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Potential control of *Aedes aegypti* (Diptera: Culicidae) with *Piper aduncum* L. (Piperaceae) extracts demonstrated by chromosomal biomarkers and toxic effects on interphase nuclei

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ABSTRACT. Dillapiol, a phenylpropanoid isolate from essential oils of leaves of *Piper aduncum* (Piperaceae), has insecticidal, fungicidal and antimicrobial activities. The insecticidal activity of dil-

lapiol was tested *in vivo* on the larvae and pupae of *Aedes aegypti*, the mosquito vector of dengue. Specifically, the effect of dillapiol on the formation of micronuclei and chromosome aberrations was analyzed. Dillapiol treatments comprised two concentrations of 200 and 400 µg/mL, dissolved in well water, and a pure well water control used to rear four generations of mosquitoes. Micronuclei occurred in mitotic diploid and tetraploid chromosomes of larvae; nuclear abnormalities also occurred in interphase, metaphase, telophase, and single nucleus cells of pupae. Mortality, oviposition, chromosome breakage, and anaphase bridges were significantly greater in the extract treatments than in controls. The genotoxic effects of dillapiol described here suggest that this natural product may be a useful alternative for the control of *A. aegypti*.

Key words: Dengue; Dillapiol; Micronucleus test; Chromosome aberrations

INTRODUCTION

Aedes (*Stegomyia*) *aegypti* (Linnaeus) is the main urban vector of dengue and yellow fever in many parts of the world, affecting 60 million people with 30,000 deaths annually (WHO, 2003). Dengue is an arboviral disease transmitted as four serotypes (*Flavivirus*, DEN-1 through DEN-4) (Halstead et al., 1963; Halstead, 1992). Resistance to pesticides is a continuing problem for mosquito control programs; consequently, *A. aegypti* resistance to pesticides is monitored as a key part of vector control program (WHO, 2003). In Brazil, the *A. aegypti* Insecticide Resistance Monitoring Network (MoReNAa) (FUNASA, 1999) Program for Dengue Control was developed by the Brazilian Ministry of Health as a consequence of this resistance to pesticides (FUNASA, 2001). Today, control of *A. aegypti* relies heavily on organophosphates (malathion, fenithrothion and temephos) and pyrethroids (deltamethrin and cypermethrin), to which the mosquito has developed resistance (Luna et al., 2004; Ministério da Saúde, 2006). Thus, interest is growing in alternatives for mosquito control, such as larvicides and growth regulators, especially natural controls from plant extracts.

Larvicides, such as *Bacillus thuringiensis israelensis*, are also used, but have short effective lives in the environment, as compared to the insecticide temephos, for example (Lima et al., 2003). Insect growth regulator and the selective larvicides methoprene (Dame et al., 1998) and pyriproxifen (Slama et al., 1974) have also been used, but they are not recommended for use near food items. Also, *A. aegypti* can develop a variety of forms of resistance to methoprene, as has occurred in various regions of Brazil. Plant extracts also have been reported to have pesticidal qualities for the *Aedes* genus (Sharma et al., 1998) including extracts from the genus *Piper* (Piperaceae), with >1000 species throughout the neotropics. Leaves of *Piper* spp naturally produce several types of bioactive products, including phenylpropanoids, lignoids and flavonoids (Bernard et al., 1995). One specific insecticidal phenylpropanoid is dimethoxy-4,5-methylenedioxy-allylbenzene (dillapiol) (Gottlieb et al., 1981). Extracts from *Piper aduncum*, known in Brazil as *pimenta longa*

(long pepper), are larvicidal (Bernard et al., 1995; Pohlit et al., 2004).

Cytogenetics is a useful tool for assays of genotoxicity when testing products *in vitro* and *in vivo*. Formation of micronuclei and chromosome aberrations are two important cytogenetic and detrimental consequences of genotoxicity that can readily be used for product evaluation (Krishna et al., 1991; Zanoni et al., 2005). Micronuclei are thought to arise from both chromosome breakage (clastogenics), and chromosome division lagging during cell division (aneugenic) effects. Chromosomal aberrations, detrimental rearrangements of chromosomes through breakage-fusion-bridge mechanisms (McClintock, 1940 apud Gisselsson et al., 2000), are visible due to variability in chromosome structure. Studies on dillapiol suggest that point mutations and chromosomal aberrations and aneuploidy are induced in higher organisms (Richard, 1994), but similar studies in insects are few and do not include evaluation of genotoxicity.

The potential pesticidal effects of dillapiol was tested to better understand its potential for control of *A. aegypti*. Specifically, two important levels of effects were examined. First, its influence on reproduction (oviposition) and survival. Second, whether it causes chromosomal damage (chromosomal aberrations and micronuclei).

MATERIAL AND METHODS

Mosquitoes

Larvae and pupae of *A. aegypti* were collected from discarded and water-filled tires (following recommendations by SUCEN, 1997) in three different areas within the city of Manaus (3°1' - 3°7' S, 9°54' - 59°58' W; from July 2005 to March 2007). Upon collection, they were placed in cooled, airtight containers and taken to the National Research Institute of Amazonia in Manaus for study. At this institute they were reared following standard mosquito rearing procedures (Santos et al., 1981).

Dillapiol

Dillapiol was obtained from volatile oils extracted from leaves of *Piper aduncum*. Young dried leaves of *P. aduncum* were submitted to hydrodistillation in a modified clevenger apparatus for 4 h. The oil was dried with $NaSO_4$ (anhydrous) and purified by open column chromatography over silica gel eluted with a gradient of hexanes and ethyl acetate. Pure dillapiol (>95%) was obtained and confirmed by gas chromatography analysis.

We used three experimental treatment concentrations. First, the control treatment contained only well water (0 µg dillapiol/mL). Next, pure dillapiol was first dissolved in DMSO (dimethyl sulfoxide, $(CH_3)_2SO$, used for its solvent qualities) and then diluted in well water to the desired two treatment concentrations: 200 and 400 µg/mL. The final DMSO concentration was 1% in both. Future reference to treatments will be control, 200 and 400.

Bioassays for reproduction, survival and genomic alterations

Bioassays were tested for interactions between dillapiol concentration and repro-

duction and survival of the mosquitoes. Larvae and pupae, collected as described above, were taken from original 24 pairs that were reared for four generations in well water. Larvae and pupae were then placed in water with one of the three experimental concentrations (control, 200, 400) for 24, 36 and 42 h. After adult emergence, males were fed *ad libitum* on a sugar solution; all adult females were fed *ad libitum* on hamster (*Mesocricetus aureatus*) blood, following routine mosquito rearing procedures at the Malaria and Dengue Vector Laboratory at INPA. Under these conditions, breeding was spontaneous and after oviposition, 840 eggs were collected from each of four generations (4 x 840 = 3360 eggs). Eggs were divided into 28 plastic receptacles per treatment, each with 40 eggs in each receptacle (for a total of 1120 eggs per treatment). Receptacles had 40 mL water (the three concentration treatments) for growth of larvae. The effects of dillapiol were tested and compared among treatments after 36 h of exposure.

Slide preparation, nuclear abnormalities and chromosomal aberrations

Slides were prepared of brain ganglial smears from fourth-instar larvae (N = 200) and of ovarian smears from pupae (N = 200) (French et al., 1962; Kumar and Collins, 1994; Rafael and Tadei, 1998) from individuals reared in the three concentration treatments. Slides were processed with 2% acetic-orcein and Ag-NOR staining (Howell and Black, 1980), with minor modifications following Rafael et al. (2003).

Slides prepared as described above were photographed under phase contrast using optovar 1.25X and 1.6X lens on a Zeiss-Axioplan microscope. Nuclear abnormalities, micronucleus frequency and chromosome aberrations were counted from 1000 random cells on each slide. Counts were then compared among the dillapiol concentration treatments.

Statistical analysis

Micronucleus frequency and the number of nuclear alterations were compared between treatments (generation and dillapiol concentration) using analysis of variance (ANOVA) followed by the Tukey test. We specifically tested two predictions. First, that dillapiol has a negative effect (causes/abnormalities) and, second, that the effect increases with concentration. Statistical significance was considered when $P \leq 0.05$.

RESULTS

Survival and reproduction

Larval mortality was similar in both dillapiol treatments and much greater than in controls. A total of 67% of the larvae died at 400 µg/mL, 53% at 200 µg/mL and only 4% died in controls ($G > 25.0$, d.f. = 1, $P < 0.05$; Figure 1). Larval mortality was independent of generation (generation, $P = 0.40$). Adult survival was approximately the same in all treatments (56.3%). Egg production was greatest in females of the control treatment (mean = 82.7), followed by 200 (52.2) and 400 (42.4); all treatments differed ($F_{2,357} = 92.8$, $r^2 = 0.34$, $P < 0.05$, Tukey test; Figure 2).

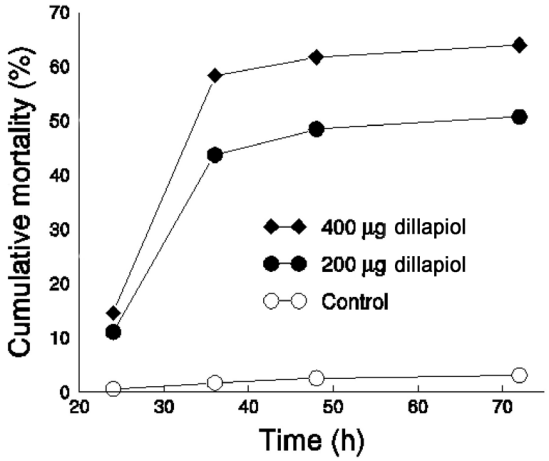

Figure 1. Comparison of mortality in *Aedes aegypti* in the three dillapiol treatment concentrations (control, 200 and 400 µg/mL) at 24, 36, 48 and 72 h after exposure. At all time intervals after 24 h, 400 > 200 > control (all $G > 25.0$, d.f. = 1, $P < 0.05$).

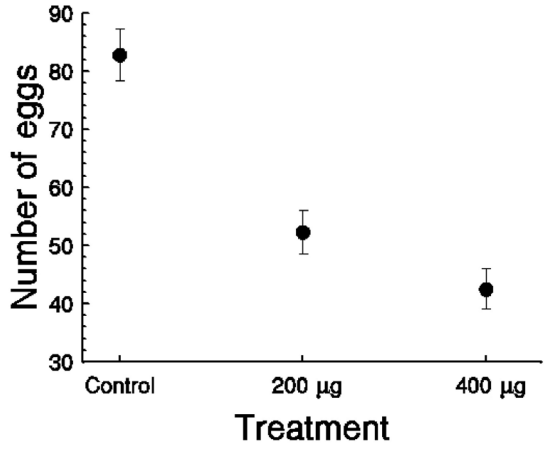

Figure 2. Comparisons of egg production in the three dillapiol treaments. Means ± 95% confidence intervals are shown, illustrating that all treatments are different ($P < 0.05$, Tukey test).

Nuclear abnormalities and micronucleus frequency

Both larvae and pupae exposed to dillapiol had greater frequencies of nuclear alterations than controls (larvae: $F_{2,249} = 12.54$, $P < 0.05$; pupae: $F_{2,249} = 12.86$, $P < 0.05$; Tables 1 and 2). In larvae and pupae that were exposed to dillapiol (but not in controls), cerebral ganglia had mononucleated (Figure 3A), binucleated (Figure 3B), polynucleated (Figure 3C) cells, nuclear "buds" (Figure 3D), and nuclear abnormalities (Figure 3E). Interphasic (Figure 4A) and metaphasic mitotic cells in both dillapiol treatments had micronuclei in larvae and pupae (Table 1) with diploid (Figure 4B) and tetraploid (Figure 4C) nuclei (Tables 1, 2).

Table 1. Proportion of larval and pupal cells with nuclear changes.

Stage	Average proportions				Dillapiol treatments (µg/mL)
	A	P	B	M	
Larvae	0.064	0.059	0.012	0.017	0 (control)
	0.089	0.082	0.028	0.029	200
	0.090	0.083	0.028	0.032	400
Pupae	0.066	0.063	0.012	0.017	0 (control)
	0.093	0.085	0.028	0.032	200
	0.097	0.090	0.033	0.035	400

Nuclear alterations (A), polynuclear (P), buds (B), and micronuclei (M).

Table 2. Total number of chromosomal aberrations in cerebral ganglia and ovaries of *Aedes aegypti* compared among dillapiol treatments.

Immature stage	Chromosomal aberrations	Dillapiol treatment (µg/mL)		
		Control (0)	200	400
Larvae	Anaphasic bridge	5122.5[a]	6392.5[b]	7013.0[b]
	Chromosomal delay	5984.0	6271.5	6272.5
	Chromosome breakage	4890.0[a]	6423.5[b]	7214.5[b]
Pupae	Anaphasic bridge	3668.0[a]	5109.5[b]	5418.5[b]
	Chromosomal delay	4508.0	4760.5	4927.5
	Chromosomal breakage	3486.0[a]	5229.0[a]	5481.0[a]

Superscript letters indicate different values as tested by the Tukey test with $P \leq 0.05$.

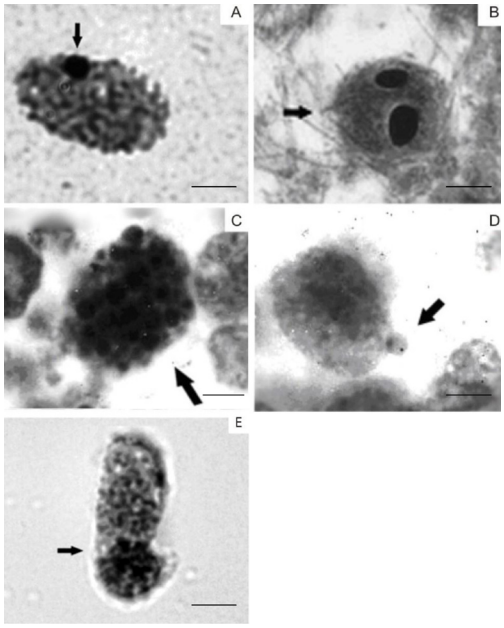

Figure 3. Cerebral ganglion interphase cells from larvae and ovarian nurse cells from pupae impregnated with silver nitrate (A) and stained with orcein (B, C, D). Mononucleate (A), binucleate (B), polynucleate (C), buds (D), and nuclear alterations (E) are all visible (arrows). Bars = 10 µm.

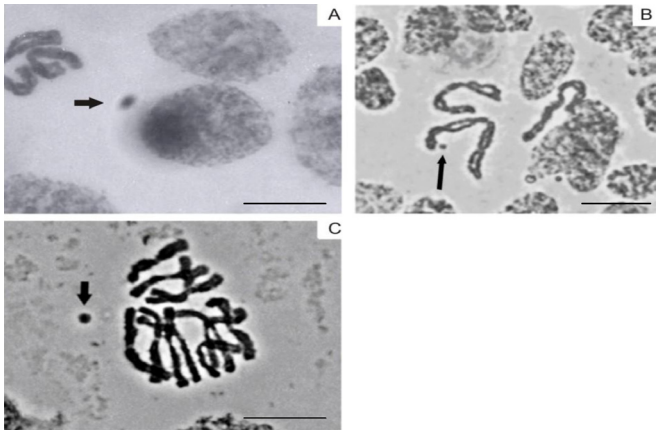

Figure 4. Ganglial and ovarian cells of *Aedes aegypti* stained with orcein. Interphase micronuclei (A), diploid mitotic metaphase (B) and tetraploid nuclei (C) (arrows). Bars = 10 µm.

Chromosome aberrations in larvae and pupae

Control larvae and pupae had undamaged anaphasic chromosomes (Figure 5A). In contrast, chromosomes in both dillapiol treatments had anaphasic bridges (Figure 5B), delayed chromosomes in anaphase (Figure 5C) and chromosomal breaks and gaps (Figure 5D, E). Chromosomal aberrations were more frequent in both dillapiol treatments in larvae and pupae (Table 2) than in the control treatment.

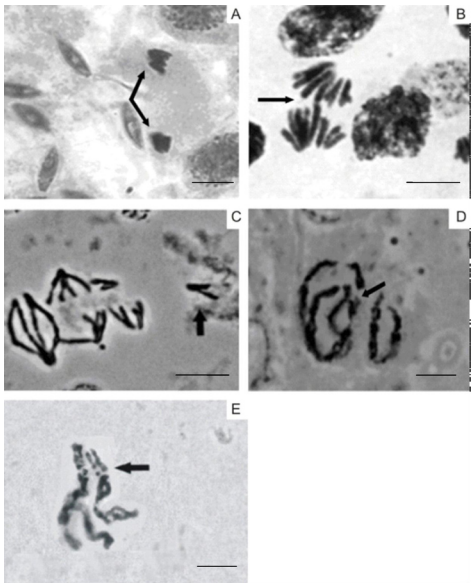

Figure 5. Cerebral ganglia from larvae, ovaries from pupae of *Aedes aegypti* stained with orcein. Normal anaphase (A), anaphasic bridge (B), chromosomal break in metaphase (C), chromosomal delay in anaphase (D), and a break in the bivalent pachytene No. 3 (E) in dillapiol-treated individuals (arrows). Bars = 10 µm.

DISCUSSION

Dillapiol was found to be toxic for larvae and pupae of mosquitoes; it also caused a decrease in egg production, showing its potential for use as a natural control agent. Insecticidal extracts of other plants have also been tested for on *Aedes* mosquitoes in many parts of the world (Sharma et al., 1998). For example, 25 plant extracts, including ethanol and hexane extracts and lyophilized compounds, such as those from *Allium sativum*, *Jatropha curcas*, *Mikania schenkii*, *Poinciana regia*, and *Spatodea campanulata*, were tested on oviposition in *Aedes fluviatilis* (Lutz) (Diptera: Culicidae) in the laboratory (Consoli et al., 1989). Repellent effects on females were found for some extracts at 100 ppm. For example, other species with monoterpene groups in the family Piperaceae were tested in Argentina, Bolivia, Brazil, and Peru and were shown to have toxic effects on *A. aegypti* (95%) (Chantraine et al., 1998) and other extracts from the genus *Piper* (*P. divaricatum*, *P. aduncum*, *P. marginatum* variety *anisatum*, *P. callosum*, *P. marginatum* variety *marginatum*) were tested on *A. aegypti* larvae and the malaria vector *Anopheles marajoara*. *Piper aduncum* caused 73-75% mortality in 24 and 48 h, respectively (Souto, 2006). It was also tested on the beetle *Cerotoma tingomarianus* Bechyné (Coleoptera: Chrysomelidae) and found CL50 = 0.6 mL oil cm^{-2} and DL50 = 0.02 mL oil cm^{-2} (Fazolin et al., 2005). In the present study, dillapiol treatment caused much greater mortality at both 200 and 400 µg/mL than the control in all generations and for both larvae and pupae. Extracts from *P. aduncum* are rich in dillapiol, which, due to its volatile composition and its rapid degradation due to light, heat and humidity, has a short shelf life (Simões and Spitzer, 1999). This short shelf life could be related to the decline in activity after 36 h (Figure 1).

Aedes mosquitoes have 2n = 6 chromosomes, with three pairs that are slightly different in size (McDonald and Rai, 1970). However, no information is yet available to our knowledge on the number of micronuclei, nor of aberrations induced by toxic compounds in mosquitoes. Our study demonstrates that physical changes of the nuclei and chromosomes occurred after application of dillapiol. Micronuclei, also called satellites or extra-chromosomal elements, occur naturally in mosquitoes: *Orthopodomyia* and *Toxorhynchites* (Breland, 1961; Kitzmiller, 1963), *Aedes atropalpus* (Rai, 1963), *A. vittatus* (Rai, 1966), *Psorophora signipennis* (Mukherjee and Rees, 1970), and *A. aegypti* and *A. fluviatilis* treated with synthetic insecticides (Lima-Cattelani and Bicudo, 1994, 1995; see also our Figure 3). Apparently micronuclei disappear after prophase due to chromosomal condensation in metaphase (Breland, 1961). However, micronuclei were found during telophase in *Aedes dorsalis* (Mukherjee and Rees, 1970). In the present study, the chromosomal aberrations in *A. aegypti* exposed to dillapiol may be due to despiralization and breaking of the DNA molecule. Such damage was observed in *A. aegypti*, associated with secondary constriction of fragile chromosome No. 3 (Mukherjee and Rees, 1970; Bianchi et al., 1972).

The data reported here showed that dillapiol can reduce survival and reproduction in *A. aegypti* (Figures 1 and 2). It also induced chromosomal damage and micronuclei, demonstrating cytotoxic effects (Figures 3-5). Moreover, nuclear abnormalities may inhibit cell division, successful reproduction or cause other abnormalities due to secondary metabolites, such as terpenes and monoterpenes and their analogues, which are common components of essential oils in many higher plants (Prates and Santos, 2002). It is important to understand the varying effects of dillapiol at different concentrations to recommend dosages and methods as potential alternatives for mosquito control. Since dillapiol can be relatively easily extracted,

we recommend further study, especially to discover possible residual effects, selectivity and forms of use for the control of *A. aegypti* and other mosquitoes.

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