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# LARVICIDAL EFFECT OF *EUCALYPTUS GRANDIS* ESSENTIAL OIL AND TURPENTINE AND THEIR MAJOR COMPONENTS ON *AEDES AEGYPTI* LARVAE

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ABSTRACT. In the search for new alternatives for the control of *Aedes aegypti* the larvicidal activity of *Eucalyptus grandis* essential oil and pine resin essential oil (turpentine) and their major components (α- and β-pinene and 1,8-cineole) was determined. Gas chromatography–mass spectroscopy analysis of *E. grandis* essential oil revealed that its major components are α-pinene and 1,8-cineole. Similar analysis of turpentine obtained by distillation of the resin pitch of conifers showed that α- and β-pinene are the only major components. Third and early 4th instars of the CIPEIN-susceptible strain of *Ae. aegypti* were exposed to acetonic solutions of *E. grandis* essential oil, turpentine, and their major components for 24 h. Turpentine, with an $LC_{50}$ of 14.7 ppm, was more active than the essential oil of *E. grandis* ($LC_{50}$: 32.4 ppm). Larvicidal activity of the essential oil components showed that α- and β-pinene present low $LC_{50}$ values (15.4 and 12.1 ppm, respectively), whereas pure 1,8-cineole showed an $LC_{50}$ of 57.2 ppm. These results suggest that α-pinene in *E. grandis* and α- and β-pinene in turpentine serve as the principal larvicidal components of both oils. Results obtained on larvicidal effects of essential oil of *Eucalyptus grandis* and turpentine could be considered a contribution to the search for new biodegradable larvicides of natural origin.

**KEY WORDS** *Aedes aegypti*, larvicidal activity, essential oil, *Eucalyptus*, turpentine

## INTRODUCTION

The mosquito *Aedes aegypti* (L.) is considered to be among the main vectors of the viral diseases dengue fever and dengue hemorrhagic fever (Gubler and Clark 1995). Dengue fever and dengue hemorrhagic fever are the most important and serious mosquito-borne diseases in Argentina (Carbajo et al. 2001). Until the 1950s, *Ae. aegypti* inhabited most of the American continent and had a global distribution between latitudes 35°S and 45°N (Christophers 1960).

During the 1950s and 1960s, more than 20 countries in Latin America were able to eradicate *Ae. aegypti* (Garin et al. 2000), but in the last decade almost all of these countries, including Argentina, have become reinfested (Boffi 2002). In 1997 dengue infection was identified in northern Argentina (Aviles et al. 1999). Currently the distribution of *Ae. aegypti* is similar to what it was before its eradication (Boffi and Schweigmann 1998), and, in the absence of a vaccine, control of the vector is regarded as essential for stopping epidemics in tropical areas.

Application of the organophosphate insecticide temephos as sand granules (Abate® 1% SG) has been used to control *Ae. aegypti* larvae in large-scale treatments. Although this larvicide provides good larvicidal effect for several weeks or months in the treated containers and is of very low cost, it is frequently rejected by inhabitants for application in their water containers because of its strong smell and slight turbidity. Besides, this insecticide has the disadvantage of nontarget effects. Although there are concerns about nontarget effects on beneficial aquatic arthropods and vertebrates, there is also concern about the development of resistance in *Aedes* larvae (Mulla et al. 1986). *Aedes aegypti* exhibits resistance to various insecticides and has already spread widely in America (World Health Organization [WHO] 1992, Pereira da-Cunha et al. 2005). In Brazil, an acquired resistance to temephos was reported (Lima et al. 2003, Macoris et al. 2003, Braga et al. 2004). Biological larvicides such as *Bacillus thuringiensis israelensis* (*Bti*) and insect growth regulators such as methoprene or novaluron could be considered useful alternatives for the control of mosquito larvae (Chavasse and Yap 1997, Mulla et al. 2003), but they are expensive products for large-scale treatments.

Another alternative to conventional insecticides is the use of natural products from plants that produce chemical defense mechanisms against microorganisms and predators and that could be candidates as new products to control *Ae. aegypti* (Chantraine et al. 1998, Ciccia et al. 2000, Yang et al. 2002, Cheng et al. 2003, Barreira Cavalcanti et al. 2004). Essential oils are particular plant products made up of volatile substances found in a variety of species (Weinzieri et al. 1994, Weinzieri 2000). The active components are isoterpenoid compounds, mainly mono- and sesquiterpenes, and are carriers of the odor of aromatic plants (Franzios et al. 1997).

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Some phytochemicals act as general toxicants to all life stages of mosquitoes, whereas others interfere with growth and reproduction or act on the olfactory receptors, eliciting responses of attractancy or repellency (Sukumar et al. 1991). The mechanisms of toxic action of terpenoids have not been discovered and are still obscure; however, the onset of toxic signs is usually rapid (Enam 2001), and this rapid effect on some pests is indicative of a neurotoxic mode of action (Isman 2006). For example, Grundy and Still (1985) and Ryan and Byrne (1988) reported that these oils were competitive inhibitors of acetylcholinesterase, and others suggested that another possible target for essential oil activity is the octopaminergic system of insects (Enam 2001, Kostyukovsky et al. 2002); and Priestley et al. (2003) showed interference with GABA-gated chloride channels.

*Eucalyptus* oils are obtained by hydrodistillation of the leaves of *Eucalyptus* and have an aroma characteristic of the particular species used. The genus *Eucalyptus* is native to Australia and consists of over 500 species of trees. (Coppen 1995, Nishimura and Sato 1999). In northeastern Argentina, *Eucalyptus* plantations occupy more than 130,000 ha (Carpineti et al. 1995), with *E. grandis* (Hill) ex Maiden the primary species. Initially, *Eucalyptus* was used for cellulose industries, but now the principal use is in sawmills (Aparicio et al. 2005). *Eucalyptus grandis* is one of the forest species of major economic importance for Uruguay and Argentina, but the essential oil industry has not been developed commercially in the region.

Turpentine is a natural product obtained as the volatile oil distilled from pine resin gathered by tapping trees of the genus *Pinus* (Coppen 1995). The chemical composition of turpentine can differ significantly depending on the species from which it is harvested. In some pines, the terpene composition is relatively simple and consists mainly of α- and β-pinene (Coppen 1995). Turpentine is used in an unprocessed form in the manufacture of soaps, papers, paints, and varnishes (Cielsa 1998). α-pinene is an important component of different essential oils used in the production of insecticides (Galeffi and Marini Bettolo, 1988)

Taking into account the necessity of developing new mosquito larvicides with more favorable environmental properties and new modes of action to avoid resistance, we studied botanical products for *Ae. aegypti* larvae control. In this study we present results about the chemical composition of *E. grandis* essential oils and turpentine and their bioactivity against *Ae. aegypti* larvae. The larvicidal activity of their major components also was determined in an attempt to establish their role in the larvicidal activity of both essential oils.

## MATERIALS AND METHODS

### Plant material

Plants 1 year old and 15 cm long were purchased from a forest tree nusery, Paul Forestal SRL (INASE Register Number J/5 188), Argentina, and planted in an experimental plot in our Center in Buenos Aires, Argentina (34°33′22″S, 58°30′52″W). Each individual plant was certified for quality and origin. After 18 months, fresh leaves were collected from *E. Grandis*.

### Essential oils

*Eucalyptus grandis* essential oils were extracted using fresh leaves by hydrodistillation in an all-glass standard distillation setup in laboratory scale for 70 min. Later, the oil was dehydrated with anhydrous sodium sulfate and stored at −4°C until use.

Turpentine was purchased from Química Oeste Co. (Buenos Aires, Argentina).

### Essential oil analysis

The chemical composition of turpentine and *E. grandis* essential oil was determined by gas chromatography–mass spectrometry (GC-MS) with the use of a QP 5050 A (Shimadzu, Japan) instrument. In the electron impact mode (70 eV), samples were analyzed on a capillary column HP-1 (cross-linked methyl silicone gum) (50 m × 0.32 mm × 0.52 μm). The GC column was maintained initially at isothermic temperature of 50°C for 10 min, programmed temperature of 50–68°C (1°C/min), programmed temperature of 68–75°C (0.5°C/min), programmed temperature of 75–250°C (20°C/min), and final isothermic temperature of 280°C for 10 min. The injector and interface was maintained at 250 and 280°C for turpentine and *Eucalyptus* essential oil, respectively, and split ratio of 13:1. Helium was used as the carrier gas (1.3 ml/min). A hexane solution (0.4 μl) of the turpentine and *E. grandis* essential oil (1 mg/ml) was injected.

Compounds identified in the samples were confirmed by comparing the GC retention times with authentic compounds when possible and by comparison of the mass spectra with available NIST or Wiley mass spectral library resident in the system. Quantification of essential oil components (expressed in relative percentage on total area of chromatogram) was carried out by peak area normalization measurements.

### Chemicals

α-pinene 98% and β-pinene 98% were purchased from Sigma Aldrich (Buenos Aires, Argentina) and 1,8-cineole 99% from the

Table 1. Chemical composition of essential oils obtained from leaves of *Eucalyptus grandis* and turpentine.

| Peak | Components | Retention time (min) | Area (%)[1] | |
|---|---|---|---|---|
| | | | Turpentine | *Eucalyptus grandis* |
| 1 | α-pinene | 17.574 | 45,3 | 52.71 |
| 2 | β-pinene | 21.773 | 47,5 | – |
| 3 | p-cymene | 26.785 | – | 9.70 |
| 4 | 1,8-cineole | 27.766 | – | 18.38 |
| 5 | γ-terpinene | 31.701 | – | 5.00 |
| 6 | α-campholene aldehyde | 39.842 | – | 1.18 |
| 7 | *trans*-pinocarveole | 42.710 | – | 1.93 |
| 8 | borneol | 44.257 | – | 2.95 |
| 9 | 4-terpineol | 44.690 | – | 1.04 |
| 10 | α-terpineol | 45.122 | – | 5.67 |
| | Unidentified compounds | | 7.2 | 2.85 |

[1] Relative percentage of total area in the chromatogram.

Fritzsche Co. (Buenos Aires, Argentina). Solvents used were of analytical grade.

### Biological material

A susceptible CIPEIN strain of *Aedes aegypti* (L.), originating from the Rockefeller strain from Venezuela, was used for all bioassays. The laboratory colony has been maintained since 1996 at 25–30°C and 80–90% relative humidity under a photoperiod of 12:12 h. This colony is maintained free of exposure to pathogens, insecticides, or repellents. Under these conditions, the full development from egg to adult occurs in about 1 wk. Eggs were collected over a wet filter paper and kept in these conditions for 48 h, and then dehydrated at room temperature and stored at least 30 days. They were rehydrated in dechlorinated water (500 eggs per 2 liters of water) at 25 ± 2°C; 24 h after rehydration, 1st instars were observed. Larvae were fed on a mixture of rabbit pellet and yeast and used as 3rd or early 4th instars for bioassays.

### Larvicidal bioassay

The larvicidal bioassay was performed according to the protocol established in a meeting of the Latin American Network for Vector Control held in Iguazú (Misiones, Argentina) in December 2004 and can be found on the Web site www.relcov.org. One milliliter of an acetonic solution of the essential oil to be tested was added to 224 ml of distilled water in a 500-ml plastic jar, which was shaken lightly to ensure a homogeneous test solution. Then 20 late 3rd or early 4th instars of *Ae. aegypti* in 25 ml of distilled water were transferred to that jar. The control solution was made with 1 ml of acetone mixed with 249 ml of distilled water, and the untreated solution contained 250 ml of distilled water only. No food was offered to the larvae. All biossays were conducted in a 27 ± 2°C regulated chamber and 80–90% relative humidity and 12:12-h photoperiod. Mortality and survival were recorded after 24 h exposure. The moribund and dead larvae in 4 tests were combined and expressed as a percentage of larval mortality for each concentration. Larvae were considered dead when they failed to move. Moribund larvae were those incapable of rising to the surface (within a reasonable period of time) or showing the characteristic diving reaction when the water was disturbed.

To evaluate the presence of any possible toxic effects based on the hydrophobic properties of water-insoluble essential oils, we performed a positive control with the use of an acetone solution of vegetable oil with a final concentration of 100 ppm.

### Statistical analysis

Dose-mortality data from each *Ae. aegypti* pool were subjected to probit analysis (Litchfield and Wilcoxon, 1949). Lethal concentration 50% ($LC_{50}$) with 95% confidence intervals were obtained by means of Micro Probit 3.0 software and were expressed as parts per million final concentration. Values were considered to be significant different if the 95% confidence limits did not overlap.

## RESULTS

Data on the essential oil obtained from *E. grandis* leaves collected in our experimental plot are shown in Table 1. The most abundant components in terms of relative percentage of total area in the chromatogram were α-pinene (52.71%) and 1,8-cineole (18.38%). The analysis by GC-MS showed that the turpentine used in this study is composed mostly of both pinenes in a concentration of 45.3% α-pinene and 47.5% β-pinene, respectively (Table 1).

Table 2. Larvicidal activity of *Eucalyptus grandis* essential oil, turpentine, and major components against *Aedes aegypti* larvae after 24 h of exposure.

| Botanical product | $LC_{50}$ (ppm) (95% CI)[1] | Slope |
|---|---|---|
| *E. grandis* essential oil | 32.4 (30.4–34.6) | 5.8141 |
| Turpentine | 14.7 (13.4–16.0) | 5.4663 |
| α-pinene 98% | 15.4 (14.0–16.8) | 5.6069 |
| β-pinene 98% | 12.1 (11.2–13.2) | 6.6371 |
| 1,8-cineole 99% | 57.2 (40.6–86.9) | 3.9390 |

[1] *n*: 80.

The larvicidal effect of the major components of *E. grandis* essential oil and turpentine was determined (Table 2). *Eucalyptus grandis* essential oil presented an $LC_{50}$ of 32.4 ppm, whereas pure samples of α-pinene, β-pinene and 1,8-cineole showed $LC_{50}$ 15.4; 12.1, and 57.2 ppm, respectively. The larvicidal activity of turpentine essential oil assayed ($LC_{50}$: 14.7 ppm) was similar to pure α-pinene and β-pinene.

## DISCUSSION

Different compositions in the major components of *E. grandis* essential oil have been reported by different authors. According to Dagne et al. (2000), *E. grandis* essential oil contains α- and β-pinene as major components, although Mora Martinez et al. (2002) found mainly α-pinene and 1,8-cineole, and Estanislau et al. (2001) determined γ-terpinene, O-cimene, and β-pinene as major components. In all the analyses performed by our laboratory, no β-pinene was detected in the samples of *E. grandis* essential oil collected from the species grown in Buenos Aires.

Larvicidal bioassay showed that *E. grandis* essential oil showed an $LC_{50}$ of 32.4 ppm, while both pinenes presented higher toxicity ($LC_{50}$: 15.4 and 12.1 ppm, respectively), but 1,8-cineole presented a lower actvity ($LC_{50}$: 57.2 ppm). Turpentine oil's larvicidal effect was similar to that established for pure α- and β-pinene (14.7 ppm). No larval mortality was observed in the control solution or in the positive control.

Other essential oils have been reported in the literature with larvicidal properties against *Ae. aegypti*. Our results showed that *E. grandis* essential oil presented an $LC_{50}$ of 32.4 ppm, a value that is lower than the $LC_{50}$ of 63 ppm reported for *Lippia sidoides* (Carvalho et al. 2003) and the 69 ppm reported for *Cymbopogon citratus* (Sukumar et al. 1991).

This study offers some promise of a new rational approach to optimize the larvicidal activity of natural compounds on the basis of the knowledge of their biological effects against mosquitoes. It is known that by hybridization of the plants, the yield and composition of essential oils can be modified. Consequently, new *Eucalyptus* hybrid species with a higher content of the mosquitocidal α- and β-pinene could be developed. Further research on the characterization of the chemical composition in relationship with the larvicidal effect of new hybrid species of *Eucalyptus* is now in progress in our laboratory.

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