestion to check whether the inhibitor is proteinaceous or not.

2. Proteinase K treatment of plant extracts

Proteinase K treatment was done for 11 plant extracts with more than 50% trypsin inhibition. The results are shown in table 2.

Table 2: Proteinase K treatment of plant extracts with more than 50% trypsin inhibition

Sl no.	Plant name	Without proteinase K Mean % inhibition ± SE	With proteinase K Mean % inhibition ± SE	p value	Proteinaceous inhibitor
1	*Calopogonium mucunoides*	87.99 ± 0.38	18.97 ± 0.12	0.002	+
2	*Lantana camara*	85.35 ± 1.36	82.44 ± 0.41	0.101	-
3	*Mucuna pruriens*	70.03 ± 0.46	17.06 ± 0.05	0.005	+
4	*Coffea arabica*	69.85 ± 1.87	15.89 ± 0.19	0.010	+
5	*Ocimum tenuiflorum*	62.10 ± 0.76	17.51 ± 0.03	0.005	+
6	*Globba sessiliflora*	61.74 ± 2.91	17.91 ± 0.08	0.021	+
7	*Mussaenda erythrophylla*	56.05 ± 1.76	53.94 ± 0.03	0.223	-
8	*Eryngium foetidum*	55.09 ± 3.74	12.45 ± 0.38	0.025	+
9	*Cinnamomum cassia*	52.81 ± 1.45	44.05 ± 0.11	0.056	-
10	*Plectranthus amboinicus*	52.81 ± 1.45	48.71 ± 0.34	0.085	-
11	*Zingiber officinale*	51.96 ± 1.70	41.69 ± 0.93	0.080	-

Values are mean ± standard error. Plus, sign (+) indicates that the inhibitor is proteinaceous in nature while minus sign (-) indicates non-proteinaceous nature.

Trypsin inhibition assay of plant extracts done after proteinase K digestion. Of the 11-plant extract with more than 50% trypsin inhibition, 6 extracts showed reduction in their inhibition after digestion with proteinase K confirming that the inhibitor present in these plant extracts is protein in nature. There was no significant reduction in trypsin inhibition of remaining 5 plant extracts confirming their non-proteinaceous nature. *C. mucunoides* extract contain a proteinaceous trypsin inhibitor with highest inhibition trypsin (87.99 ± 0.38 %) among the plants tested.

3. Purification of *Calopogonium mucunoides* trypsin inhibitor

Differential fractionation of *C. mucunoides* crude extract with a protein concentration 11.94 µg/µL was done using ammonium sulphate precipitation. Toxicity to *A. albopictus* larvae was checked with crude and ammonium sulphate fraction equating their protein to a final concentration of 7.2 µg/ml. Observations after 48 hours gives 100 % mortality in crude and 0-30% fraction. In 30-50% ammonium sulphate fraction, 53.33 ± 3.33 % mortality was observed and 23.33 ± 3.33 % mortality in 50-70% fraction. These results are represented in figure1. The 0-30% ammonium sulphate fraction showing highest percentage mortality in *A. albopictus* larvae was further purified in SEC 650 gel

filtration column (supplementary fig. 1). Protein concentration and percentage trypsin inhibition of elutes were calculated. Elute 43 had the highest protein concentration and percentage trypsin inhibition of 0.50 µg/µl and 63.11 ± 0.33% respectively. The protein concentration and percentage trypsin inhibition of elutes is given in supplementary data (Fig. 2). The elutes 42,43,44 and 45 were pooled together and concentrated to get a final protein concentration of 1.11 ± 0.01 µg/µl and percentage trypsin inhibition of 58.29 ± 0.43 and used for further purification on a 1 ml source Q column using FPLC system. Proteins eluted from 600-750 mM NaCl concentration gave a prominent peak (supplementary fig. 2) and its protein estimation and percentage trypsin inhibition were calculated (Fig. 3). The elutes 4,5,6 with 48.66 ± 0.12, 58.39 ± 0.21 and 51.70 ± 0.44 % trypsin inhibition were pooled and concentrated and subjected to final purification using trypsin affinity chromatography by batch method. Trypsin affinity chromatography yields elute with 22.54 ± 0.27 % trypsin inhibition.

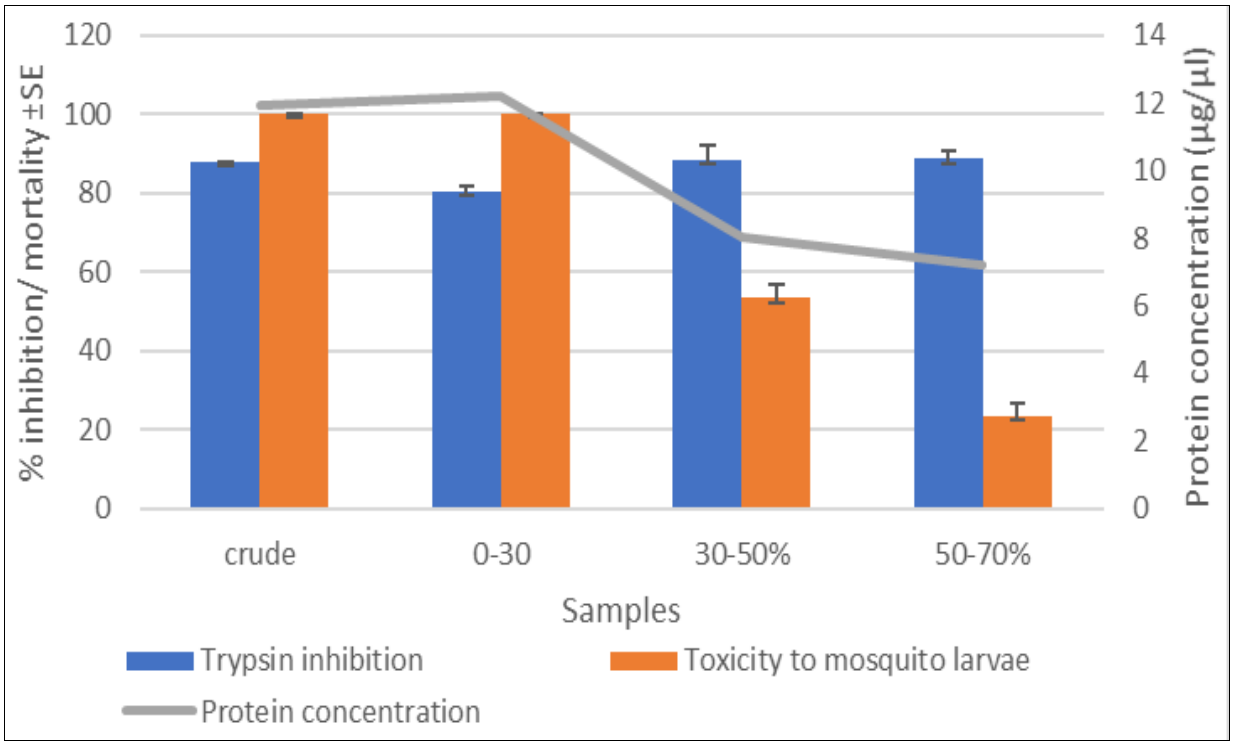

Fig 1: Protein concentration, trypsin inhibition and toxicity of crude and ammonium sulphate fractions. The line graph represents protein concentration of the samples. Histogram represents percentage trypsin inhibition and percentage mortality to *A. albopictus* larvae.

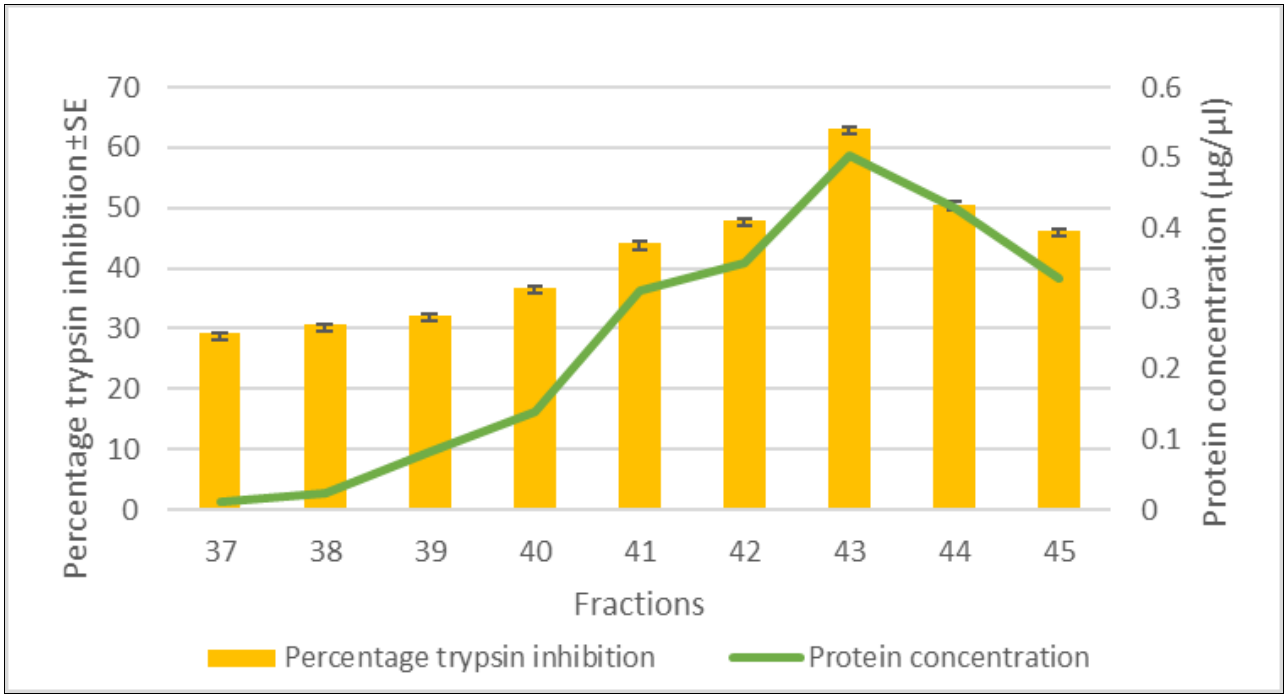

Fig 2: Protein concentration and trypsin inhibition of gel filtration fractions. Histogram represent the percentage trypsin inhibition with standard error of gel filtration fractions and the line graph represents protein concentrations.

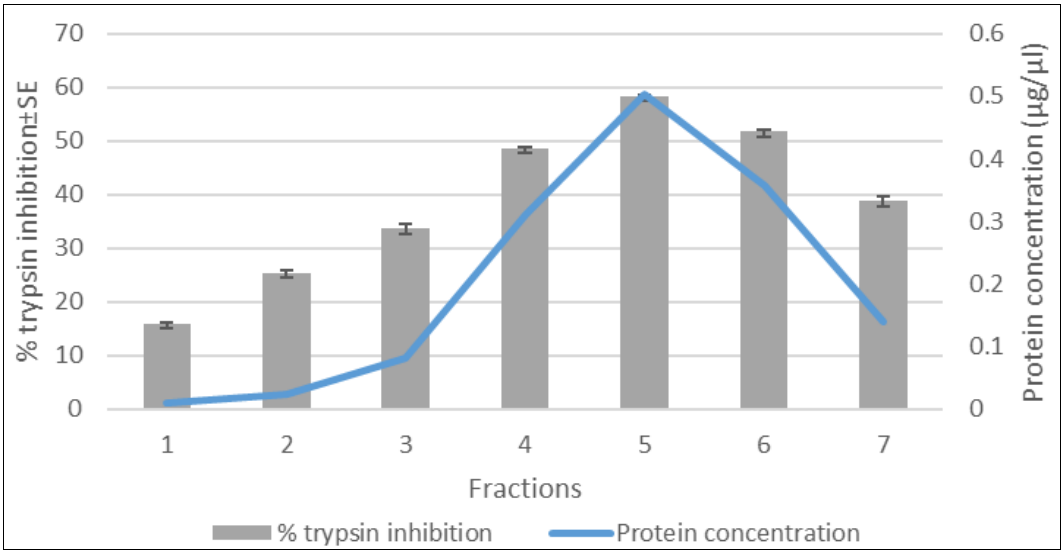

Fig 3: Protein concentration and trypsin inhibition of ion exchange fractions. Histogram represents the percentage trypsin inhibition and line graph represents protein concentration of fractions collected.

4. Protein concentration and trypsin inhibition of fractions during different stages of purification

Protein was estimated by Bradford's dye binding method and the inhibition of trypsin was assessed by trypsin inhibition assay during each stage of purification (Table 3). As the purification progressed, lower concentrations only required for inhibiting trypsin. The yield of the purified protein was 0.1 µg per gram of seed.

Table 3: Protein concentration and percentage trypsin inhibition of *C. mucunoides* fractions

Sample	Protein concentration (µg/µl)	Percentage trypsin inhibition (Mean ± SE)
Crude	11.94	87.99 ± 0.38
0-30% ammonium sulphate fraction	12.20	80.45 ± 1.35
Concentrated gel filtration fraction	1.11	58.29 ± 0.43
Concentrated ion exchange fraction	0.50	54.08 ± 0.37
Trypsin affinity elute	0.04	22.54 ± 0.27

Protein concentration and trypsin inhibition of fractions during purification. Percentage trypsin inhibition expressed in mean± SE, n=3.

5. SDS-PAGE analysis

Proteins purified in each step of the purification was subjected to SDS-PAGE to assess the level of purification obtained in each stage. As the purification progressed number of protein bands decreased from crude extract to trypsin affinity purified fraction (Fig. 4). The purified inhibitor is a 22 kDa protein, a molecular weight in the range of Kunitz type trypsin inhibitors.

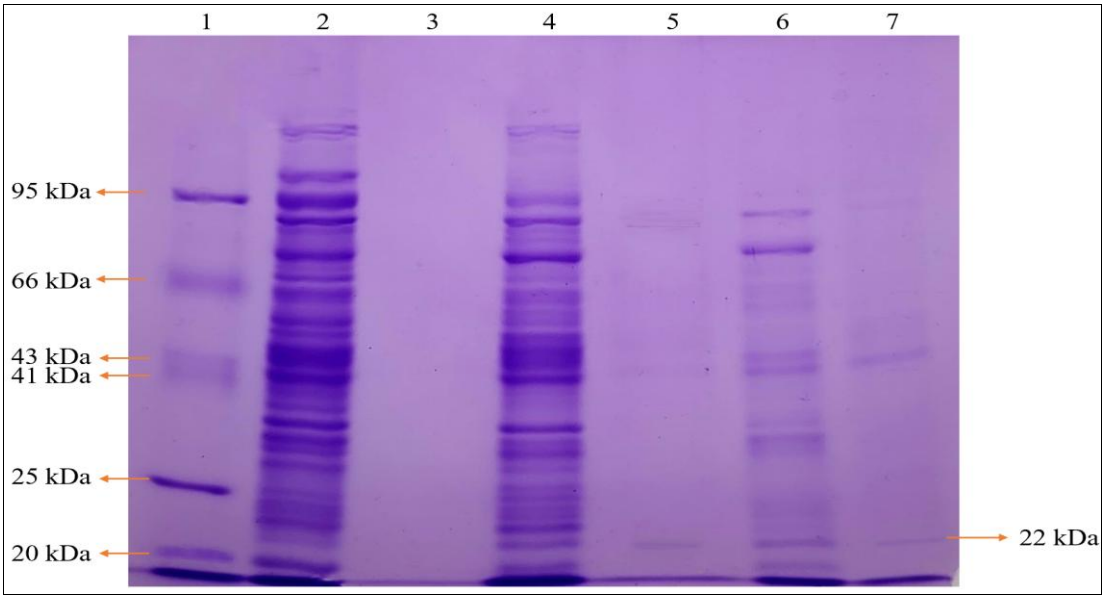

Fig 4: SDS-PAGE showing purification of trypsin inhibitor from *Calopogonium mucunoides*. Lane 1. Protein marker, Lane 2. Crude extract (50µg), Lane 3. Dye alone, Lane 4. 0-30% ammonium sulphate fraction (50µg), Lane 5. Elute from trypsin affinity (10µg), Lane 6. Concentrated gel filtration elutes (10µg), Lane 7. Concentrated ion exchange elutes (10µg)

6. Toxicity of gel filtration fraction of *Calopogonium mucunoides* to second instar *Aedes albopictus* larvae

When the toxicity of crude extract, 0-30% ammonium sulphate fraction and GFF to *A. albopictus* larvae studied, 100% mortality was observed for crude and 0-30% ammonium sulphate fraction at protein concentrations 3.23 ±0.0 and 2.83 ±0.0 µg/ml respectively (Fig. 5). GFF gave 77.33 ±1.33 % larval mortality at 1.1 µg/ml protein concentration. LC_{50} (48 hours) for crude, 0-30% ammonium sulphate and GFF was found to be 0.50 ± 0.01, 0.48 ± 0.01 and 0.32 ± 0.00 µg/ml respectively.

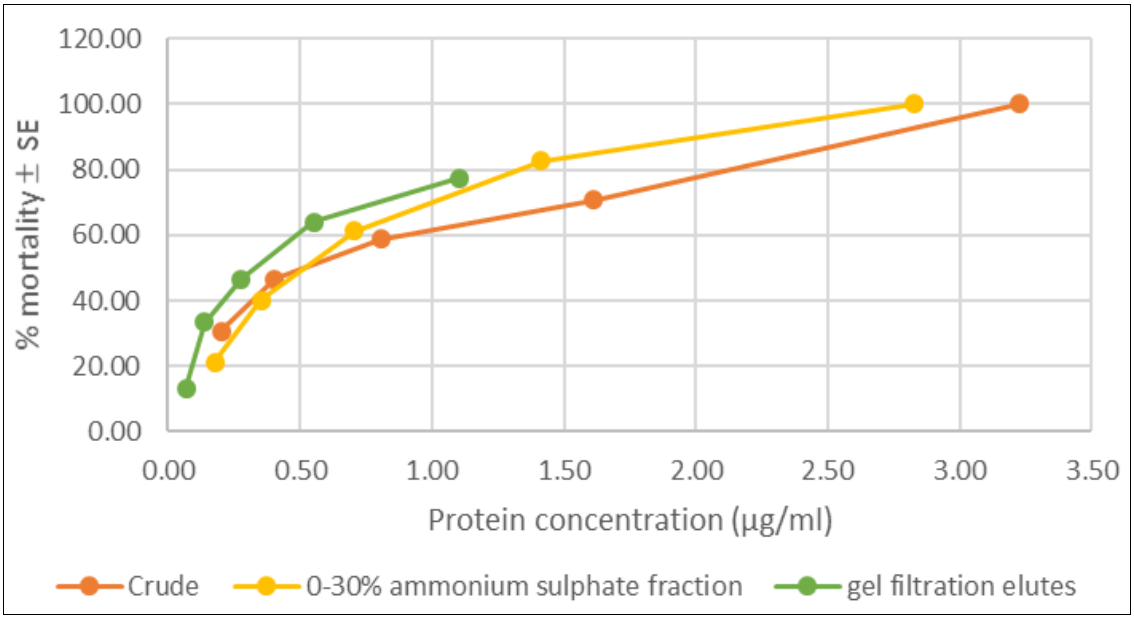

Fig 5: Toxicity to *Aedes albopictus* larvae. The line graphs show toxicity of *C. mucunoides* fractions to larvae of *A. albopictus*.

Discussion

Proteases are hydrolytic enzymes crucial for protein metabolism and are essential for digestion of proteins and supply of essential nutrients such as amino acids, which serve as the fundamental building blocks of an organism's body. Being key enzymes of protein metabolism, they should be regulated. Protease inhibitors are naturally occurring molecules found in plants, animals, and microorganisms (Valueva & Mosolov, 2004; Christeller, 2005; Haq *et al*., 2004; Supuran *et al*., 2002; Mosolov & Valueva, 2005; Mosolov *et al*., 2001) [5, 12, 21, 22, 32, 34] that could interfere in protease activity. In plants, several kinds of protease inhibitors are described varying in their biological functions or domains. These inhibitors may be constitutive as in storage organs (De Leo *et al*., 2002) [6] or can be induced in response to herbivory by insect pests (Ryan, 1990) [27]. Plant protease inhibitors have been found to be effective in managing phytophagous pest populations (Lawrence & Koundal, 2002) [17]. Plant protease inhibitors with potential for pest control have been isolated and characterized (Patinhara Parambil & Vadakkadath Meethal, 2022; Sajitha & Vadakkadath Meethal, 2024) [25, 28]. The replication of DENV-2 particles in *Aedes aegypti* mosquitoes was reportedly slower when treated with soybean trypsin inhibitors (Molina-Cruz *et al*., 2005) [21]. Trypsin is the main gut protease in the larvae of mosquitoes. Inhibition of trypsin activity disrupts food digestion and nutrient availability, leading to the death of mosquitoes. Several studies have identified plant-derived trypsin inhibitors with potent larvicidal effects against mosquitoes (Sara Prakash *et al*., 2020; N *et al*., 2021) [29]. For example, trypsin inhibitors from *Cassia leiandra* (Dias *et al*., 2017) [7], *Allium sativum* (Shamsi *et al*., 2018) [31], and *Enterolobium contortisiliquum* (Tabosa *et al*., 2020) [33] have been shown to have mosquito larvicidal activity on *A. aegypti*. The identification and purification of such proteins offer a promising, eco-friendly alternative to traditional chemical control methods for managing mosquito population.

In this study, 45 plant extracts were screened to identify potential trypsin inhibitors. Trypsin inhibition assay using azocasein as substrate was employed to quantitatively assess the inhibitory activity of the extracts. The nature of the inhibitors may vary, with some being proteinaceous, while others could be non-protein plant metabolites. To identify proteinaceous inhibitors, Proteinase K digestion was performed on 11 plant extracts that exhibited more than 50% trypsin inhibition. Of these, six extracts were confirmed to contain proteinaceous inhibitors and they are *Calopogonium mucunoides*, *Mucuna pruriens*, *Coffea arabica*, *Ocimum tenuiflorum*, *Globba sessiliflora*, and *Eryngium foetidum*. Among these six, *Calopogonium mucunoides* exhibited the highest level of trypsin inhibition and was selected for further purification. An ammonium sulphate fraction (0-30%) gave a trypsin inhibition of 80.45 ± 1.35 % with 100% larvicidal activity at a protein concentration of 7.2 µg/ml to *A. albopictus* second instar larvae. The 0-30% ammonium sulphate fraction was further purified by gel filtration chromatography, ion exchange chromatography, and trypsin affinity chromatography. The purified trypsin inhibitor was found to have 22.54 ± 0.27 percentage trypsin inhibition at a protein concentration of 0.04 µg/µl. The purified inhibitor is a 22 kDa protein with a yield of 0.1 µg per gram seeds. There are reports of purified Kunitz type proteinase inhibitor from *Theobroma cacao* having a size of 23 kDa (do Amaral *et al*., 2022) [8], *Archidendron ellipticum* seeds with a size of 20 kDa (Bhattacharyya *et al*., 2006) [2] and from *Hibiscus acetocella* seeds with a size of 21 kDa (Sajitha & Vadakkadath Meethal, 2024) [28]. Since the yield of purified inhibitor was

very low, we used concentrated gel filtration fraction for toxicity studies in *A. albopictus* larvae. The LC_{50} (48 hours) value of concentrated gel filtration fraction against *A. albopictus* larvae was 0.32 ± 0.00 µg/ml. These LC_{50} values were significantly lower compared to the purified inhibitor from *Allium sativum*, which showed an LC_{50} of 50.83 µg/ml against *Aedes aegypti* larvae (Shamsi *et al*., 2018) [31]. Certain protease inhibitors are known for their antimicrobial (Kim *et al*., 2009) [15], anti-inflammatory (Li *et al*., 2011), antioxidant (Arbogast *et al*., 2007) [1], and anti-cancerous (Mehdad *et al*., 2016) [20] properties. But when tested we could not find anti-microbial or anti-cancer activity for the crude extract from *C. mucunoides* (data not shown).

The trypsin inhibitor purified from *C. mucunoides* is toxic to the larvae of *A. albopictus.* Thus, the trypsin inhibitor from *C. mucunoides* will find applications in mosquito control. For this the gene coding for the inhibitor can be cloned and expressed in bacteria or yeast to make a mosquito larvicidal preparation.

Conclusions

In this study, 45 plant extracts were screened for identifying trypsin inhibitors and found six proteinaceous trypsin inhibitors with more than 50% percentage trypsin inhibition. A new trypsin inhibitor was purified from the seeds of *Calopogonium mucunoides* by ammonium sulphate precipitation, gel filtration chromatography, ion exchange chromatography and trypsin affinity chromatography. The purified trypsin inhibitor has a size of 22 kDa and at a protein concentration of 0.04 µg/µl inhibited trypsin to the extent of 22.54 ± 0.27 percentage. The yield of purified inhibitor from *C. mucunoides* was 0.1µg per gram of seed. The crude, 0-30% ammonium sulphate fraction and concentrated gel filtration fraction were toxic to larvae of *Aedes albopictus* larvae with an LC_{50} (48 hours) of 0.50 ± 0.01, 0.48 ± 0.01, and 0.32 ± 0.00 µg/ml, respectively. Thus, the trypsin inhibitor purified from *C. mucunoides* will find applications in mosquito control.

Funding Information

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Conflict of Interest

The authors declared that there is no conflict of interest, financial or other.

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Data Availability

Data will be made available on request.

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