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SHORT COMMUNICATION

Larvicidal effects of a flabelliferin saponin from palmyrah flour on dengue mosquito *Aedes* sp.

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Abastract: The ethanolic extractives of palmyrah (*Borassus flabellifer* L.) flour were toxic to the dengue mosquito (*Aedes albopictus* and *Aedes aegypti*) larvae with LD_{50} value of 60 mg/L to 76 mg/L depending on larval molting stage. A single compound was isolated from the ethanolic extractives by medium pressure liquid chromatography, by bioassay directed fractionation at a fold purification of >1000. This compound had the chemical characteristics of a saponin belonging to the group flabelliferin. It had a molecular weight (MW) of 868 and confirmed 1 Glc and 2 Rha by mass spectroscopy. The pure compound was found to be lethal to dengue mosquitoes. The lethality is due to the above saponin causing a physical barrier visible only by froth on shaking. The saponin can also lower the surface tension of water surface. Thus, the *Aedes* mosquito larvae cannot hang on to the water surface to expose their breathing siphons into the air. *Culex quinquefasciatus*, with long breathing siphons is not effected. The application of these compounds to free aquatic environment is limited as the compounds showed toxicity to a lung breathers *Micoglanis iheringis* (anntena catfish) and also for a gill breather *Poecilia reticulata* (guppy). However, it could be used as a remedy in controlling dengue mosquito larvae in vases, containers and other discard household utensils in which this dengue mosquito often breed.

Keywords: *Aedes albopictus, Borassus flabellifer* L., flabelliferins, lethality, mosquito larvae, palmyrah flour.

INTRODUCTION

Palmyrah flour, which is obtained from dried seed shoots of *Borassus flabellifer* L. is used in the North East of Sri Lanka as a substitute for wheat and rice flour. A neurotoxic factor was reported in palmyrah flour as far back as 1971[1].This finding has been supported by many other workers[2, 3]. Another study claimed early pupation effects and lethal effects on *Aedes albopictus* mosquito larvae[4]. *Aedes* sp. are the vectors of the deadly disease dengue, especially in urban areas. Compounds having lethal effects on these mosquito larvae can be helpful in controlling the dengue mosquito. It has been proven that the large numbers of bioactivities of both the flour and fruit pulp of palmyrah are due to a family of 16 - 20 steroidal saponins called flabelliferins[5, 6]. Flabelliferins usually contain â-sitosterol as the aglycone and differ from each other depending on the carbohydrate moiety attached to the aglycone[6].

In this study an attempt was made to investigate (i) the effects of palmyrah flour extractives on early pupation and lethality to dengue mosquito larvae[4] (ii) to purify and identify the compounds responsible for lethality (iii) to investigate the effects of the above compounds on other aquatic organisms (iv) to examine the ultra-structures of treated and untreated mosquito larvae and to determine whether chromosomal damages have occured.

METHODS AND MATERIALS

Palmyrah flour: Palmyrah flour samples were obtained from Kalpitiya through the Palmyrah Development Board. Dried flour of palmyrah shoots were packed in plastic bags and stored at room temperature until analysis.

Extraction and separation of palmyrah flour compounds: An Ultra Turrax T-25 homogenizer was used in extraction of palmyrah flour (10 g) constituents with ethanol (50 mL). Medium pressure liquid chromatography (MPLC) apparatus with FMI lab pump (QD) and Chromatotron (Harrison Research) were employed in the separation of flabelliferins as previously described using petroleum ether, dichloromethane, ethyl acetate methanol

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and water gradient systems[7-10]. Separated fractions were subjected to normal and reverse phase (thin liquid chromatography) TLC and pooled together using anisaldehyde as spray reagent. Analytical grade solvents were used in the separations and traces of solvent were removed by rotary evaporation under reduced pressure before the final freeze-drying process to form solid powders. The solid powders dissolved in water were used for bioassays.

Mosquito larvae: Mosquito larvae were obtained from prepared sites in the University of Sri Jayewardenepura Gangodawila, Kohuwala and Horana areas. Mosquito larvae were separated using identification keys based on characteristic hair pattern, structure of the siphon, pectin teeth and molting stages[11-13]. They were grouped randomly for trials. Controls were observed upto the emergence of the adult mosquitos to confirm the identification of the species. Larvae were fed with "Serilac" infant milk powder. *Aedes albopictus* larvae were identified from a single upper and lower head hair and abdominal hairs 2-2-2-1 (Segment III – VI) in 3rd and 4th instar larval stages[13]. The identity of larvae and adults of *Culex quinquefasciatus* and *Aedes aegypti* were confirmed before being used in bioassays.

Observation of lethality: Solvent free methanolic extractives, which were rotor evaporated to dryness and freeze-dried at each stage in different concentrations, were dissolved in water. Equal numbers of mosquito larvae (n = 8) were placed in boiling tubes in the tests and controls. Time taken to result death of larvae were observed. Number of dead larvae vs. time graphs were plotted and LD_{50} values for different larval stages were obtained. Since this is an "all or none" effect of death SD cannot be calculated.

Microscopic studies: The Olympus BX50 light microscope and the TEM-1200 EXII (Jeol make) transition electron microscope were used to observe the mosquito larvae. *A. albopictus* mosquito larvae were exposed to methanol extractives of palmyrah flour and immediately after death, the larvae were transferred to 2% glutaraldehyde. Phosphate buffer was used to remove glutaraldehyde and then the samples were treated with OsO_4 and subsequently washed with alcohol in a stepwise manner to remove OsO_4. A latex embedding was carried out. After sample processing was complete, suitable sections were selected using a light microscope. Finally ultra sections were obtained using a diamond head microtome. The sections were mounted on the Cu grid mesh and stained using uranyl acetate. These samples were subjected to transition electron microscopy and ultra structures of the test and the control larvae were compared.

Extraction of palmyrah flour from different solvents: Palmyrah flour (5 g) was stepwise extracted using petroleum ether (60°-80°C), methylene chloride, ethyl acetate, ethanol and water using 25 mL in each case and homogenised at 17500 rpm for 3 min. Supernatants were separated by 3000 rpm centrifugation and solvents were evaporated. Before weighing, samples were freeze-dried. Compounds were subjected to the larval assays and froth test.

Medium pressure liquid chromatography(MPLC): Ethanolic extracts (131 mg) obtained from 10 g palmyrah flour were mixed with silica in 1:3 ratio. This pre-adsorbed silica was packed in to MPLC (FMI Lab Pump QD) column and clamped against gravity. Petroleum ether: methylene chloride: ethyl acetate: methanol gradient with a flow of 18 mL/min was employed in eluting the compounds. Samples were pooled according to the well-established TLC patterns observed using butanol: ethanol: ammonia; 7:3:4 with anisadehyde spray reagent[6]. On repeating MPLC with smaller volume fractionations, a single compound was obtained which was highly active against dengue mosquito larvae. A chromatatron was employed with an isocratic solvent system of methanol water (1:1) to remove the UV binder[6]. The active compound was further purified by re-crystallization five times. A water, ethanol and ethyl acetate mixture was used as solvent system and ethyl acetate was added drop wise to obtain cloudiness. Fine crystals were obtained after keeping overnight at low temperature. Crystals were separated using suction filtration and subjected to vacuum drying and finally subjected to a freeze drying process.

Spectroscopy: Purity of the dissolved compound in D-DMSO was ensured by monitored recoding 300 MHz spectrums from Bruker 300 spectrophotometer. Mass spectrum was obtained by using 1mg samples dissolved in MeOH and using Bruker microTOF mass spectrophotometer.

Froth test: Fractionated palmyrah flour extractives were tested for froth forming ability. A sample of 1mg/mL concentration was shaken manually for 1 min and the froth height was recorded in milimetres. The samples were left at room temperature to monitor the stability of froth formed.

Effects on aquatic organisms: Micoglanis iheringis (Antenna catfish) and *Poecilia reticulata* (guppy) (total n = 18) were divided into test and control samples. The test group was treated with 260 mg/L concentrations of solvent free methanolic extractives of palmyrah flour and the effects were observed. The effects of the above extractions on *Hydrilla* plants were also observed.

RESULTS

Experiments to study the increased rate of pupation were replicated out ten times using different larval stages. No enhancement of the rate of pupation was observed. There was an indication of lowering rate of pupation but it was not statistically significant. Although we employed more than 1300 mosquito larvae in our experiments, only few representive selected tables of results are given to illustrate the effects.

The weights of extractives obtained by the sequential extraction of 5 g of the Kalpitiya palmyrah flour with petroleum ether, methylene chloride, ethyl acetate, ethanol and water resulted in 7 mg, 28 mg, 1020 mg, 32 mg and 1101 mg respectively. Although higher yield extractives were obtained using ethylacetate and water, the ethanolic extractive was the most lethal to *A. albopictus* mosquito larvae (Table 1). All solvent free extractives except the water extract resulted in retardation of active locomotive motion of mosquito larvae. Only the ethanolic extractive resulted in a long-lasting froth (5 mm) for 1 mg/mL concentration of extractives. The LD_{50} values determined for different mosquito larvae and different larval stages are shown in Table 2. Another experiment indicated that the solvent free methanolic extractives are lethal to 4th instar *A. albopictus* even at a concentration as low as 6 mg/L. There was no effect on the filarial mosquito *C. quinquifaciatus* larvae at a concentration as high as 333 mg/L.

Lethality observed with the separated fractions of palmyrah flour is given in Table 3. The MPLC fractions 33 to 43 contained flabelliferins, which are steroidal saponins[6,7]. These fractions, after evaporation of solvents appeared as white crystals and were lethal to *A. albopictus* larvae to different extents. Fraction 38, when subjected to the froth test resulted in 17 mm high froth at 500mg/L concentration. Fraction 38, resulted from ethanolic extract was 9 mg after MPLC. This resulted in a 1111 fold purification based on palmyrah flour. The mass spectrum for the mosquito larvicidal compound is given in Figure

Table 1: Effects of different solvent extractions on 3rd instar *Aedes albopictus* larvae

Solvent	No. of larvae at time 0	No. of larval deaths 24 hours	48 hours	72 hours
Control 1	9	0	0	0
Control 2	9	0	0	0
Petroleum ether (60 –80°C)	9	3	5	5
CH_2Cl_2	9	4	7	7
Ethyl acetate	9	2	3	3
Ethanol	9	8	9	9
H_2O	9	0	0	0

Note: 330 mg/L concentrations were prepared from different solvent extractives. The experiment was repeated once.

Table 2: LD_{50} obtained for different mosquito larvae, at 3rd and 4th instar larval stages.

Mosquito	Larval stage	LD_{50} *
Aedea albopictus	3rd instar larvae	68.8 mg/L
	4th instar larvae	75.8 mg/L
Aedes aegypti	3rd instar larvae	N/D
	4th instar larvae	60.0 mg/L
Culex quinquifaciatus	3rd instar larvae	No death effect
	4th instar larvae	was observed

Note: Crude ethanolic extracts were used.
* LD_{50} was determined by graphical method for each point n = 8 larvae.
N/D – not done.

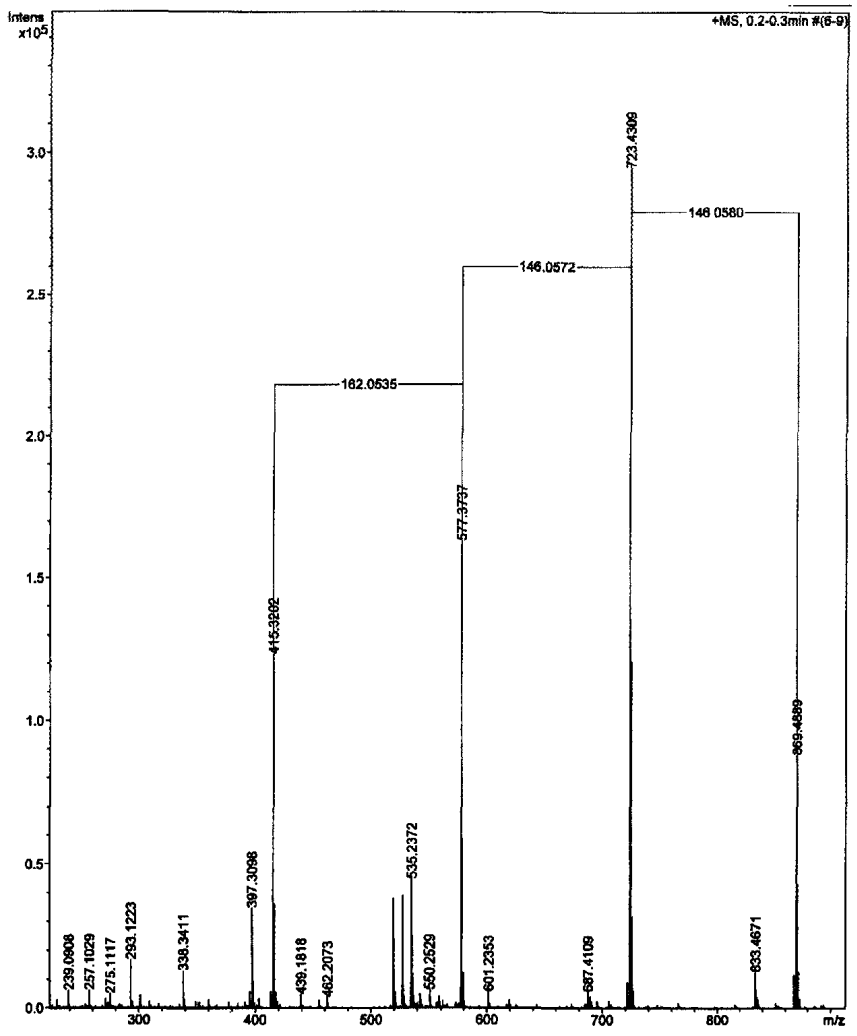

Figure 1: MicroTOF mass spectrum obtained for mosquito larvicidal compound

1. It resulted in a peak at MW of 868 (M^++ H at 869). The peak at 415 indicated the aglycone. Fragmentation pattern attached to the aglycone resulted Glc Rha Rha moieties with linear sequence.

Addition of solvent free methanolic extractives of palmyrah flour at the level toxic to mosquito larvae in water affected the behavior of the selected fish instantly. Time taken for death was less than 10 minutes for *Poecilia reticulata* (guppy) a gill breather and less than one hour to *Micoglanis iheringis* (Antenna catfish), which is a lung breather. No effects of the above extractives were observed on *Hydrilla*.

DISCUSSION

From the mosquito larvae experiments we could not obtain any evidence for stimulation of pupation from palmyrah flour extractives[4]. Further, any chromosomal changes, that give a clastrogenic effect which may induce pupation was ruled out by the electron microscopy. Absence of chromosomal changes confirmed the absence of any clastrogenic effect.

Apart from that we observed a slight insignificant delay of pupation at low concentrations. It is well known that the rate of pupation depends on the water temperature and the nutritional state of larvae[11]. During our experiment the water temperature varied between 28 - 35 °C for both test and control. Thus it is possible to speculate that the larvae food intake is affected at low concentrations of the compound.

Results of the present study indicate that the ethanolic extractive had the highest larvaecidal activity with LD_{50} ranging from 60 mg/L to 76 mg/L depending on the larval stage. The purified compound resulted from this extract (fraction 38) was shown to have the highest activity against mosquito larvae (Table 3). The chemical characteristics of this compound indicated that the compound is likely to be a saponin. Using MS data this compound was confirmed to be a steroidal triglycoside.

The compound was lethal to *A. albopictus* and *A. aegypti*, but was not effective against filarial mosquito larvae. The lethality of the compound to larvae is due to the effect on the breathing process. It is known that saponins form a layer of compound on the surface of the water. It was therefore likely that the dengue mosquitos having a shorter breathing siphon (< 1mm) are unable to penetrate this saponin layer. For contrast, the filaria mosquito *C. quinquefasciatus*, have a much longer (~ 2 mm) breathing siphon, and thus they can survive even at higher concentration of the compounds. However, a question arises as to how such a small quantity of extractive could form such a formidable surface layer, which prevents mosquito larvae from breathing. It was seen from our experiments that the addition of the compound resulted in slight turbidity in water. This shows that all the compounds including the effective compound are only partially soluble in water. After the point of saturation, it is likely that the excess flabelliferins (amphipathic compounds) are arranged on the surface of water. This is facilitated by the hydrophilic and hydrophobic nature of flabelliferins. In addition, the ability of flabilliferins to bind with other molecules may also facilitate the enhancement of the thickness of the above layer[14]. All the above processes can lead to decreased levels of surface tension of water[15]. Thus, the death of *Aedes* mosquito larvae occurs due to non-availability of oxygen. It was observed that the 4th instar larvae required a higher concentration of the compound than the 3rd instar larvae for lethality (Table 2). This may be due to the maturity of the larvae.

These particular compounds have no visible significant effect on aquatic plants like *Hydrilla* sp. However, they have severe effects on both lung and gill breathers. When the solvent free methanol extractive was tested against *P. reticulata* (Guppy) and *M. iheringis* (Antenna catfish) and the time taken for death was less than 10 minutes and less than one hour respectively. This is possible due to the action of the saponin on the oxygen transfer apparatus of the lungs and gills of fish species[16]. Thus this compound cannot be used in a free aquatic environment. Since dengue mosquito larvae are found in clear water containers like vases, pots etc. this compound could be used effectively to kill the *A. albopictus* and *A. aegypti* larvae, which breed in household containers.

Studies on the recrystalised isolate and MS analysis of the compound confirmed it to be a steroidal saponin of molecular weight of 868 with a linear sequence of Glc-Rha-Rha. (Rha, Rha + H^+ peak at 293 peak observed eliminating a branched structure). The aglycone has a MW 414 and ^{13}C NMR indicated that it may be β-sitosterol or an isomer. *(Unpublished data: Keerthi and Patoomarathana)*. This structure can be distinguished from the branched flabelliferin F_B (MW 868) as the compound does not have the anti-microbial activity

Table 3: Effect of MPLC fractions on 3rd instar *Aedes albopictus* larvae

Fraction No.	No. of larvae at time 0	No. of larval deaths 24 hours	48 hours
Control 1	10	0	0
Control 2	7	0	0
Fr 4-13*	10	8	8
Fr 14-24	10	0	0
Fr 25-32	10	0	0
Fr 33	6	5	5
Fr 35	6	0	2
Fr 38	6	5	6
Fr 40	6	0	2
Fr 44	6	0	0
Fr 46-56	10	1	2

330 mg/L concentrations were used in this experiment.

*Fraction 4 –13 was repeated and found to be non-lethal.

Fraction 33 - 38 claimed the lethal flabelliferins.

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