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Larvicidal efficacy of leaf extracts of *Heliotropium Indicum* and *Mukia maderaspatana* against the dengue fever mosquito vector *Aedes aegypti*

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Abstract
Aedes aegypti is a vector of dengue fever, which is a public health problem in the world. During epidemics, emphasis is laid on the use of insecticides for controlling mosquitoes. The application of easily degradable plant compounds is considered to be one of the safest methods to control insect pests and vectors as an alternative to synthetic pesticides. A study was made to monitor the effect of plant extract, *Heliotropium indicum* and *Mukia maderaspatana*, on different larval instars and pupae of mosquito vector of *A. aegypti*. Bio-assay was performed using the solvent acetone to find out the median lethal concentration. The study indicated that essential compounds were the only chemical used for the control of mosquito larvae while extract was used as the control of adult mosquitoes. The results suggest a potential utilization of the extracts of these two plant species for the control of *A. aegypti*.

Keywords: *Aedes aegypti*, larvicidal activity, mosquito control, phyto-compounds, *H. indicum* and *M. maderaspatana*

1. Introduction
Dengue fever is endemic over large areas of tropics and subtropics. Outbreaks of dengue have repeatedly occurred in world over the last 10 years. Despite of the fact that dengue outbreaks in the tropics have been worldwide for over 200 years, it is still the most important mosquito transmitted viral disease affecting man. The etiological agent is an arbovirus and the major vector is the *Aedes aegypti* mosquito, which is found in 3600 Brazilian municipalities. While most patients are asymptomatic, reinfection with different serotypes of dengue viruses may lead to hemorrhagic fever with high mortality. During outbreaks, public health authorities in Brazil have standardized the use of aerolized pyrethroid insecticides that can cause allergies. This measure only partially controls the mosquito population since it eliminates the adult flying insects but does not eliminate the breeding places. In these breeding sites, the larvicide used is usually the organophosphate Temephos, although very slightly toxic may cause headaches, loss of memory and irritability (NICC 2003) [14].
Dengue is a viral disease that has major public health consequences in many parts of the world. The principal vector of dengue fever, including the haemorrhagic form, is the mosquito *Aedes aegypti* (WHO 2010) [35]. Presently, mosquito control primarily depends worldwide on continued applications of conventional toxic synthetic insecticides to which resistance has been reported in many areas where it is widely used (Macoris *et al*. 2003; Magalhaes *et al*. 2010) [6, 7]. This has stimulated the investigation of natural insecticides as an alternative control, focused on plant-derived compounds, including volatile chemical constituents (essential oils), as potentially bioactive substances against mosquito larvae.
Aedes aegypti (Diptera: Culicidae) is an arbovirus vector responsible for yellow fever in central and south America and in West Africa. It is also the vector for dengue hemorrhagic fever (DHF), endemic to south-east Asia, the Pacific Islands, Africa and the Americas. It is estimated that 2.5 billion people are currently at risk for dengue fever (DF), DHF, and dengue shock syndrome (DSS) (The Center for Disease Control 2007). The size and spread of the dengue pandemic, the unpredictability of the epidemic occurrences and the circulation of virulent and non-virulent strains make DHF/DSS a model for emerging infectious disease. Despite of this challenge, the development of dengue virus vaccines is still a long way to be of

any use due to several obstacles (National Institute of Allergy and Infectious Diseases 2007).

Heliotropium indicum, a very toxic herb, member of the Boraginaceae family, popularly named Indian heliotrope can be referenced under the symbol HEIN, is a rare medicinal plant which has a very long history of medicinal use, though it is little used by present-day herbalists. It is an aromatic tonic herb that stimulates the antiulcer, reduces inflammation, controls bacterial infections and promotes healing. The flowering herb, with or without the root, is anti-inflammatory, antiulcer, diaphoretic, emmenagogue, febrifuge, oxytonic and stimulant (Srinivas *et al*. 2000 and Ramamurthy *et al*. 2010) [28, 21]. The plant contains a complex of acids so called "organic acids" which stimulate white blood cell activity and speeds the healing of wounds if it is used in correct concentration. Externally it is used in the treatment of slow-healing cuts, eczema, infected toe and fingernails etc., but internal consumption can cause damage to the kidneys and uterine bleeding.

Mukia maderaspatana belongs to the family Cucurbitaceae. The plant is a tendril climber/prostrate herb (The Wealth of India 2003) [32]. The plant was reported to have activities such as hepatoprotective (Thabrew *et al*. 1995) [30], antirheumatic, antiflatulent, antiinflammatory, antidiabetic, expectorant, diuretic, stomachic, and is used for toothache and recommended in vertigo and biliousness.

In the present work alcoholic extract of *Heliotropium indicum* and *Mukia maderaspatana* leaf extracts were investigated for potential larvicidal activity. To identify and characterized the compounds of therapeutic value extracted from *H. indicum* and *M. maderaspatana.* The analytical methods chosen are Gas Chromatography/Mass Spectrometry (GC/MS). The methods were applied to characterize the infusion prepared from this plant and to make a comparison between the alcoholic extracts of the leaves.

2. Materials and Methods

2.1 Plant material and oil distillation

The medicinal plants *H. indicum* and *M. maderaspatana* were collected from in and around area of Pattukkottai, Thanjavur District, Tamil Nadu and South India. The plants were identified with the help of flora presidency, Tamil Nadu and Karnatic flora (Gamble 1967; Matthew 1983) [10] and standard references (Krtikar and Basu 1935) [5]. A voucher specimen has been preserved in our laboratory. The leaves were dried and powdered. 50 g powdered sample was extracted with ethanol using Soxhlet apparatus and concentrated *in-vacuo*. Approximately, 5 g of extract was obtained from 100 g of dried powder material. The extracts were dried in an air conditioned room at 25 °C, milled and submitted to hydro distillation in a Clevenger-type apparatus for 4 hours. The extracts were dried in anhydrous sodium sulphate, filtered, stored in amber glass bottles in a refrigerator (4 °C) for investigation of chemical constituents and larvicidal activity.

2.2 GC-MS analysis

The extracts of *H. indicum* and *M. maderaspatana* were washed with sterile distilled water and they were shade dried and powdered by using Mortar and Pestle for the alcoholic extracts (96% alcoholic solution). The sample was prepared by mixing leaves of the plant with a 50% alcoholic solution for 30 days. The infusion was also prepared by mixing parts of the plant with hot water for 20 min and the alcoholic extracts by mixing the fresh parts of the plant with a 96% alcoholic solution for 12 days.

The dry fractions (20 g) were dissolved in 75 ml of alcohol and then soaked for 24 hrs. After soaking, filtrate was collected and evaporated under liquid nitrogen. Then the filtrate was concentrated for GC-MS analysis.

For the GC-MS analysis a 30 m x 0.25 mm I.D x 1.0 µm df fused Elite-1 (100% Dimethyl Poly Siloxane) column; GC Clarus 500 Perkin Elmer gas chromatograph with Mass detector- Turbo mass gold- Perkin Elmer, Software- Turbo mass 5.1. The samples (1 µl) were introduced *via* an all – glass injector working in the split mode (10:1), with Helium as the carrier gas.

2.3 Oven temperature programme

110 deg-2 min hold, upto 280 deg at the rate of 5 deg/ 9 min hold. Injector temperature: 250 deg C. GC time – 45 mins.

2.4 MS Programme

Inlet line temperature: 200 ^{o}C, Source temperature: 200 ^{o}C, Electron energy: 70eV, Mass scan: (m/z) 45-450. MS time – 46 mins. The identification of the constituents was performed by computer library search, retention indices and visual interpretation of the mass spectra. Compounds were identified by comparing their mass spectrum to those of the database of the GC-MS (NIST 62.lib), literature (McLafferty and Stauffer 1989) [11] and retention indices (Adams 2007) [2].

2.5 Collection and storage of experimental animals

Larvae of *A. aegypti* were obtained from a permanent colony. The larvae were cultured and maintained in the laboratory at 27 ± 2 ^{0}C and 50 - 75% relative humidity. Larval forms were maintained in tray by providing dog biscuit and yeast powder in the ratio 3:1.

2.6 Test for Larvicidal activity (WHO, 1996)

The laboratory colonies of *A. aegypti* were used for the larvicidal activity. The instar II and instar IV larvae and pupae of the selected mosquito species were kept in 1 litre glass beaker and different concentrations of selected plant extract were added to find out LC_{50}.

2.7 Larvicidal bioassay

Different concentrations of extract (0.300, 0.250, 0.200, 0.150, 0.100, 0.75, 50 and 0.25 mg/ml) were prepared using distilled water. The mosquito larvae were treated with extract by using the method of WHO (1981) [33]. Ten larvae of *A. aegypti* were introduced in different test concentration of both plant extracts along with a set of control containing distilled water without any test solution. After adding the larvae, the glass dishes were kept in laboratory at room temperature. By counting the number of dead larvae at 24 hrs of exposure, the mortality rate and the median lethal concentration were obtained. Three replications were maintained for each treatment. Dead larvae were removed as soon as possible in order to prevent decomposition which may cause rapid death of the remaining larvae. The water used for the study was analyzed by using the method of APHA (1996) [3]. Mortality was recorded after 24 h of exposure during which no nutritional supplement was added. The experiments were carried out 27 ± 2 ^{0}C. Each test comprised of three replicates with four concentrations (300, 250, 100, 50 and 25 µg/ml).

2.8 Statistical Analysis

Data were evaluated through regression analysis. From the regression line, the LC_{50} values were read representing the lethal concentration for 50% larval mortality of *A. aegypti*.

3. Results and Discussion

Physical and chemical characteristics of water used for the study, like temperature 27 ± 0.5 ^{0}C, pH 7.3 ± 0.5, dissolved oxygen 3.6 ± 0.5 mg/l, dissolved carbon dioxide 1.2 ± 0.5 mg/l, salinity 1.5 ± 0.5 ppt and alkalinity 125 ± 0.5 mg/l were within the permissible limits throughout the study period.

The 24h bioassay is a major tool for evaluating the toxicity of phytochemicals and a number of researchers have been applying this method to assess the toxic effect of different plant extracts against mosquitoes (Sakthivadivel and Daniel 1999) [24]. The mosquito larvae exposed under plant extracts showed significant behavioral changes. The changes were observed within 30 minutes of exposure. The most obvious sign of behavioral changes observed in *A. aegypti* was inability to come on the surface. The larvae also showed restlessness, loss of equilibrium and finally death. Remia and Logaswamy (2010) [23] reported that these behavioral effects were more pronounced in case of *Catharanthus roseus* than *Lantana camara* extracts after exposure. These effects may be due the presence of neurotoxic compounds in both the plants. In the present study the behavioral effects were more pronounced in case of *H. indicum* than *M. maderaspatana* extracts after exposure. No such behavioral changes were obtained in control groups.

Results of the experiment conducted for evaluating the larvicidal efficacy of both plants showed that they are toxic to e *A. aegypti* larvae. Three replicates of each extract and control were performed in order to ascertain the consistency of the results (Tables 1-3).

Table 1: Larvicidal effects of ethanolic extracts of *Heliotropium indicum* and *Mukia maderaspatana* on larvae of *A. Aegypti* after a 24 h treatment at room temperature

S. No	Concentration of the extract (mg/ml)	No. of larvae Dead/No. exposed (*H. indicum*)	No. of larvae Dead/No. exposed (*M. maderaspatana*)	Mortality
1	Control	0/30	0/30	0
2	0.25	3/30	3/30	10
3	0.50	6/30	6/30	20
4	0.75	9/30	9/30	30
5	0.100	12/30	12/30	40
6	0.125	15/30	15/30	50
7	0.150	18/30	18/30	60
8	0.200	24/30	24/30	70
9	0.250	30/30	30/30	80
10	0.300	30/30	30/30	100

Table 2: Phyto-components of extract of *H. indicum* identified by GC-MS study

S. No	Components	Formula
1	Benzene acetaldehyde	C_8H_8O
2	5H-1-Pyrindine	C_8H_7N
3	2-Furancarboxaldehyde, 5-(Hydroxymethyl)-	$C_6H_6O_3$
4	Benzene acetic acid	$C_8H_8O_2$
5	Dodecanoic acid	$C_{12}H_{24}O_2$
6	Phenol, 3-Isopropoxy-5-Methyl-	$C_{10}H_{14}O_2$
7	3`-Acetyllycopsamine	$C_{17}H_{27}NO_6$
8	Squalene	$C_{30}H_{50}$
9	Octanoic acid, Ethyl ester	$C_{10}H_{20}O_2$
10	Benzaldehyde, 3-Hydroxy-4-Methoxy-	$C_8H_8O_3$
11	Benzaldehyde, 4-Hydroxy-3, 5-Dimethoxy-	$C_9H_{10}O_4$
12	4-((1E)-3-Hydroxy-1-propenyl)-2-Methoxy Phenol	$C_{10}H_{12}O_3$
13	Benzaldehyde, 4-Hydroxy-	$C_7H_6O_2$
14	Butanoic acid, 2-Methyl-	$C_5H_{10}O_2$
15	Nonanoic acid	$C_9H_{18}O_2$
16	Benzene acetic acid, 2,5-Dihydroxy-	$C_8H_8O_4$
17	3,7,11,15-Tetramethyl-2-Hexadecen-1-ol	$C_{20}H_{40}O$
18	Phytol	$C_{20}H_{40}O$
19	(Z)6,(Z)9-Pentadecadien-1-ol	$C_{15}H_{28}O$
20	1-(+)-Ascorbic acid 2,6-Dihexadeconate	$C_{38}H_{68}O_8$
21	Phytol	$C_{20}H_{40}O$
22	9,12-Octadecadienoic acid (Z, Z)-	$C_{18}H_{32}O_2$
23	9,12-Octadecadienoic acid, Ethyl Ester	$C_{20}H_{36}O_2$
24	Squalene	$C_{30}H_{50}$
25	Methyl Salicylate	$C_8H_8O_3$
26	1-(+)-Ascorbic acid 2,6-Dihexadeconate	$C_{38}H_{68}O_8$

+**:** Present; -: Absence

Table 3: Phyto-components of extract of *M. maderaspatana* identified by GC-MS study

S. No	RT	Name of the Compound	Molecular Formula
1	3.95	Benzene, 1,2,3-trimethyl-	C9H12
2	11.32	Undecanoic acid	C11H22O2
3	13.03	E-7-Tetradecenol	C14H28O
4	13.79	Tetradecanoic acid	C14H28O2
5	16.61	n-Hexadecanoic acid	C16H32O2
6	16.89	Hexadecanoic acid, ethyl ester	C18H36O2
7	18.89	Phytol	C20H40O
8	19.31	9,12-Octadecadienoic acid (Z,Z)-	C18H32O2
9	19.61	Oleic Acid	C18H34O2
10	23.12	Eicosane, 2-methyl-	C21H44
11	23.48	Oxirane, tetradecyl-	C16H32O
12	25.18	1,2-Benzenedicarboxylic acid, diisooctyl ester	C24H38O4
13	27.41	Heptacosane	C27H56
14	29.51	Squalene	C30H50

Source: Dr. Duke's Phytochemical and Ethnobotanical Databases

Table 4: Percentage larval and pupal mortality of *A. aegypti* for different concentrations of extract of *H. indicum* and *M. maderaspatana* following 24 h exposure

Plants used	Stages of exposure	Parameters	Effective concentration in µg/ml					
M. maderaspatana	II instar	Larval mortality (%)	Control	50	100	150	200	250
			0	5	24	37	50	64
	IV instar	Larval mortality (%)	Control	160	180	200	220	240
			0	16	24	32	48	55
	Pupae	Pupal mortality (%)	Control	200	225	250	275	300
			0	10	19	27	53	66
H. indicum	II instar	Larval mortality (%)	Control	25	50	75	100	125
			0	15	22	31	42	55
	IV instar	Larval mortality (%)	Control	50	100	150	200	250
			0	14	35	52	63	75
	Pupae	Pupal mortality (%)	Control	200	225	250	275	300
			0	29	42	53	66	81

The crude extract of *M. maderaspatana* was found to be active against the IV instar larvae of *A. aegypti*. The larvicidal activity varied with the concentration and exposure. The larvicidal activity of *M. maderaspatana* was comparable to that of *H. indicum*. *H. indicum* has been reported to contain sufficient amount of tetranortriterpenoids (Pegel and Rogers 1990, Siddiqui *et al*. 2000) [18, 25] responsible for the larvicidal activity. The observed mosquito larvicidal effects could possibly be due to these compounds.

The results from *A. aegypti* larvicidal assay using two different plants are shown in Table 4. The most active essential compounds against third instar larvae of A. *aegypti* were those of *H. indicum* and *M. maderaspatana*. Sukumar *et al*. (1991) [37] reported that *C. citratus* causes significant growth inhibition and mortality in later developmental stages of *A. aegypti*. The analysis of the essential oil of this plant from the state of Ceará, showed that its major components are geranial (60.3%) and neral (39.7%). *Lippia sidoides* essential oil and its main constituent thymol were shown to be very active against *A. aegypti* larvae (Carvalho *et al*. 2003). [36] Sukumar *et al*. (1991) [37] studied *Ocimum americanum* and showed that solvent extracts from the whole plant have ovipositional deterrence against *A. aegypti*. Matos (2000) reported that *O. gratissimum* essential oil displays antifungal (*Aspergillus* and *Trichoderma*) and antibacterial (S*taphylococcus*) activities. *O. gratissimum* oil presented antihelmintic activity against *Haemonchus contortus*, the main nematode of ovines and caprines in Northeastern Brazil (Pessoa *et al*. 2002) [19]. The citrus oils, although they have insecticidal activities (Ezeonu *et al*. 2001) [38] and *Hyptis suaveolens* that is used as mosquito repellent (Palsson and Jaenson 1999) [15] were not effective in the larvicidal test. Supavarn *et al*. (1974) [29] tested 36 vegetable extracts on *A. aegypti* and found that 11.1% were capable of producing mortality at a concentration of 500 ppm but only 2.8% produced the same effect at a concentration of 100 ppm.

The use of vegetable oil presents a better option in comparison to chemical pesticides for the larval mosquito control, as chemicals may cause environmental hazards proving troublesome in the long run (Ranapukar *et al*. 2001) [22]. Extensive research has been carried out on the effect of botanical derivatives of the neem tree and its derivatives (Mulla and Su 1999) [12].

Methanolic extract of the leaves of *Atalantia monophylla* (Rutaceae) were evaluated for mosquitocidal activity against the immature stages of mosquitoes, *Culex quinquefasciatus, Anopheles stephensi* and *A. aegypti* in the laboratory (Sivagnaname and Kalyanasundaram 2004) [27]. A survey of literature on control of different species of mosquito revealed that assessment of the efficacy of different phytochemicals obtained from various plants has been carried out by a number of researches on the field of vector control *Ageratina adenophora* (Spreng.) showed toxic effects on the mosquito species of *A. aegypti* and *C. quinquefasciatus* (Rajmohan and Ramaswamy 2007) [20]. *Albizia amara* and *Ocimum sanctum* showed larvicidal and repellant properties

against *A. aegypti* and neem seed kernel extracts showed higher larvicidal activity of *A. aegypti* (Palsson and Janeson 1999; Sakthivadivel and Daniel 1999) [15, 24]. A detailed laboratory study on extracts of fruit of *Piper nigrum* against larvae of *C. pipines, A. aegypti* and *A. togoi* was carried out (Park *et al*. 2002). The authors determined the LC_{50} and observed the behavioural changes and mortality in the larvae. Similar observations were noticed in the present study supporting the potential application of these herbs in mosquito control measures.

Molluscicidal and mosquito larvicidal efficacy of *Heliotropium indicum* and mosquito larvicidal property of *Momordica charantia* have already been reported (Manisha Srivastava *et al*. 2007 and Singh *et al*. 2006) [8, 26] as safe for human health. In conclusion the leaf extract of *H. indicum* and *M. maderaspatana* are highly toxic even at low doses thus proving to be effective larvicide. Further analysis is required to isolate the active principles and optimum dosages, responsible for larvicidal and adult emergence inhibition activity in *A. aegypti*. The product of these plants can be well utilized for preparing phytochemicals from which all the non-target organisms can be rescued from harmful vectors. These plants may serve as eco-friendly and suitable alternative to synthetic insecticides as they are relatively safe, inexpensive and readily available in many areas of the world.

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