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Identification of plant extracts inhibiting trypsin-like larval gut proteases of *Aedes albopictus* (Diptera: Culicidae) larvae

Anju Sara Prakash[1*], Ashitha Kormath[2], Kannan Vadakkadath Meethal[3]
[1-3] Department of Zoology, University of Calicut, Thenhipalam, Kerala, India

Abstract
Major proteases in the gut of mosquito larvae are trypsin-like serine proteases. Plants produce protease inhibitors and they play an important role in the defense of plants against pests and pathogens. In this study we screened different plant extracts to identify extracts containing inhibitors against trypsin-like gut proteases of 4th instar larvae of *Aedes albopictus*. Initial screening was done using bovine trypsin and the extracts showing greater than 50% trypsin inhibition was further tested against the gut proteases of *Aedes albopictus* larvae. From 27 plant extracts selected for initial screening, 19 plant extracts showed trypsin inhibition greater than 50%. These plant extracts were used to test the gut protease inhibition of *A. albopictus* larvae and 7 plant extracts showed greater than 50% inhibition. Purification and characterization of plant protease inhibitors from these plant extracts can be exploited for mosquito control.

Keywords: trypsin, proteases, plant protease inhibitors, *Aedes albopictus*, gut proteases

1. Introduction
Mosquitoes are vectors of many diseases. *Aedes* mosquitoes transmit serious diseases, including Dengue fever, Yellow fever, Chikungunya and Zika. The two most important species that transmit disease causing viruses are *Aedes aegypti* and *Aedes albopictus*. *Ae. albopictus* also known as Asian tiger mosquito, is primarily a forest species that has become adapted to rural, suburban and urban human environments. It is characterized by black and white striped legs and small black and white striped body.
Plants developed defensive mechanism against pests by producing plant protease inhibitors. Many lepidopteran pests have serine proteases as their major digestive enzyme [1]. Serine protease inhibitors exhibit anti-nutritional effects against many lepidopteran insects [2]. Protease inhibitors prevent digestion of protein in the larvae, leading to non-availability of amino acid for the larval growth and development resulting in death of the larvae. Digestive enzymes present in *Ae. aegypti* larvae include trypsin-like, chymotrypsin-like and elastase-like serine proteases [3]. The major proteases in the gut of *Culex pipiens* mosquito larvae are trypsin-like serine proteases [4]. The most abundant digestive enzymes present in the midgut of *Ae. aegypti* larvae are trypsin and chymotrypsin [5].
Plant protease inhibitors are tested for their anti-nutritional effect on mosquito larvae. For example, the trypsin inhibitor from *Moringa oleifera* flowers (MoFTI) interferes with the survival and development of *Ae. aegypti* larvae. Mortality of newly hatched larvae, reduction in survival of pupae and growth of bacteria in the midgut of fourth instar larvae were observed when treated with *M. oleifera* flower extract and MoFTI [6]. When *Ae. aegypti* larvae were treated with *Adenanthera pavonina* seed proteinase inhibitor (ApTI), a decrease in weight gain, survival and proteinase activities in the midgut extracts of the larvae were observed [7]. Also, trypsin inhibitor from *Leucaena leucocephala* seeds is known to delay and disrupt the development of *Ae. Aegypti* larvae [8]. Thus, it is clear that plant protease inhibitors are capable of inhibiting the gut proteases of *Ae. aegypti* mosquito larvae and have detrimental effect on the development of larvae. In this study we screened plant extracts to identify extracts containing protease inhibitors against trypsin-like gut protease of *Aedes albopictus* larvae.

2. Materials and Methods
Nα-Benzoyl-DLArginine-P-NitroAnilide (BAPNA) was purchased from Sigma Aldrich, USA. Trypsin (Bovine) was purchased from HIMEDIA, India. All other chemicals and reagents used were of analytical grade.

2.1 Collection of plant parts and preparation of plant extracts
Plant parts (seeds and leaves) were collected from Nilambur, Kerala, India, Kodanchery, Calicut, Kerala, India and University of Calicut Botanical garden, Calicut, Kerala, India. Plant parts were soaked in bicarbonate buffer, pH 9.0 (1g tissue/5ml buffer). Seeds were soaked overnight and leaves were soaked for 2 hours. Plant extract was prepared by homogenizing the seeds/leaves in bicarbonate buffer, pH 9.0 and centrifuged at 9400 xg for 10 minutes at 4ºC. The supernatant containing soluble protein was collected and frozen until use. This supernatant was used for protease inhibition assay.

2.2 Collection and maintenance of mosquito larvae
The larvae of *Aedes albopictus* mosquito were collected from Nilambur, Kerala, India and from the Botanical garden of University of Calicut, Kerala, India. The larvae were identified and kept in plastic beakers containing well water. The larvae were fed with yeast granules until they reached 4th instar stage.

2.3 Preparation of larval gut extract
The 4th instar larvae of *Ae. albopictus* were chilled and transferred onto a glass slide and midgut was dissected out using needles. Larval gut from sixty larvae homogenized in

100µl bicarbonate buffer pH 9.0. The homogenate was centrifuged at 9400 xg for 10 minutes at 4ºC. The supernatant containing soluble protein was collected and frozen until use and this supernatant was used for the protease assay/protease inhibition assay.

2.4 Protease assay
The protease assay was carried out in a total volume of 300µl, by mixing 5µl of bovine trypsin (1.25 mg/ml in 1mM HCl) or 5µl gut extract, 20µl 0.9% NaCl, 175µl 200 mM Tris buffer pH 7.8 with 20 mM Calcium chloride and 100 µl N-α-Benzoyl-DLArginine-P-NitroAnilide (BAPNA) (1mg/ml) as substrate. A blank was also done by adding 5µl 1mM HCl instead of trypsin or 5µl 0.1M bicarbonate buffer instead of gut extract. Proteolytic activity was determined by continuous spectrophotometric rate determination method using UV Spectrophotometer, by measuring the increase in absorbance at 405nm for 5 minutes. Experiments were done in duplicates and repeated three times.

2.5 Protease inhibition assay
Protease inhibition assay was done as detailed in protease assay except that 10µl of plant extract was pre-incubated with 5µl of bovine trypsin (1.25 mg/ml) (for protease inhibition assay with trypsin) or with 5µl of gut extract (for protease inhibition assay with gut extract) for 10 minutes, before adding 100 µl N-α-Benzoyl-DLArginine-P-NitroAnilide (BAPNA) (1mg/ml) as substrate and 165 µl 200 mM Tris buffer pH 7.8 with 20 mM Calcium chloride was added instead of 175 µl buffer. Proteolytic activity was determined by continuous spectrophotometric rate determination method using UV Spectrophotometer, by measuring the increase in absorbance at 405nm for 5minutes. The absorbance of control was taken as 100% enzyme activity for calculating the percentage inhibition in presence of the inhibitor. Protease activity in the plant extract, if any, was also taken into account for calculating inhibition. Experiments were done in duplicates and repeated three times.

2.6 Statistical analysis
Statistical analysis was done using R-program.

3. Results
Initially extracts from 27 plants were screened to identify the plant extracts containing inhibitors against trypsin. Out of the 27 plants screened, 19 of them showed greater than 50% inhibition against trypsin (Table 1). These plant extracts were used to test their inhibition towards gut protease activity of 4th instar larvae of *Ae. albopictus.* Seven plant extracts gave greater than 50% inhibition of gut protease activity of *Ae. albopictus* larvae (Table 2).

Table 1: List of plants showing greater than 50% inhibition of Trypsin

Sl. No.	Scientific name of Plant	Common name	Plant part used	Trypsin inhibition (Mean percentage ± SE)
1.	*Vigna radiata*	Green gram	Seed	99.66± 0.33
2.	*Anacardium occidentale*	Cashew	Seed	99.5± 0.17
3.	*Artocarpus heterophyllus*	Jackfruit	Seed	97.52 ± 0.26
4.	*Nephelium lappaceum*	Rambutan	Seed	95.34 ± 0.13
5.	*Plectranthus amboinicus*	Indian borage	Leaf	91.37 ± 0.69
6.	*Curcuma longa*	Curcumin	Leaf	91.2± 0.31
7.	*Phaseolus vulgaris*	Beans	Leaf	84.73± 0.63
8.	*Coffea arabica*	Coffee	Leaf	78.63± 0.55
9.	*Tectona grandis*	Teak	Leaf	74.0± 1.89
10.	*Catharanthus roseus*	Madagascar Periwinkle	Leaf	72.26± 0.46
11.	*Santalum album*	Sandal	Leaf	65.47± 0.61
12.	*Eryngium foetidum*	Culantro	Leaf	65.3± 0.09
13.	*Hibiscus surattensis*	Wild sour	Leaf	64.24 ±1.23
14.	*Anthurium andraeanum*	Anthurium	Leaf	63.0± 1.06
15.	*Ricinus communis*	Castor bean	Seed	62.89 ± 1.48
16.	*Datura stramonium*	Thorn apple	Seed	61.9 ± 1.03
17.	*Croton hirtus*	Hairy croton	Leaf	55.94 ± 1.08
18.	*Calliandra rosea*	Powder puff	Leaf	51.04 ± 2.33
19.	*Abelmoschus esculentus*	Lady's finger	Seed	50.66± 8.72

Table 2: List of plant extracts showing greater than 50% inhibition of gut protease activity of 4th instar larvae of *Ae. albopictus*.

Sl. No.	Name of the plant	Common name	Plant part used	Mean % inhibition ± SE
1.	*Curcuma longa*	Curcumin	Leaf	79.9 ± 0.60
2.	*Plectranthus amboinicus*	Indian borage	Leaf	71.87 ± 1.80
3.	*Artocarpus heterophyllus*	Jackfruit	Seed	67.52 ± 0.71
4.	*Anacardium occidentale*	Cashew	Seed	61.3 ± 0.65
5.	*Eryngium foetidum*	Culantro	Leaf	60.5 ±0.84
6.	*Santalum album*	Sandal	Leaf	57.07±1.40
7.	*Ricinus communis*	Castor oil seed	Seed	55.48 ± 0.87

4. Discussion
Among the plant extracts tested, the highest trypsin inhibition was obtained for *Vigna radiata* (99.66 ± 0.33%) and the other five plant extracts gave trypsin inhibition greater than 90% were *Anacardium occidentale* (99.5± 0.17%), *Artocarpus heterophyllus* (97.52 ± 0.26%), *Nephelium lappaceum* (95.34 ± 0.13%), *Plectranthus amboinicus* (91.37 ± 0.69%) and *Curcuma longa* (91.2 ± 0.31%). An inhibitor which inhibits the trypsin or chymotrypsin activity of vertebrates was already reported from the seeds of *Artocarpus heterophyllus.* This inhibitor was purified and was found to be a glycoprotein with a

molecular weight of 26 kDa [9]. From *Ricinus communis* also a protease inhibitor has been isolated [10]. Similarly, greater than 50% inhibition of trypsin was reported from extracts of *Datura stramonium, Ricinus communis, Plectranthus amboinicus, Santalum album, Phyllanthus amarus* and *Croton hirtus* [11]. From *Vigna radiata* which showed the highest percentage of trypsin inhibition (99.66± 0.33%) among the plant extracts screened, a trypsin inhibitor has already been reported. It was extracted and purified from the mung bean seeds grown in Thailand [12]. Trypsin inhibitor from *Nephelium lappaceum* was isolated from fresh seeds of *N. lappaceum,* which reduced the proteolytic activity of trypsin and α-chymotrypsin [13]. To the best of our knowledge this is the first report of trypsin inhibitor from plant extracts of *Hibiscus surattensis, Calliandra rosea, Eryngium foetidum and Anthurium andraeanum.*

The plant extracts form 19 plants which showed greater than 50% inhibition against trypsin were used to test their inhibition towards gut protease activity of 4th instar larvae of *Ae. albopictus.* Out of these, seven plant extracts gave greater than 50% inhibition of gut protease activity of *Ae. Albopictus* larvae. The highest inhibition of gut extract was shown by *Curcuma longa* (79.9 ± 0.6%), followed by *Plectranthus amboinicus* (71.87 ± 1.80%), *Artocarpus heterophyllus* (67.52 ± 0.71%), *Anacardium occidentale* (61.3 ± 0.65%), *Eryngium foetidum* (60.5 ± 0.84%), *Santalum album* (57.07± 1.40%), and *Ricinus communis* (55.48 ± 0.87%). This is the first report of presence of protease inhibitor against the larval gut protease of *Ae. albopictus* from these plants. Though some of the plant extracts like that from *Vigna radiata* showed very high trypsin inhibition (99.66± 0.33), but failed to inhibit the larval gut protease greater than 50%. This may be due to the inactivation of the inhibitor in the gut extract or due to the presence of proteases insensitive to the inhibitor.

The seed extracts of *A.occidentale* was found to inhibit the gut protease activity of *Spodoptera mauritia* (73.6± 0.35%) [14]. Also extracts from *Artocarpus heterophyllus* was reported to inhibit the gut protease of S*podoptera mauritia* larvae [15]. Studies also proved that many plant extracts have the capacity to delay and disrupt the growth and development of mosquito larvae [6,7,8]. As extracts from *Curcuma longa, Plectranthus amboinicus, Artocarpus heterophyllus, Anacardium occidentale* and *Eryngium foetidum* are showing greater than 60% inhibition of gut protease activity of *Ae. Albopictus* larvae, further investigation is necessary to purify and characterize the plant protease inhibitor from these plants and to test their *in vivo* effects on mosquito larvae for exploiting them for the control mosquitoes. Cloning and expressing the protease inhibitor in organisms upon which the mosquito larvae feeds is a viable option.

5. Conclusions

Out of the 27 plant extracts screened for trypsin inhibition, 19 extracts showed greater than 50% inhibition against trypsin. These 19 plant extracts when tested to check their inhibition against the gut protease activity of 4th instar larvae of *Ae. albopictus*, seven plant extracts showed greater than 50% inhibition of the gut protease activity. These plant extracts are promising candidates for mosquito control as they give relatively higher inhibition of the larval gut protease activity. Further investigation is necessary to purify and characterize the plant protease inhibitors from these plants and their *in vivo* toxicity need to be investigated for their use in mosquito control.

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7. References

1. Houseman JG, Philogene BJR, Downe AER. Partial characterization of proteinase activity in the larval midgut of the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae). Canadian journal of zoology. 1989; 67(4):864-868.
2. Applebaum SW. Biochemistry of digestion. Comprehensive Insect Physiology, Biochemistry and Pharmacology *in* G.A. Kerkut and L.I. Gilbert (eds.), Pergamon Press, New York, 1985; 4:279-311.
3. Soares TS, Watanabe RM, Lemos FJ, Tanaka AS. Molecular characterization of genes encoding trypsin-like enzymes from *Aedes aegypti* larvae and identification of digestive enzymes. Gene. 2011; 489(2):70-75.
4. Amrutha M, Shiji TM, Kannan Vadakkadath Meethal. Serine proteases represent the predominant protease in the gut of *Culex pipiens* mosquito larvae. International Journal of Entomology Research. 2018; 3(1):92-94.
5. Yang YJ, Davies DM. Trypsin and chymotrypsin during metamorphosis in *Aedes aegypti* and properties of the chymotrypsin. Journal of insect physiology. 1971; 17(1):117-131.
6. Pontual EV, de Lima Santos ND, de Moura MC, Coelho LCBB, Navarro DMDAF, Napoleão TH, *et al*. Trypsin inhibitor from *Moringa oleifera* flowers interferes with survival and development of *Aedes aegypti* larvae and kills bacteria inhabitant of larvae midgut. Parasitology research. 2014; 113(2):727-733.
7. Sasaki DY, Jacobowski AC, de Souza AP, Cardoso MH, Franco OL, Macedo MLR, *et al*. Effects of proteinase inhibitor from *Adenanthera pavonina* seeds on short-and long-term larval development of *Aedes aegypti*. Biochimie. 2015; 112:172-186.
8. Almeida Filho LC, de Souza TM, Tabosa PM, Soares NG, Rocha Bezerra LC, Vasconcelos IM, *et al*. Trypsin inhibitor from *Leucaena leucocephala* seeds delays and disrupts the development of *Aedes aegypti*, a multiple disease vector. Pest management science. 2017; 73(1):181-187.
9. Bhat AV, Pattabiraman TN. Protease inhibitors from jackfruit seed (*Artocarpus integrifolia*). Journal of Biosciences. 1989; 14(4):351-365.
10. Soomro FA, Dahot MU, Rehman AU. Isolation and characterization of crude protease inhibitors from *Ricinis communis* (Castor Beans). Pak. J Biotechnol. 2007; 4(1-2):93-99.
11. Divya C, Sreedharan KS, Parambath BP, Meethal KV. Identification of plant extracts expressing trypsin inhibitor. Acta Biologica Indica. 2014; 3(1):522-526.
12. Klomklao S, Benjakul S, Kishimura H, Chaijan M. Extraction, purification and properties of trypsin

inhibitor from Thai mung bean (*Vigna radiata* (L.) R. Wilczek). Food chemistry. 2011; 129(4):1348-1354.
13. Fang EF, Ng TB. A trypsin inhibitor from rambutan seeds with antitumor, anti-HIV-1 reverse transcriptase, and nitric oxide-inducing properties. Applied biochemistry and biotechnology. 2015; 175(8):3828-3839.
14. Remya PP, Sameena U, Parambath BP, Meethal KV. Identification of plant extracts containing protease inhibitors against the gut proteases of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). Acta Biologica Indica. 2013; 2(2):451-455.
15. Lakshmanan AP, Meethal KV. Jackfruit seed and areca nut extracts inhibit gut protease activity of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). Acta Biologica Indica. 2012; 1(1):129-131.