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Effects of *Dysoxylum malabaricum* Bedd. (Meliaceae) extract on the malarial vector *Anopheles stephensi* Liston (Diptera: Culicidae)

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Abstract

In recent years, use of environmentally friendly and biodegradable natural insecticides of plant origin have received renewed attention as agents for disease vector control. Methanol extracts of leaves from the Indian white cedar *Dysoxylum malabaricum* Bedd. (Meliaceae) were tested against mature and immature *Anopheles stephensi* Liston (Diptera) mosquitoes under laboratory conditions. The extract showed strong larvicidal, pupicidal, adulticidal, and antiovipositional activity. The maximum leaf extract concentration tested in this study was 4%, which produced pronounced effects. In general, first and second instars were more susceptible to leaf extract than older insects. Clear dose–response relationships were established, with the highest dose of 4% plant extract causing 97% mortality of first instars.

Keywords: Malarial vector; *Anopheles stephensi*; *Dysoxylum malabaricum*; Larvicide; Pupicide; Adulticide

1. Introduction

Continued use of synthetic chemical insecticide-based intervention measures for vector control has resulted in lower efficacy of such insecticides in controlling medically important disease vectors. The selective pressure imposed by conventional insecticides is enhancing resistance in mosquito populations (Brown, 1986; Pates and Curtis, 2005; Senthil Nathan et al., 2005a) increasing the demand for new products that are environmentally safe, target-specific, and degradable. Worldwide, malaria is the most important epidemic disease, and vector borne diseases are a major threat to human health. Although biological control has an important role to play in modern vector control programs, it does not provide a complete solution alone. Chemical control methods still are necessary in situations of epidemic outbreak and sudden increases of adult mosquitoes. Insecticides are known for their rapid action and effective control of mosquito populations during epidemics, so they are the preferred control agent regardless of their side effects (Pates and Curtis, 2005; Senthil Nathan et al., 2005a).

Recently, there has been a major effort to promote the use of botanicals as environmentally friendly pesticides, microbial sprays, and insect growth regulators in combination with other control measures such as beneficial insects in an integrated control program (Ascher et al., 1995). Insect growth regulators (IGR) have attracted attention for possible use in the selective control of insects of medical and veterinary importance.

The Meliaceae plant family contains a variety of compounds with insecticidal, antifeedant, growth regulating and development modifying properties. Most of the

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Meliaceous plants are recognized for their insecticidal properties. In general, products from these plants have insecticidal, antifeedant, growth regulation, and oviposition repellent and deterrent effects in a variety of test insect species. These insects include Coleoptera, such as the bruchids, *Callosobruchus maculates* Fabricius, *C. analis* Fabricius, *C. chinensis* L., *Trogoderma granarium* Everts (Yadav, 1985; Jood et al., 1996); Lepidoptera, such as the cabbage webworm, *Crocidolomia binotalis* Zeller (Fagoonee, 1981), the tobacco caterpillar, *Spodoptera litura* Fabricius (Joshi, 1987), the Afro-Asian cotton bollworm, *Helicoverpa armigera* Hubner (Saxena and Rembold, 1984), the rice leaffolder *Cnaphalocrocis medinalis* Guenée (Senthil Nathan et al., 2004), Diptera, such as the blowfly, *Luciliac uprina* Wied. (Rice et al., 1985) and the mosquitoes *Anopheles stephensi* Liston, *A. culicifacies* Giles (Dhar et al., 1996), and *Culex quinquefasciatus* Say (Zebitz, 1984, 1987).

Several investigators have demonstrated biological effects on insects of limonoids isolated from different genera in the Meliaceae, i.e., *Azadirachta indica* A. Juss (Butterworth and Morgan, 1971), *Melia azederach* L. (Morgan and Thornton, 1973), *Khaya ivorensis* A. Chev. (Vanucci et al., 1992), *Trichilia* sp. (Xie et al., 1994), *Dysoxylum spectabile*, Hooke f., (Russell et al., 1994), and *Aglaia* sp. (Satasook et al., 1994). *Dysoxylum malabaricum* Bedd. (Meliaceae), known as Indian white cedar, is a critically endangered and economically important tree of Western Ghats, Southern India. This is a large tree with the leaves that contain several limonoids. Leaf extracts of *D. malabaricum* affect insects in a variety of ways, acting as an antifeedant, and causing growth retardation, reduced fecundity, moulting disorders, morphogenetic defects, and changes in behavior (Govindachari et al., 1999; Hisham et al., 2001). Many other investigators have isolated limonoids from *Dysoxylum* species (Singh et al., 1976; Mulholland and Naidoo, 2000; Hisham et al., 2001; Luo et al., 2002) but their bioactivity against mosquitoes remains unexplored.

A. stephensi Liston (Diptera: Culicidae) has a wide distribution, and is a major disease vector in India as well as in some West Asian countries. It is directly responsible for about 40–50% of the annual malarial incidence. This mosquito predominantly breeds in wells, overhead or ground level water tanks, cisterns, coolers, roof gutters, and artificial containers. Secondary metabolites of plants, many of them produced for protection against microorganisms and insect predators, are natural candidates for the discovery of new products to combat *A. stephensi*. Several studies have focused on insecticidal, larvicidal and repellent properties of natural products for controlling *Anopheles* mosquito, but have reported varied results (Saxena et al., 1993; Rajni and Bhat, 1994; Pushpalatha and Muthukrishnan, 1999; Muthukrishnan and Pushpalatha, 2001; Ansari et al., 2000a,b; Jeyabalan et al., 2003; Prajapati et al., 2005). Extracts from neem and other Meliaceae plant seeds and leaves have shown excellent insecticidal properties against the mosquito vector (*A. stephensi* Liston, *A. culicifacies* Giles (Dhar et al., 1996), and *Culex quinquefasciatus* (Zebitz, 1987)), and at the same time were very eco-friendly (Schmutterer, 1990). The present investigation was undertaken to study the effect of *D. malabaricum* against larvae and adults of *A. stephensi* in a search for effective natural products to be used in the control of malaria.

2. Methods

2.1. Mosquito culture

A. stephensi eggs were collected in and around the Chonbuk National University campus and larvae were reared in plastic and enamel trays containing tap water. They were maintained, and all the experiments were carried out, at 27 ± 2 °C and 75–85% RH under a 14:10 L/D photoperiod. Larvae were fed a diet of Brewers yeast, dog biscuits and algae collected from ponds in a ratio of 3:1:1, respectively. Pupae were transferred from the trays to a cup containing tap water and placed in screened cages ($23 \times 23 \times 32$ cm) where adults emerged. Adults were maintained in $30 \times 30 \times 30$ cm glass cages. Adults were continuously provided with 10% sucrose solution in a jar with a cotton wick. On day 5 post-emergence adults were deprived of sugar for 12 h, then provided with a mouse placed in resting cages overnight for blood feeding by females. Adult mosquitoes were maintained under the same environmental conditions as the larvae.

2.2. Methanolic extracts of D. malabaricum leaves

D. malabaricum leaves (mature leaves) were collected from five trees approximately 28 months old in natural forests in Kakachi, Western Ghats, India. The leaves were picked out during morning times. Methanol extracts of leaves were obtained according to the procedure of Warthen et al. (1984). First, the plant leaves were crushed to a fine particle size and dried in the shade at room temperature. One hundred grams of crushed and dried plant materials were stirred for 3 h in 1000 ml of methanol. After leaving the solution to rest overnight, it was filtered through Whatman no. 40 filter paper. The solid filtration residue was extracted again following an identical procedure, and the two filtrates were combined. The solvent was removed by vacuum evaporation in a rotary evaporator, and a dark green residue from leaves was obtained. This crude extract was used to prepare a stock solution.

2.3. Preparation of stock solution

Crude extract was dissolved in methanol to 500 ml of volume. The stock solution (500 ml) was serially diluted with water to prepare test solutions of 0.10%, 0.25%, 0.50%, 1.0%, 2.0% and 4.0% crude extract. One drop of emulsifier (Tween 20, Sigma Chemical Company) was added to ensure complete solubility of the material in water.

2.4. Larvacidal assay

Bioassays were performed on first to fourth instars of *A. stephensi* using concentrations of 0.25%, 0.5%, 1.0%, 2.0% and 4.0% *D. malabaricum* extract. Methanol (0.1%) served as a control. A minimum of 20 larvae per concentration was used for all the experiments and the experiments were replicated five times (total, $n = 100$). The effective concentration (EC_{50}) was calculated using Probit analysis (Finney, 1971).

For mortality studies, 20 larvae each of first, second, third, and fourth instars, and pupae were introduced to a 250-ml glass beaker containing various concentrations 0–4% (0.5–4%) of the *D. malabaricum* extract supplemented with 50 mg/l of yeast extract. A control was also maintained with methanol. The treatments were replicated five times, and each replicate set contained one control (total, $n = 100$). Percentage mortality in the treatments was corrected when necessary for mortality in the controls using Abbott's (1925) formula.

2.5. Adulticidal assay

Ten *A. stephensi* fresh adults were exposed to filter paper (90 mm, Advantec Toyo, Japan) treated with various concentrations of *D. malabaricum* extract. The treatments (3 ml of 0–4%) were coated on filter paper and air dried. The paper was kept inside the beaker. Control insects were exposed only to methanol (0.1%) treated paper. Mortality counts were taken after 24 h. The experiment was repeated five times (total, $n = 50$).

2.6. Oviposition assay

Different concentrations (0–4%) of the *D. malabaricum* extract were mixed thoroughly with 200 ml of rearing food in 250 ml glass jars to obtain the concentration desired for the tests with *A. stephensi*. Ten gravid females were given a choice between treated and control jars for oviposition. Methanol dilutions served as controls (five replicates, total, $n = 50$). During the tests, groups of 10 females were kept separate for 48 h in cages measuring $25 \times 25 \times 30$ cm. After the eggs were counted, the oviposition activity index (OAI) (Hwang et al., 1982) was calculated using formula (1):

$$\mathrm{OAI} = \frac{Nt - Ns}{Nt + Ns} \times 100 \quad (1)$$

where Nt = total number of egg rafts in the test solution, and Ns = total number of egg rafts in the control.

2.7. Larval and pupal stages assay

To assay the effects of *D. malabaricum* extract on the stages of *A. stephensi* larval and pupal stages, test solutions of sublethal concentrations of 0.10%, 0.25%, 0.5% and 1.0% crude extract were prepared in a $30 \times 25 \times 5$ cm enamel tray. Fifty eggs were released in treated water and allowed to hatch. Total larval duration (days) was recorded from hatching to pupation. Pupae were placed in a small container closed with a transparent mesh. Pupal duration (days) was calculated from the molt into the pupal stage to adult emergence. The experiment were replicated five times.

2.8. Fecundity and adult longevity

The fecundity experiments were conducted with 10 male and 10 female *A. stephensi* that emerged from the control and treatment concentrations in the experiment described in Section 2.7. They were allowed to mate in $30 \times 30 \times 30$ cm cages. Three days after the blood meal, eggs were collected daily from the water in oviposition traps. Fecundity was calculated as the number of the eggs laid in the oviposition traps divided by the number of females in the cage. Adult longevity was recorded for males and females. Eggs collected from the fecundity tests were placed in an enamel tray and observed for successful hatching.

2.9. Statistical analysis

Data from biology, mortality, oviposition deterrence and effective concentrations were analyzed with ANOVA of arcsine transformed percentages followed by Tukey's multiple range test ($P \leqslant 0.05$) (Snedecor and Cochran, 1989; SAS Institute, 2001). Mortality was corrected using Abbott's (1925) formula.

3. Results

3.1. Effect of D. malabaricum on mortality of A. stephensi

Exposure to *D. malabaricum* extract in the larval diet increased mortality of first to fourth instar *A. stephensi* in a concentration-dependent manner (Table 1). The 4% concentration of leaf extract killed more than 97% of first instars, 92% of fifth instars, 93% of pupae and 91% of adults. Thus, lethal effects on larvae appear to greatly reduce survival of adult mosquitoes. An EC_{50} value of *D. malabaricum* extract against *A. stephensi* is shown in Fig. 1. First instar larvae were most susceptible in bioassay experiments with the lowest EC_{50} (0.9%).

3.2. Effect of D. malabaricum on biological parameters of A. stephensi

Adult longevity and fecundity decreased markedly by the *D. malabaricum* extract treatment (Figs. 2 and 3). In addition to significantly lower survivorship, larval duration was lengthened. The duration of larval instars and total developmental time were prolonged even when larvae were fed on lower concentrations of extract (0.10% and 0.25%). Larval duration was approximately 8 days for control larvae and gradually increased with *D. malabaricum* extract concentration. Pupal duration increased and adult

Table 1
Percentage mortality of *A. stephensi* after treatment with *D. malabaricum* extracts at different concentrations

Treatments (% concentration)	Larval mortality (%)				Pupal mortality (%)	Adult mortality (%)
	Instar					
	1	2	3	4		
Control	2.5 ± 0.2^{e}	1.9 ± 0.1^{e}	1.0 ± 0.1^{e}	0.9 ± 0.1^{e}	1.0 ± 0.1^{e}	1.2 ± 0.1^{e}
0.50	23.6 ± 2.5^{d}	21.6 ± 1.8^{d}	19.8 ± 1.5^{d}	16.2 ± 1.9^{d}	18.6 ± 1.5^{d}	21.2 ± 1.6^{d}
1.0	48.3 ± 3.9^{c}	43.2 ± 4.7^{c}	39.8 ± 4.4^{c}	35.4 ± 4.1^{c}	37.2 ± 4.0^{c}	40.1 ± 4.2^{c}
2.0	64.7 ± 5.6^{b}	62.1 ± 5.9^{b}	59.1 ± 6.2^{b}	57.2 ± 6.0^{b}	59.5 ± 6.5^{b}	59.4 ± 6.0^{b}
4.0	96.4 ± 4.6^{a}	95.3 ± 5.6^{a}	93.8 ± 7.0^{a}	91.6 ± 7.5^{a}	92.4 ± 7.4^{a}	91.3 ± 7.2^{a}

Means (±SE) within columns followed by the same letter are not significantly different (Tukey's test, $P \leqslant 0.05$).

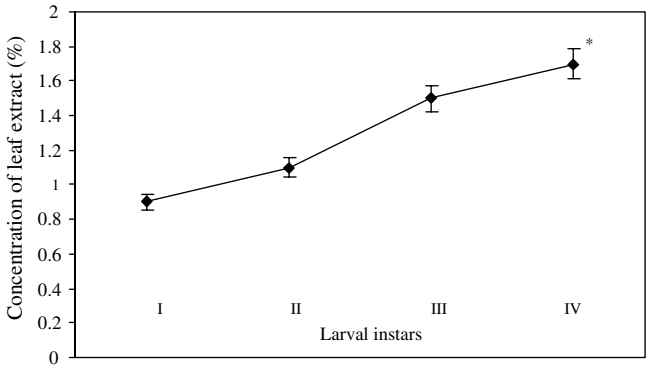

Fig. 1. Effective concentrations (EC_{50}) of *D. malabaricum* against *A. stephensi*. *Values are mean of five replicates and ±standard error.

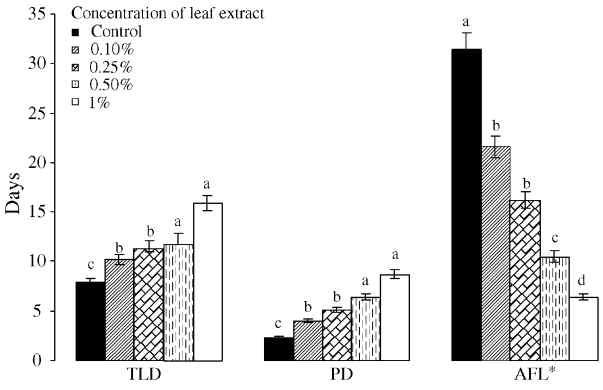

Fig. 2. Means (±SE) duration of larval and pupal stages, adult longevity of *A. stephensi* after treatment with different concentrations of *D. malabaricum*. Means (±SE, standard error) followed by the same letters above bars indicate no significant difference ($P \leqslant 0.05$) according to a Tukey test. *TLD, total larval duration; PD, pupal duration; AFL, adult female longevity.

longevity decreased with exposure to increasing concentration of *D. malabaricum* extract (Fig. 2).

The greatest ovipositional activity index was observed in the 4% *D. malabaricum* extract treatment (Fig. 4). Larvae treated with the lower dose moulted into subsequent instars. But late third and early fourth instars displayed morphological deformities, which impeded their development. The treated larvae were not able to pupate normally. Larval–pupal intermediates were generated with pupal

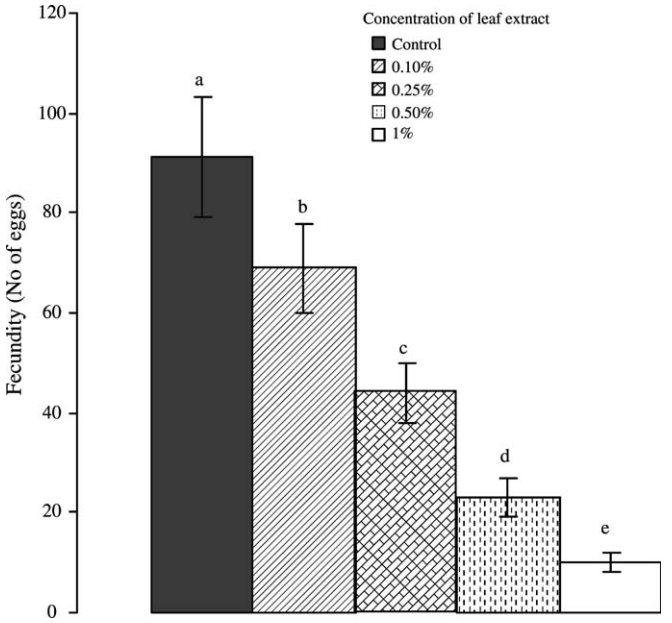

Fig. 3. Mean number of eggs (fecundity) laid by female *A. stephensi* after treatment with *D. malabaricum*. Means (±SE, standard error) followed by the same letters above bars indicate no significant difference ($P \leqslant 0.05$) according to a Tukey test.

cuticle covering the posterior part of the abdomen, while the fore body retained typical larval characters.

4. Discussion

One of the greatest drawbacks of some chemical insecticides is their persistence in the environment, promoting the development of resistance in the insect. Development of resistance is more a function of frequency of use and persistence. Consequently, there is a need for alternative insecticides, which are effective, but with fewer side effects and rapid degradation, reducing the likelihood of resistance development (Nivsarkar et al., 2001).

4.1. Effect of D. malabaricum on biology and adult mortality of A. stephensi

The plant extracts tested in the present study are considered eco-friendly and are not toxic to vertebrates

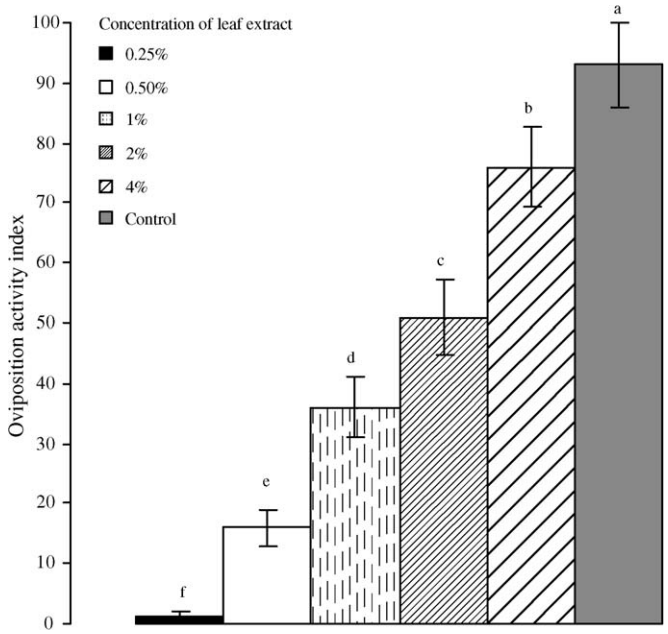

Fig. 4. Oviposition activity index (%) for *A. stephensi* after treatment with indicated concentrations of *D. malabaricum* extract. Means (±SE, standard error) followed by the same letters above bars indicate no significant difference ($P \leqslant 0.05$) according to a Tukey test.

(Govindachari et al., 1999). Crude or partially purified plant extracts containing compounds exhibiting synergistic or potentiating interactions have higher and longer-lasting effects on target insects (Chockalingam et al., 1992). Effects of *D. malabaricum* extracts at 1% or above on the mortality, reproduction, and egg hatchability reported in this study confirm their potential for control of mosquito populations.

Chemicals produced by plants in the Meliaceae often exhibit IGR activity (Saxena et al., 1984; Jacobson, 1987; Schmutterer, 1990; Senthil Nathan et al., 2005b,c), and is the most important physiological effect of *D. malabaricum* extracts on insects. Thus, the family Meliaceae has emerged as a source of insecticides. Exposure of *A. stephensi* larvae to sub-lethal doses of neem leaf extract in the laboratory prolonged larval development and reduced pupal weight (Murugan et al., 1996). In the field, delayed development of surviving larvae and reduced pupal weight are commonly observed after treatment with neem (Zebitz, 1984, 1987; Dhar et al., 1996). Our results indicate that plant-based compounds such as liminoids (compounds present in Meliaceae seed) may be an effective alternative to conventional synthetic insecticides for control of *A. stephensi*. Plant extracts drastically reduced female fecundity and longevity, with only a few adults surviving. Undoubtedly, plant derived toxicants are an invaluable source of potential insecticides and could replaced current chemical control programs. These and other naturally occurring insecticides may play a more prominent role in mosquito control programs in the future (Mordue and Blackwell, 1993), as vector control is progressively compromised by the emergence of resistance in vector mosquitoes to conventional synthetic insecticides, warranting either countermeasures or development of alternative insecticides.

4.2. Effect of *D. malabaricum* on larval, pupal, adult duration, reproduction of *A. stephensi*

Application of *D. malabaricum* extract greatly affected the growth of *A. stephensi*. The lower dose treatments affected development and mortality in a dose dependent manner. After treatment with a higher dose of *D. malabaricum* extract, the larvae became abnormal and irregular in movement, dying before reaching the pupal stage. Our results clearly indicate that application of *D. malabaricum* extract can disrupt normal feeding and physiology. Oviposition deterrence increased significantly with extract concentration (Fig. 4). According to the definitions of Dethier et al. (1960) and Isoe et al. (1995), oviposition repellents are volatile compounds that cause gravid females to move away from the oviposition medium after detection from a distance, while deterrents are non-volatile contact chemical stimuli that inhibit oviposition when present in an otherwise suitable oviposition site. One or more active components in the *D. malabaricum* extract (Dymalol, Nymania-3, and other triterpenes) act as an oviposition repellent and/or deterrent to *A. stephensi* (Govindachari et al., 1994, 1999; Hisham et al., 2001). These results suggest that at concentrations exceeding a certain threshold, repellents can act as insecticides (Jeyabalan et al., 2003).

Larval development was delayed and mortality rate elevated by *D. malabaricum* at concentrations 1% and above. These toxic effects may be independent of each other due to the differences in the way the larvae were exposed to the plant extracts. The results of this study will contribute to the goal of reducing the use of synthetic insecticides, and fostering further investigation of natural control tactics botanical pesticides of various medically important pests. Because botanical pesticides are often active against a limited number of species, less expensive, easily biodegradable, and potentially suitable for use in mosquito control programs (Alkofahi et al., 1989; Su and Mulla, 1999), they represent potentially safer insect control agents. Plant allelochemicals may be quite useful in increasing the efficacy of biological control agents because plants produce a large variety of compounds that increase their resistance to insect attack (Berenbaum, 1988; Murugan et al., 1996; Senthil Nathan et al., 2004, 2005a,b,c,d).

In conclusion, *D. malabaricum* leaf extract produced more than 90% mortality of all instars of *A. stephensi* at a concentration of 4% of leaf extract. Though larvicidal activity was observed at higher doses, lower doses greatly inhibited the reproductive potential of adults. These results are very promising in developing new, effective and affordable approaches to control *Anopheles* mosquitoes, and thus malaria. Studies on mode of action and synergism with biocides under field conditions are in progress.

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