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Toxicity of plant extracts containing trypsin inhibitor to the larvae of *Aedes aegypti*

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Abstract
Mosquitoes are vectors of many pathogens and can transmit a range of potentially fatal diseases. Some of these diseases spread via mosquitoes belonging to the genus Aedes include Dengue, Chikungunya, Yellow fever, Zika etc. Conventional methods of mosquito control are not always completely successful. Therefore new strategies need to be adopted for mosquito control. Plant protease inhibitors are promising candidates as they prevent larval growth by inhibiting gut proteases of mosquito larvae. In this study we screened 15 plant extracts containing trypsin inhibitors for their toxicity to the larvae of *Aedes aegypti*. Leaf extracts from *Lawsonia inermis* showed the highest mortality with 100% mortality after 48 hours in second, third and in fourth instar larvae. The treatment with extract from *Annona muricata* leaf led to 100, 96 and 66% mortality after 48 hours in second, third and fourth instar larvae respectively. After Proteinase K treatment of *Lawsonia inermis* leaf extract, a reduction in the in vitro inhibition of larval gut protease activity from 82 to 6% and a decrease in mortality from 80 to 50% were observed compared to untreated. This revealed that the mortality, at least in part, is due to the presence of a protenaceous plant protease inhibitor in the extract. Expressing the gene coding for such protease inhibitors in micro organisms or algae upon which the mosquito larvae feeds will serve as an alternate method for control of mosquito population.

Keywords: trypsin inhibitor, *Aedes aegypti*, *Annona muricata*, *Lawsonia inermis*, mosquito control

1. Introduction
Human health is affected by many factors including diseases. According to World Health Organization, vector-borne diseases like Malaria, Dengue, Yellow fever, Chagas disease etc account for more than 7,00,000 deaths per year. Mosquitoes transmit many anthropogenic diseases which require no animal host and are transmitted from human to human by mosquito vectors. In the tropical and sub tropical areas of the world, approximately 45% of the world's population is susceptible to Malaria which is transmitted by Anopheles species of mosquitoes. There are about 41 genera of mosquitoes with approximately 3500 species [1]. Anopheles, Culex and Aedes are the most important genera among them as they transmit many deadly diseases.

Aedes mosquitoes are distinctive visually because of their black and white marking on the abdomen and on legs. They transmit diseases like Dengue, Chickungunya, Yellow fever, Zika virus etc. Among the many species, *Aedes aegypti* and *A. albopictus* are very important. *Aedes aegypti* originally from Africa, now globally distributed in tropical and sub tropical regions [2]. *Aedes albopictus* also called as Asian tiger mosquito or Indian forest mosquito, though a forest species is rapidly spreading in urban areas.

Various mosquito control strategies are adopted to control mosquitoes and these include physical methods, such as use of traps, mosquito bats, nets, repellant creams etc, chemical methods such as insect growth regulators, synthetic organophopsphates, organochlorine compounds and biological control methods like the use of natural enemies like predator fishes, bacteria, fungi and viruses. The harmful effects of using synthetic chemical insecticides including development of resistance in mosquitoes, environmental pollution and health hazards led to the current interest in search for plant based insecticide products that are

environmentally safe and effective. Over 2000 species of plants have shown insecticidal activity [3]. Some of the plants that have been tested against mosquito larvae include *Cleome viscose*, *Ocimum basilicum*, *Vitex negundo*, *Delonix regia* and *Oligochaeta ramose* etc [4, 5].

Plant protease inhibitors targeting the gut proteases of mosquitoes is a new alternative. Plant protease inhibitors are mainly proteins and bind to insect gut proteases and thereby inhibit the enzyme [6]. The inability to digest the ingested proteins leads to deficiency of amino acids and affect their growth and survival [7]. Amrutha *et al* have showed that serine proteases are the major proteases in the gut of Culex mosquito larvae [8]. In the genome of *A. aegypti* mosquito hundreds of serine protease- like genes are present but only five of them are expressed in the mid gut [9]. Trypsin and chymotrypsin are the most abundant digestive enzyme present in *A. aegypt* larval mid gut [10, 11]. Protease inhibitor purified from the seed extract of *Adenanthera pavonia* showed significant reduction in the midgut protease activity as well as survival of *A. aegypti* larvae [12]. Thus protease inhibitors inhibiting the gut proteases of mosquito larvae can be of use in the control mosquito population. In the present study we examined the toxicity of plant extracts containing trypsin inhibitor against the larvae of *Aedes aegypti.*

2. Materials and Methods

The eggs of *Aedes aegypti* were obtained from Bio-control Research Laboratory of PCL India Ltd, Bangalore. They were cultured in a tray containing dechlorinated tap water. The eggs hatched out into larvae within two or three days and were fed with yeast granules. The second, third and fourth instar larvae were used for experiments.

2.1 Preparation of Trypsin

Trypsin solution was prepared by dissolving 2.5 mg bovine trypsin in 2.0 ml of 1 mM HCl and frozen until use.

2.2 Collection and preparation of plant extracts

Plants extracts containing trypsin inhibitor was used for the study. Plant parts were collected from Calicut university Botanical garden. Leaves and seeds were used for the experiments. Plant parts were soaked in bicarbonate buffer of pH 9.0 (1g tissue/3ml buffer). Leaves were soaked for 2-3 hours and seeds were soaked overnight. The extract was prepared by homogenizing the plant parts in bicarbonate buffer and centrifuged at 9400 x g at 4 ˚C for 10 minutes. The supernatant was collected and frozen until use.

2.3 Preparation of gut extract from larvae for protease assay

Fourty larvae were collected and cold anaesthetized and dissected out the gut, homogenized in 100 ul bicarbonate buffer pH 9.0 and centrifuged at 9400 x g for 10 minutes at 4 ˚C. The supernatant was collected and protease assay done as described in protease assay using 5µl of gut extract.

2.4 Protease assay

In order to check the protease activity in the gut extract protease assay was conducted. For the protease assay, 5µl of trypsin (1.25 µg/µl) or 5ul of gut extract was incubated with 5.2µl azocasein (15 µg/µl), at 37 ˚C for 30 minutes in a total volume of 20.2µl bicarbonate buffer pH 9.0. The reaction was stopped by adding 80µl of 5% TCA and was centrifuged at 9400 x g at 4 ºC for 10 minutes. Fifty micro liter of the supernatant was mixed with 150µl of 0.5M NaOH and the absorbance was measured at 440nm in a micro plate reader.

2.5 Protease inhibition assay

In protease inhibition assay the plant extract was pre incubated with gut extract for 10 minutes before adding substrate and proceeded the same way as described in protease assay.

2.6 Proteinase K treatment

To identify the proteinaceous nature of the protease inhibitor in extract, proteinase K digestion of the extract followed by protease inhibition assay was done. For this, 90 µl plant extract was incubated with 10 µl of proteinase K (2.3µg) at 56 °C over night. The proteinase K was inactivated by heating the mixture at 96 °C for 10 minutes and then the mixture used for protease inhibition assay.

2.7 Protein estimation

The protein concentration of plant extracts were estimated by Bradford's dye binding method using Bovine serum albumin as standard [13].

2.8 Study of gut protease activity of larvae treated with plant extract

Sixty 4th instar larvae were incubated with plant extracts at a concentration of 15% (v/v) for two hours. The live larvae were collected and cold anaesthetized and dissected out the gut, homogenized in bicarbonate buffer pH 9.0 and centrifuged at 9400 x g for 10 minutes at 4 ˚C. The supernatant was collected and protease assay done using 5µl of gut extract as described earlier. .

2.9 Study of effect of plant extracts on mortality of *Aedes aegypti* larvae

Second, third, and fourth instar larvae were used for the experiments. Larvae of the same instars were introduced into de-chlorinated water and toxicity testing was done as per WHO protocol (WHO, 2005). A control was also kept without the addition of plant extract. Yeast granules were given as food. A total of 15 different plant extracts were used for the experiments. Number of live and dead larvae noted after 24 and 48 hours.

2.10 Statistical analysis

The statistical analysis was done using R program and values are mean of 3 separate experiments with each experiment done in duplicate.

3. Results and Discussion

Trypsin like and chymotrypsin like serine proteases are the two major classes of secreted endoproteases present in the midgut of the blood fed *A. aegypti* [14-18]. Trypsin-like enzymes are also the major proteases in the gut of mosquito larvae. Therefore trypsin inhibitors can be used to inhibit larval gut proteases and may result in larval mortality. In the present study we examined the toxicity of trypsin inhibitor containing plant extracts to the larvae of *A. aegypti.*

3.1 Trypsin inhibition by plant extracts

Fifteen different plant extracts containing trypsin inhibitors were used after assessing their trypsin inhibition using

azocasein as substrate. The highest percent of trypsin inhibition was shown by *Carica papaya* leaf extract (92.7±2.2%), followed by *Globba sessiliflora* leaf extract (87.9±0.6%), *Santalam album* leaf extract (87.7 ± 0.9) and *Lawsonia inermis* leaf extract (81.7 ± 1.3) (Table 1). Out of 15 extracts 9 of them showed more than 50% inhibition of trypsin activity (Table 1). Azarkan *et al* also reported 24 kDa Kunitz type protease inhibitor from *Carica papaya* [19]. A papain inhibitor also reported from latex of *Carica papaya* [20]. Trypsin inhibition was shown by the ethanolic tuber extract from *Globba bulbifera* [21] but no trypsin inhibitor was reported from the *Globba sessiliflora* leaf extract.

Table 1: Inhibition of trypsin by plant extracts

Sl. No	Name of plant	Plant part used	Percentage inhibition Mean ±SE
1	*Carica papaya*	Leaf	92.7 ± 2.2
2	*Globba sessiliflora*	Leaf	87.9 ± 0.6
3	*Santalam album*	Leaf	87.7 ± 0.9
4	*Lawsonia inermis*	Leaf	81.7 ± 1.3
5	*Simarouba glauca*	Leaf	65.2 ± 0.2
6	*Plectranthus amboinicus*	Leaf	54.9 ± 1.5
7	*Annona muricata*	Seed	51.8 ± 1.3
8	*Murraya koenijii*	Leaf	49.9 ± 0.3
9	*Tectona grandis*	Leaf	49.5 ± 0.1
10	*Annona muricata*	Leaf	44.9 ± 1.3
11	*Catheranthus roseus*	Leaf	42.4 ± 1.4
12	*Calotropis gigantea*	Leaf	41.4 ± 1.1
13	*Datura stramonium*	Seed	37.6 ± 1.8
14	*Kopsia fruticosa*	Leaf	35.6 ± 1.1
15	*Averrhoa carambola*	Seed	33.7 ± 1.8

Leaf extract from *Santalum album* showed 87.7 ± 0.9% trypsin inhibition (Table 1). Divya *et al* reported trypsin inhibitor from *Santalum album* leaf extract [22]. *Lawsonia inermis* and *Simarouba glauca* leaf extracts gave trypsin inhibition of 81.7± 1.3% and 65.2 ± 0.2% respectively (Table 1). A Kunitz type trypsin inhibitor was purified and characterized from the seeds of *Lawsonia inermis* [23]. Santhosh *et al*., reported antioxidant activity from *Simarouba glauca* extract [24]. To our knowledge no protease inhibitor is reported from the *Simarouba glauca* but anti-feedent and insecticidal activity was reported from the bark and leaves [25]. Leaf extract from *Plectranthus amboinicus* showed 54.9 ± 1.5% trypsin inhibition and Divya *et al* also reported trypsin inhibitory activity from the same plant [22]. The seed extract and leaf extract of *Annona muricata* showed trypsin inhibition of 51.8 ± 1.3% and 44.9 ± 1.3% respectively (Table 1). Protease inhibitor activities were reported from the seed extract of *Annona muricata* [26].

Leaf extract of *Murraya koenijii* showed 49.9 ± 0.3% trypsin inhibition (Table 1) and Shee and Sharma purified a 27 kDa trypsin inhibitor from the seeds of *M. koenijii* [27]. *Tectona grandis*, *Catheranthus roseus* and *Calotropis gigantea* leaf extracts showed 49.5± 0.1%, 42.4 ±1.4% and 41.4± 1.1% trypsin inhibitions respectively (Table 1). Prakash *et al*., reported trypsin inhibition from the leaf extracts of *Tectona grandis* and *Catheranthus roseus* leaf extract [28]. Cystein protease inhibitor was reported from the *Calotropis procera* latex and it showed antiplasmodial property in mice [29]. Seed extract from *Datura stramonium* showed 37.7 ± 1.8% inhibitions towards trypsin. Divya *et al* also reported the presence of trypsin inhibitor from the seeds of *Datura stramonium* [22]. The extract from the leaf of *Kopsia fruticosa* and seeds of *Averrhoa carambola* inhibited trypsin to the extent of 35.6 ± 1.1% and 33.7 ± 1.8% respectively (Table 1). High total alkaloid content was observed in *Kopsia fruticosa* leaf extract by Wong *et al* [30]. To our knowledge no trypsin inhibitor was reported from *Kopsia fruticosa* and *Averrhoa carambola*. Thus out of the 15 extracts used in the study, trypsin inhibition was reported for the first time for plant extracts from *Globba sessiliflora*, *Simarouba glauca* , *Calotropis gigantea*, *Kopsia fruticosa* and *Averrhoa carambola*.

3.2 Toxicity of trypsin inhibitor containing plant extracts to larvae of *Aedes aegypti*.

Table 2: Percentage mortality of various larval instars after treatment with plant extracts

Name Of Plant	Second Instar Larvae		Third Instar Larvae		Fourth Instar Larvae	
	After 24 hours Mean±SE	After 48 hours Mean±SE	After 24 hours Mean±SE	After 48 hours Mean±SE	After 24 hours Mean±SE	After 48 hours Mean±SE
Lawsonia inermis	100±0.0	100±0.0	66.6±6.6	100±0.0	80±0.0	100±0.0
Annona muricata	100±0.0	100±0.0	80±10	96.6±3.3	53.3±6.6	66.6±3.3
Annona muricata (seed)	86.6±6.6	93.3±6.6	73.3±6.6	96.6±3.3	53.3±3.3	66.6±3.3
Calotropis gigantea	0±0.0	0±0.0	23.3±3.3	36.6±3.3	13.3±8.8	20±10

Toxicity of the plant extracts containing trypsin inhibitor were studied using three different larval instars. Second, third and fourth instar larvae were used for the toxicity studies and mortality rates were observed after 24 and 48 hours of exposure to the plant extract. The extracts from *Annona muricata* leaf, *Annona muricata* seed and *Lawsonia inermis* (henna) leaf showed very high mortality in second, third and fourth instar *Aedes aegypti* larvae (Table 2). After 24hrs of exposure of second instar larvae to extracts from *Annona muricata* leaf, *Annona muricata* seed, *Lawsonia inermis* leaf led to mortaility of 100%, 86.7%, and 100% respectively. Mortality increased to 93% for *Annona muricata* seed extracts

after 48hrs of exposure (Table 2).

For third instar larvae, the highest mortality was observed after 24hrs for *Annona muricata* leaf extracts (80%) followed by *Annona muricata* seed extracts (73%), *Lawsonia inermis* leaf extracts (66%) and *Calotropis gigantea* leaf extracts (23%). The percentage mortality increased to 100% after 48hrs for *Lawsonia inermis* leaf extract while that of *Annona muricata* leaf extracts and *Annona muricata* seed extracts the mortality rates increased to 96% (Table 2). Sesanti *et al*. observed larvicidal effects of *Carica papaya* leaf and seed extract on Anopheles species of mosquitos [32].

When fourth instar larvae were treated with plant extracts, the highest mortality was shown by *Lawsonia inermis* leaf extract (80%) followed by *Annona muricata* leaf extract (53%), *Annona muricata* seed extract (53%), *Calotropis gigantea* leaf (13%) after 24hrs of exposure. The mortality in *Lawsonia inermis* leaf extract increased to 100% and that of *Annona muricata* leaf extract and *Annona muricata* seed extract both increased to 66% after 48 hours. Parthipan *et al* also reported the larvicidal activity of the *A. muricata* seed kernal extracts [33]. Komansilan *et al*. identified and isolated biolarvicide against *Aedes aegypti* from the seeds of *A. muricata* [34]. Other remaining ten plant extracts did not produce any mortality in the larvae. For example *Carica papaya* leaf extract with 93% trypsin inhibition and *Santalam album* leaf extract with 88% trypsin inhibition did not produce any mortality in the larvae (data not shown). Trypsin inhibition by the plant extract cannot be directly correlated with mortality in many cases. This may be due to augmentation of the mortality by other components present in the extract or loss of activity of the inhibitor in the gut or production proteases insensitive to the inhibitor by the larvae.

The protein concentration of extracts used from *Annona muricata* seed, *Annona muricata* leaf and *Lawsonia inermis* leaf which produced considerable mortality were found to be 24.6±5.97, 36.93±3.04, 16.11±4.64 µg/ml respectively (Table 3)

Table 3: Protein concentration of the plant extracts showing high mortality

Name of Plant	Plant Part Used	Protein Concentration (µg/µl) Mean ±Se
Annona muricata	LEAF	24.6 ± 5.97
Annona muricata	SEED	36.93 ± 3.04
Lawsonia inermis	LEAF	16.11 ± 4.64

3.3 Protease activity in the Gut of Larvae exposed to plant extracts

In order to see whether protease activity in the gut of the larvae was affected by the treatment, gut of larvae exposed to plant extracts were dissected out and protease activity of the gut was assessed. In comparison to the protease activity in the gut of control larvae, larvae treated with *Annona muricata* leaf extract showed only 52% protease activity after exposure to the extract for 12 hours compared to control. In *Annona muricata* seed extracts, the protease activity was reduced to 11% after the treatment compared to control. For *Lawsonia inermis* leaf extracts, treatment led to a gut protease activity of 52% compared to control (Table 4).

Table 4: Protease activity in the gut of larvae exposed to extract containing plant protease inhibitors

Name of Plant	Plant Extract Used	Gut Protease Activity After Treatment With Plant Extract (%) Mean ±Se
Annona muricata	LEAF	52.4 ± 3.3
Annona muricata	SEED	11.1 ± 2.4
Lawsonia inermis	LEAF	51.8 ± 13.1

3.4 Toxicity of plant extracts to fourth instars *Aedes aegypti* larvae after proteinase K treatment

In order to assess whether protein protease inhibitors contribute to the mortality, the extract was digested with Proteinase K. For *Annona muricata* leaf extract the Proteinase K treatment did not change the protease inhibition significantly and the mortality rate remained the same (Table 5) indicating that that the inhibitor in the extract is not a protein or not degraded by Proteinase K treatment. Rodrigues *et al*. reported the larvicidal effects of *A. muricata* seed extract against *Aedes aegypti* and *A. albopictus* and Annonacin as the major component [35]. But it remains to be seen whether it is the same inhibitor in the leaf showing the larvicidal effect.

Table 5: Percentage inhibition and larval mortality after proteinase K treatment of plant extract

Name of Plant/Plant Part Used	Percentage Inhibition of Untreated Plant Extract (Mean±Se)	Percentage Inhibition of Extract Treated With Proteinase K (Mean±Se)	Percentage Mortality of Untreated Extract (After 24hr)	Percentage Mortality of Extract Treated With Proteinase K (After 24hr)
Annona muricata (leaf)	44.9 ± 1.30	54.8±0.8	53.3±6.6	55±5.0
Lawsonia inermis (leaf)	81.7± 1.35	5.5±0.1	80±0.0	50±0.0

For *Lawsonia inermis* leaf extract, after Proteinase K treatment, the inhibition decreased from 81.7± 1.4 to 5.5% and the larval mortality decreased from 80 to 50% (Table 5) indicating that, in part the proteinaceous inhibitor accounts for larvicidal effect of the extract. Das and Mariappan also observed larvicidal activity of *Lawsonia inermis* leaf extract against *Culex quinquefasciatus* [36]. A 19 kDa trypsin inhibitor was reported from the seed extract of *Lawsonia inermis* [37]. But whether the larvicidal effect is due to the same protease inhibitor also present in the leaf extract remains to be examined. Many protease inhibitors purified from plant extract showed adverse effects on the larval development and midgut proteases of *A. aegypti*. Purified trypsin inhibitor from the seed extract of *Cassia leiandra* inhibited the larval midgut proteases of *Aedes aegypti* and it impairs the larval growth and its survival [38]. Pontual *et al*. reported the larvicidal properties of a trypsin inhibitor purified from *Moringa oleifera* flower extract against *Aedes agypti* [39]. Cloning the gene and expressing such protease inhibitors in microorganisms or algae upon which the larvae feeds will be

useful for producing genetically modified organisms for the control of mosquito population.

4. Conclusions

In the present study we screened fifteen plant extract containing trypsin inhibitors of which eight of them showed above 50% trypsin inhibition. To our knowledge this is the first report of trypsin inhibitor from plant extracts from *Globba sessiliflora*, *Simarouba glauca*, *Calotropis gigantean*, *Kopsia fruticosa* and *Averrhoa carambola.* We examined the toxicity of fifteen plant extracts containing trypsin inhibitor against the larvae of *Aedes aegypti*. It was found that, exposure to extracts from *Lawsonia inermis* leaf extracts showed the highest mortality in all larval stages, 100%, in second instar, third instar and fourth instar after 48 hours followed by *Annona muricata* leaf and *Annona muricata* seed extract. We also observed that the exposure of larvae to extracts from *Lawsonia inermis* leaf, *Annona muricata* leaf, *Annona muricata* seed resulted in decreasing the gut protease activity to 52%, 52% and 11%, respectively. In the case of *Lawsonia inermis* leaf extract, the mortality in part is due to the plant protease inhibitor in the extract, as there is a decrease in protease inhibition accompanied by decrease in mortality from 80 to 50% on Proteinase K treatment of the extract. Cloning the gene and expressing such plant protease inhibitors in microorganisms or algae upon which the larvae feeds will be useful for producing genetically modified organisms for the control of mosquito population.

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