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Identification of mosquito repellent compounds in *Chloroxylon swietenia DC. b*y electrophysiological and behavioural response of *Aedes aegypti*

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Abstract
Aedes aegypti is the primary vector for Dengue transmission in Tropical and sub-tropical countries. As per WHO in 2012, dengue ranks as the most important mosquito borne viral disease in the world. The aim of the study is to explore the efficiency of the repellent from *Chloroxylon swietenia* DC plant in Mosquito Control. *Chloroxylon swietenia* DC, are found in India and Sri Lanka and it has medicinal properties. The experiment is conducted to identify the active repellent compound using gas-chromatography coupled with electroantennogram recording (GC-EAD) analysis. GC-EAD active compounds include Dictamnine, Anthracene, Naphthalene, Nitroimidazole, Methyl-2-nitroimidazole.
The identified compounds reduce the landing of female *Aedes aegypti* on humans. Based on this study it may be concluded that Plant extracts are effective and safe for human beings as a personal protection against mosquitoes.

Keywords: *Chloroxylon swietenia, Plant Extract, Aedes aegypti*, mosquito, *Repellent*, GC-*EAD*

Introduction
Aedes aegypti is highly Anthropophillic in nature and the vector for the transmission of the most deadly diseases like Dengue & Chikungunya. According to WHO [1] (World Health Organization) 50–100 million people suffer from Dengue and half of the world's population live in this region where Dengue is endemic. Disease transmission is due to female mosquitoes which feed on its host to complete their gonotrophic reproductive cycle, for which it uses its olfactory senses to locate the host. The repellent was developed many years ago. Usage of repellents has become a regular phenomenon in day to day life of human beings. Repellents are the chemical compounds which drive away the mosquitoes due to chemical interactions. Many Synthetic repellents are available which have adverse effect on humans due to dermal absorption and are non-biodegradable. Plant derived green repellents [2] have no adverse effect after its usage. According to WHO [3] more than 80% of the world population depends on traditional medicine. Plants used for traditional medicines can be used to cure several diseases and infections. Plants contain many secondary metabolites that act as defense mechanisms against phytophagous insects such as mosquitoes etc. Most are grouped under broad categories like Alkaloids, terpenoids, phenolics, proteinase inhibitors, and growth regulators.
Methods, such as electroantennogram, read the response of sensory neurons in the insect antennae to attractants and repellent compounds. This has allowed greater understanding of insects' sensory systems [4, 6].In our study we have observed that the folklore and ethnic tribes in Telangana State in India had been using *Chloroxylon swietenia* DC plant leaves as a mosquito repellent for a long time. Chloroxylon *swietenia* DC is a deciduous tree, which grows up to 18-25 m tall, with pinnate leaves and thick bark. The duration of Chloroxylon swietenia DC protection time and its repellent activity was evaluated and also determined the electrophysiological responses using (GC-EAD) Gas Chromatography Coupled – electroantennogram Detection for identification of particular constituents which are responsible for antennal response of *A. aegypti* female mosquito.

Materials and Methods
Mosquito Rearing: 2nd and 3rd Instar larvae were collected so that it should adapt to the invitro environment [7-8]. Room temperature 24°-27 °C and fed with powdered yeast and powdered dog food in 1:1 ratio [9]. The larvae were nurtured until they reached the Pupa stage. Pupas are collected and kept inside the mosquito cage. Number of pupas varied as per the size of the cage. As the pupa hatched out into adult mosquitoes and they were segregated and fed with sucrose solution as basic food and female mosquitoes were allowed to feed on nude mice. F1 generation Mosquitoes are reared in Square shaped cages (45cm x 45cm) at 27 °C, RH 80% and L:D 13:11 (D/N) and fed with 10% Sucrose solution [10-11].

Plant material: Chloroxylon swietenia DC (Rutaceae family) plant leaves were collected from Vikarabad forest, Rangareddy district, Telangana, India (17° 20'0" N, 77°54'0"East). Chloroxylon swietenia DC was taxonomically identified in the Department of Botany, Osmania University, Hyderabad, India. A voucher specimen was deposited in the Department of Zoology, Osmania University.

Plant Extract: The Leaves of Chloroxylon swietenia was shade dried and dried plant material is ground to powder [12]. Hexane is used as solvent to extract compound from plant material by using Soxhlet apparatus for 12hrs at 45 ^{0}C and kept at room temperature (27 ^{0}C) till the solvent Evaporate and stored at 4 ^{0}C for further use. One gram of crude is taken with hexane to make a different concentration extract (25%, 50%, 75%, 90%) to conduct Repellent test.

Mosquito repellency test: Repellency of Extract was conducted on human volunteer by exposing hands treated with different concentration to caged mosquitoes by the following method of [13]. In this Test the volunteers skin should be washed with soap and rinsed with water [14]. Forearm is treated with different concentrations [15] of 25%, 50%, 75%, 90%. Experiment is repeated with different concentrations after every 2 hours because the repellent vapours of the concentrations might interfere in the result.
Protection Time was calculated by inserting hands in the cage of 15 intervals and observed for 3 mins. Repellency percentage is calculated.

Mosquito Repellency Test is done using the formulae[15]

$$\text{Repellency (\%)} = \frac{A-B}{A} \times 100$$

A – Number of mosquitoes landed on the control arm, B – Number of mosquitoes landed on the treated arm

Electroantennogram Recording Technique (EAG): The Antennal Response of *Aedes aegypti* female (4 days unfed mosquitoes) were tested receptivity with plant extract which was determined by using Electroantennogram Recording Technique (EAG). For electrophysiological recordings, Syntech EAG (Syntech, Hilversum, The Netherlands) was employed and the procedure for the preparation of stimulus pipettes and methodology followed as per [16] .Plant extract was diluted in (HPLC grade)n-hexane to obtain concentration of 5µg. The extract to be tested was transferred to a 2 cm^2 Whatmann filter paper and after complete evaporation of solvent, the filter paper was inserted in to 15 cm Pasteur pipette. EAG responses were recorded at continuous flow rate of 4ml and Pulse duration of 5 seconds.

Y-Tube Olfactometer: Y-Tube Olfactometer experiment is conducted following the procedure described as in [17] to identify whether the plant extracts has Repellent or attractant property .Y-tube Olfactometer (stem, 12 cm; arms, 7.5 cm at 60° angle; internal diameter 1cm Analytical Research Systems, Florida, USA)consist of two tube of 4cm in length and 1cm in diameter of which one arm is meant for test sample and other arm is for control [18] used to conduct experiment to identify the orientation responses of *Aedes aegypti* females to plant extracts. The bioassays conducted between 14.00 – 16.00hrs at 27 ± 2 °C using 50 female *Aedes aegypti* in two replicated trials uniformly.
Chloroxylon plant extract is taken on the Whatmann filter paper of the conc -5µg of extract (which triggered the response in Electroantennogram) of the size 2–cm and placed in right arm of the 'Y' tube chambers against the air stream and Hexane in the Left arm as a control. 10 No. of female mosquitoes which were starved for 4 days were introduced into the Y-tube at the entrance of the stem and the behaviour of the mosquitoes were noted. Each experiment was triplicated using 30 insects and the Olfactometer arms were flipped around (180°) to minimize positional effect after testing of 10 insects.

Gas Chromagram coupled Electroantennogram Detector Recordings (GC-EAD):
GC-EAD Recordings were performed with GC 7900 Tech comp, gas chromatograph (GC) with a column split and an extra outlet which allows for simultaneous flame ionization (FID) and electroantennogram detection (EAD) described in detail earlier[16]. The GC was fitted with a Varian capillary column (15 m X 0.25mm i.d.; 0.25) and oven temperature 60 °C to 250 °C at 7 °C/min (held for 5 min), with an injector temperature of 275 °C. Carrier gas - Hydrogen. Injections were made in the split less mode. The column effluent was split between a flame ionization detector (FID) and EAD at a ratio of 1:1.
The column of the EAD outlet was introduced into an 8 mm diameter glass tube with a constant air stream filtered through activated charcoal. (Flow 0.5 l /min). An antenna of female *Aedes aegypti* was carefully dissected along with the head and fixed between the two probes of an electrode holder with the help of a conducting gel. The parameters are followed as described in [16]. Several GC-EAD experiments were carried out using Chloroxylon leaf extract against the antennae of *Aedes aegypti* female mosquitoes to pin point the bioactive EAD peaks that had responded.

Gas Chromatography –Mass Spectrometer GC-MS:
GC-MS analysis was done by the GC Varian 450 coupled with MS Varian 240 with the Varian Capillary column (15 m X 0.25mm), an oven temperature from 50 to 280 °C at 4 °C/min and held at this temperature for 5min; inlet and interface temperatures were 250 °C and 280 °C, respectively. Helium as Carrier Gas at the flow rate of 1.0ml/min .0.2ml of sample was injected under split of 20:1. EIMS: electron energy, 70 eV.
Co-Occurring EAD peaks and antennal responses were identified [19] using GC-MS.

Results and Discussions
Mosquito repellency test: Landing Assay of mosquitoes to the Chloroxylon swietenia extracts at different concentration was observed. Protection Time (Table 1) and Repellency Percentage (Table 2) of *Aedes aegypti* against the Plant Extract was calculated

Table 1: Shows the Landing Assay and Protection Time

Plant Extract Conc %	% Repellency (mean ± SE)		Protection time (min)	
	Sample	Control	Sample	Control
25%	35.3	69.3	1.07	0.41
50%	27	64.6	1.48	0.49
75%	17	63	2.11	0.53
90%	8.33	64.33	2.38	0.57

Table 2: Shows the Repellency Percentage

Concentration of plant Extract	Repellence Percentage
25%	47.61
50%	58.20
75%	73.01
90%	91.20

EAG:

The EAG data in (Figure 1) represents Antennae response to plant extract at the range of 1.75 – 2 Mv after subtracting the Hexane response range of 0.25 mV. This shows the significant response of female *Aedes aegypti* mosquito to the plant extract.

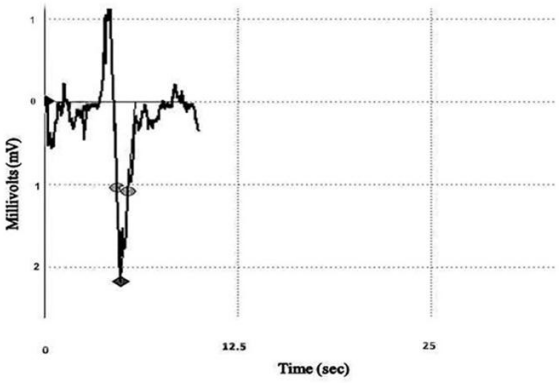

Figure 1: Antennae response of Aedes aegypti to plant extract at the range of 1.75 – 2 mV.

Fig 1: Antennae Response of *Aedes aegypti* to Plant extract at the range of 1.75-2mV

Olfactometer:

The right arm with plant extract was not preferred by female mosquitoes but few mosquitoes tend to move into the left control arm with hexane in it. Indeed, 97 % of the female mosquitoes did not select the chamber containing plant extract compared to the hexane chamber, which proved the efficacy of Chloroxylon Leaf extract has repellency properties.

GC-EAD:

GC-EAD analyses of Plant extract against female *Aedes aegypti* antennae have shown deflections. Female mosquitoes antennae consistently elicited significant and reproducible The Upper trace illustrates EAD response to eluding compounds in the Chloroxylon swietenia plant extract at particular GC retention times of 9.7,12.4,13.3 17.9,19.3,21.8,22.9,25.4 minutes towards the Plant extracts (Fig. 2). Strongest EAD responses of 0.02 mV and 2 mV were elicited by female mosquito antennae towards the 4th, 5th & 7th FID peaks eluted at 17.9,19.3 and 22.9 minutes respectively. The FID retention times (RT) of the bioactive peaks were recorded for further structural identification by GC-MS. The results provided important leads for chemical identification of expected components through GC-MS analysis.

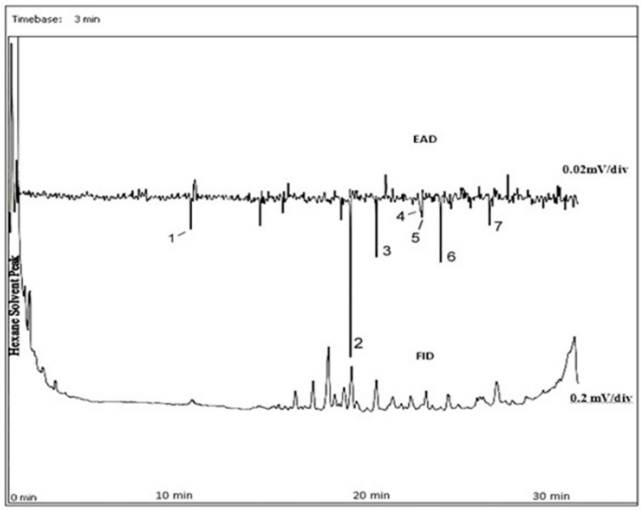

Figure 2: GC-EAD response of Aedes aegypti to plant extract at particular GC retention time

Fig 2: GC-EAD response of *Aedes aegypti* to plant Extract at particular GC Retention time

GC-MS:

Chloroxylon swietenia plant extract has been injected into GC-MS, and the compound with the retention time were compared with the GC-EAD chromatogram that the compound which triggers the response are be identified and listed out in Table 3.

Table 3: Compounds Identified using GCMS

Peak No	MW	Compound
1	127	4-Methyl-2-nitroimidazole
2	180	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol
3	199	Dictamnine
4	228	2H,8H-Benzo[1,2-b:5,4-b′]dipyran-2-one,8,8-dimethyl-
5	229	Naptho (2,3-h) quinoline
6	254	Napthalen-1(2H)-one,3,4-dihyro-2-(3-methyl-2-thenylmethylene)-
7	260	[1,1′:4′,1″-Terpheynl]-2,2″-diamine

Conclusion

This study evaluates the Chloroxylon swietenia plant extract and repellency against *Aedes aegypti* a major vector of dengue and Chikungunya in the laboratory condition. The result shows that Chloroxylon plant extract provides repellent activity against *Aedes aegypti* female mosquito. The identified compound also has the Anti-microbial [20], Anti-Fungicidal properties [21]. During experimentation, plant extract did not show any adverse effects such as skin irritation, ashes, discomfort and other allergic reactions to the volunteers. Our research is being continued for searching efficacy of effective Plant Extract with repellent properties against blood sucking mosquitoes. The further study will provide information for developing a new plant based repellent product and also alternative repellent to synthetic repellents which is Environment Friendly in nature.

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