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Original article

Effects of essential oil from leaves of *Eugenia sulcata* on the development of agricultural pest insects

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Essential oils are known for their insect control potential, which is mainly attributed to the presence of terpenes that interfere with hormonal and physiological processes of arthropods. The aim of the present study was to evaluate the effects of essential oil from the leaves of *Eugenia sulcata* Spring ex Mart., Myrtaceae, on the development of two species of agricultural pest insects, *Dysdercus peruvianus* and *Oncopeltus fasciatus*. Results showed that the essential oil induced mortality, and reduced numbers of adults. Topical treatment of *Oncopeltus fasciatus* using pure essential oil caused significant mortality rates (96.67%), while *Dysdercus peruvianus* had a higher tolerance, with 80% mortality at the end of the experiments. Results suggest that essential oil from the leaves of *Eugenia sulcata* may be used in agriculture for insect pest control.

Introduction

The use of chemical pesticides is a cost-effective method of controlling insect pests, but these chemicals are highly toxic to other species in the environment. There is a growing concern worldwide over the indiscriminate use of such chemicals, which cause environmental pollution and posit a toxicity risk to non-target organisms (Rao et al., 2003), thus, intensified efforts are being made to find safe, effective and viable alternatives. Plant-based insecticides can be less toxic to humans, readily biodegradable, safer for the environment, suitable for use by small-scale farmers, and yet capable of protecting crops from attack by a wide range of insect pests (Rosenthal, 1986).

Essential oils derived from aromatic plants are a promising new class of ecological products for insect pest's control (López and Pascual-Villalobos, 2010). They are constituted mainly by mixtures of monoterpenes, sesquiterpenes and phenylpropanoids; metabolites that confer the mixtures with organoleptic characteristics and biological activities (Stefanello et al., 2011). Despite the fact that aromatic plants have been widely used in developing countries to protect stored products against insect pests in traditionnal agricultural systems, the

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effects of essential oils on many arthropods is largely unkown. Candidate insect pests for testing essential oils include *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae), commonly known as bug walker, which is a predator of cotton (*Gossypium hirsutum* L., Malvaceae). The feeding habits of this insect cause damage to the seeds and fibers, reducing their commercial value of the plant and resulting in serious economic loss. *Oncopeltus fasciatus* (Hemiptera: Lygaeidae) is another pest insect potentially relevant for essential oils testing since it is used worldwide as a model insect in different studies (Fernandes et al., 2013) and is easy to rear.

Plants of the Myrtaceae family are rich sources of essential oils. Spherical secretory cavities containing terpenoids and other aromatic substances in vegetative and reproductive organs are synapomorphic throughout this family (Judd et al., 2009). *Eugenia* is one of its largest genera, and is represented in Brazil by 301 endemic species (Sobral et al., 2013). *Eugenia sulcata* Spring ex Mart. is an endemic species of the Atlantic Rainforest and is commonly known as "murtinha" or "murta-preta" in Restinga de Jurubatiba National Park (Santos et al., 2009). The essential oil from *E. sulcata* has anticholinesterase activity (Lima et al., 2012), which is one of the mechanisms of action of secondary metabolites from plants with insecticidal activity (Rattan, 2010); however, the biological potential of this essential oil remains almost unexplored. As part of our ongoing studies with the essential oil from the leaves of *E. sulcata*, the aim of the present study was to evaluate the effects of this oil on the development and mortality of *O. fasciatus* and *D. peruvianus*.

Materials and methods

Plant material

Aerial parts of different specimens of *Eugenia sulcata* Spring ex Mart., Myrtaceae, were collected at Parque Nacional da Restinga de Jurubatiba, RJ, Brazil (22°12'57,7"S 41°34'58,5"W) in "Clusia" scrub vegetation during October 2010 and March 2011. Authentication was performed by the botanist Dr. Marcelo Guerra Santos, and a voucher specimen was deposited at the herbarium of the Faculdade de Formação de Professores at Universidade do Estado do Rio de Janeiro, Brazil, under the register numbers RFFP 14.496 and RFFP 13.788.

Extraction of the essential oil

Leaves of *E. sulcata* were turbolized with distilled water and then the material was placed in a 5 l flask and submitted to hydrodistillation for over 4 h in a Clevenger-type apparatus. Subsequently, the oil was collected and stored at 4°C for further analyses.

Gas chromatography/mass spectrometry analysis

Immediately after collection of the leaves, the essential oil was analyzed using a GCMS-QP5000 (Shimadzu) gas chromatograph equipped with a mass spectrometer using electron ionization. The gas chromatography (GC) conditions were as follows: injector temperature, 260°C; FID temperature, 290°C; carrier gas (Helium); flow rate 1 ml/min, and split injection with a split ratio 1:40. The oven temperature was initially 60°C and then temperature was raised to 290°C at a rate of 3°C/min. Each sample (1 µl) was dissolved in CH_2Cl_2 (1:100 mg/µl) and injected in a DB-5 column (i.d. = 0.25 mm, length 30 m, film thickness = 0.25 µm). The mass spectrometry (MS) conditions were voltage 70 eV and scan rate 1 scan/s. The retention indices (RI) were calculated by interpolation to the retention times of a mixture of aliphatic hydrocarbons (C9-C30) analyzed in the same conditions (Van Den Dool and Kratz, 1963). The identification of the substances was performed by comparison of their retention indices and mass spectra with those reported in the literature (Adams, 2007). The MS fragmentation pattern of compounds was also checked with NIST mass spectra libraries. Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (CG/FID), under the same conditions as the GC/MS analysis and percentages obtained by FID peak-area normalization method.

Insect colonies

Colonies of *Oncopeltus fasciatus* and *Dysdercus peruvianus* were established in the Laboratory of Insect Biology of the Universidade Federal Fluminense, and kept at a constant temperature (26 ± 1°C), photoperiod (16L:8D) and relative humidity (60% ± 5) (Feir, 1974; Milano et al., 1999). Insects were maintained in transparent glass pots, covered with screen tissue, fed cotton seeds (*Gossypium hirsutum*) for *D. peruvianus* and sunflower seeds (*Helianthus annuus* L., Asteraceae) for *O. fasciatus* (Feir and Beck, 1963). Insects had free access to water stored in glass flasks inside the pots. Seeds were placed inside the pots during the mating and laying period and up to the first instar. From the second instar stage on, insects were transferred to a clean pot each week, in order to avoid contact of the seeds with the insect feces and facilitate cleaning. Seeds were placed on top of the netting that sealed the pots. All insecticidal activity experiments were conducted at a constant temperature of 26 ± 1°C.

Insect bioassays

The experiments were carried out as described by Fernandes et al. (2013) with some modifications. In order to evaluate the toxicity of the essential oils, 4^{th} instar insects of *O. fasciatus* and *D. peruvianus*, chosen randomly, were topically applied pure or diluted essential oil dilutions. From 1 g of the pure and fresh essential oil, a serial dilution in acetone produced final concentrations of 500, 250, 125, 62.5, and 31.2 mg of oil/ml. Subsequently, 1 µl of each dilution was applied topically to the ventral cuticle of the experimental insect groups. Another experimental group received 1 µl of the pure, undiluted essential oil. Final groups of insects acted as controls and were either untreated or received topical application of the acetone solvent without the essential oil. Biological evaluation of different treatments was performed during the entire time required for development from the 4^{th} instar nymphs to the adult stage. Insect weight, toxicity (mortality during 24 h immediately after treatment), lethality, intermoult period (range), moult and metamorphosis. All experiments were repeated three times, in groups of 10 fully engorged insects. Observation was performed from the day after essential oil topical application on 4th instar nymphs (1st day) to the last day (10th day for *D. peruvianus* and 14th day for *O. fasciatus*).

Data and statistical analysis

The significance of the results was analyzed using ANOVA and Tukey's test using Stats Direct Statistical Software, version 2.2.7 for Windows 98 (Armitage et al. 2002). Differences between treated and control insects were considered not statistically significant when $p > 0.05$. Probability levels are specified within the text and the tables.

Results

In a previous study, the essential oil from leaves of *Eugenia sulcata* Spring ex Mart., Myrtaceae, was obtained and after a gas chromatographic analysis, 22 substances were identified. Of these, sesquiterpenes were the main group found in the oil (58.2%) and β-caryophyllene was the main constituent, corresponding to 24.6% of total relative composition of the oil. The monoterpenes α-pinene and β-pinene were also found in great quantities, corresponding to 17.2% and 10.9%, respectively, of the oil (Lima et al., 2012). Treatment with the essential oil from *E. sulcata* did not interfere with the body weight increase of either *Oncopeltus fasciatus* or *Dysdercus peruvianus* when compared with the control groups. This indicated that none of the treatments tested had a feeding deterrent effect. There were also no statistically significant differences in body weight, morphology or development between the untreated insects and insects topically treated only with the acetone solvent.

Topical treatment of *D. peruvianus* 4th instar nymphs with essential oil from *E. sulcata* showed significant increases in mortality (Fig. 1). Pure essential oil caused significant mortality rates, reaching 43.33 ± 20.80% ($p < 0.001$) in 24 h (toxicity) and 80 ± 17.30% ($p < 0.0001$) in ten days of observation; while 26.67 ± 11.50% of acetone control insects and 16.67 ± 15.30% of the untreated group died by the end of the experiments. It was observed that insects treated with the 500 mg/ml dilution of essential oil, produced 66.70 ± 25.20% ($p < 0.0001$) of mortality in 24 h and 80 ± 20% ($p < 0.0001$) in ten days. A dilution of 250 mg/ml, produced 40 ± 10% ($p < 0.001$) of mortality in 24 h and 66.70 ± 5.80% ($p < 0.001$) by the end of the experiments (10 days). A dilution of 125 mg/m induced 20 ± 17.30% ($p < 0.01$) of mortality in 24 h and 53.30 ± 20.80% ($p < 0.001$) in ten days. A mortality of 6.67 ± 5.80% ($p < 0.05$) in 24 h and 23.30 ± 15.30% ($p < 0.05$) in ten days was induced by the dilution of 62.5 mg/ml. The lowest dilution of 31.2 mg/ml caused no mortality rates in 24 h and 20 ± 10% ($p < 0.05$) in ten days of observation (Fig. 1).

Essential oil from the leaves of *E. sulcata* did not interfere with moulting (inhibiting moulting) of surviving insects to 5th instar nymphs. Live insects arrive to 5th instar and to adults but some insects died during development to adults as showed in Table 1. The number of adults was lower because of the high number of dead insects in the fourth stage (Fig. 1) and during development (Table 1). Regarding untreated insects, 83.3 ± 15.3% reached the adult stage, while 20 ± 17.3% ($p < 0.0001$), 20 ± 20% ($p < 0.0001$), 33.3 ± 5.8% ($p < 0.0001$), 46.7 ± 20.8% ($p < 0.001$), 76.7 ± 15.3% ($p < 0.05$) and 80 ± 10% ($p < 0.05$) reached adult stage (survivors insects) after treatment with pure essential oil and 1 µl of its dilutions: 500, 250, 125, 62.5 and 31.2 mg/ml, respectively (Table 1).

Topical treatment of *Oncopeltus fasciatus* 4th instar nymphs with essential oil from *E. sulcata* showed significant increases in mortality (Fig. 2). Pure essential oil caused significant mortality rates, which reached 93.33 ± 5.77% ($p < 0.0001$) in 24 h (toxicity) and 96.67 ± 5.77% ($p < 0.0001$) in fourteen days of observation, while 6.67 ± 5.8% of acetone control insects and 3.33 ± 5.8% of the untreated group died by the end of the experiments (Fig. 2).

It was observed that insects treated with essential oil at the dilution of 500 mg/ml presented 53.33 ± 15.33% ($p < 0.0001$) of

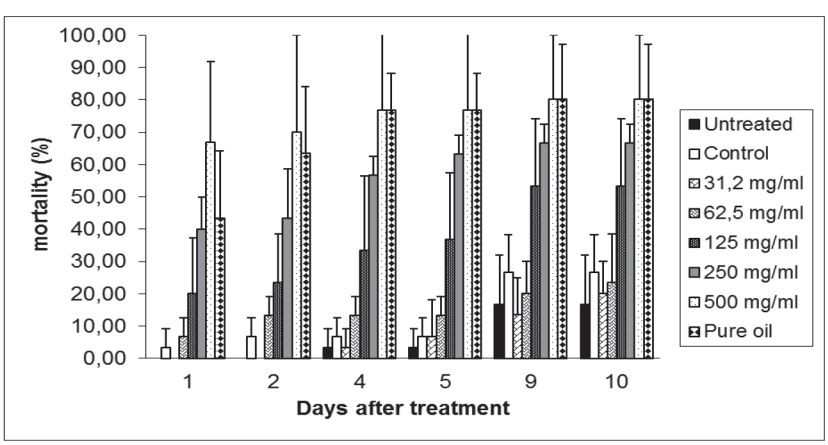

Figure 1 – Mortality after topical treatment with essential oil from leaves of *Eugenia sulcata* on *Dysdercus peruvianus* 4th instar nymphs. Untreated insects were only fed. The other control was topically applied with acetone alone.

Table 1
Analysis of survivors in adult insects after topical treatment of *Dysdercus peruvianus* with essential oil of *Eugenia sulcata* leaves.

Groups	% survival adult insects ± SD
	10 DAT
Untreated	83.3 ± 15.3 ns
Control acetone	73.3 ± 11.5 ns
Pure essential oil	20.0 ± 17.3[a]
500.0 mg/ml	20.0 ± 20.0[a]
250.0 mg/ml	33.3 ± 5.8[a]
125.0 mg/ml	46.7 ± 20.8[b]
62.5 mg/ml	76.7 ± 15.3[c]
31.2 mg/ml	80.0 ± 10.0[c]

DAT, days after treatment; SD, standard deviation; ns, no significance: $p > 0.05$; Significance - $^{a}p < 0.0001$; $^{b}p < 0.001$; $^{c}p < 0.05$; Percentage values of total insect (n = 10) was calculated in each group (in triplicates) from the beginning of experiment. Moulting period was scored with the sum of all insects from each triplicate and the range 10th day after the topical treatments.

mortality in 24 h and 100% in fourteen days. A dilution of 250 mg/ml, produced 43.33 ± 15.33% ($p < 0.001$) mortality in 24 h and 93.33 ± 17.30% ($p < 0.0001$) by the end of the experiments (fourteen days). A dilution of 125 mg/ml, induced 20 ± 20% ($p < 0.001$) of mortality in 24 h and 70 ± 17.30% ($p < 0.0001$) in fourteen days. A mortality of 40 ± 17.30% ($p < 0.001$) in 24 h and 66.67 ± 15.30% ($p < 0.0001$) in fourteen days was induced by the dilution of 62.5 mg/ml. The lowest dilution of 31.2 mg/ml, caused no mortality in 24 h and 46.67 ± 5.80% ($p < 0.001$) in fourteen days of observation (Fig. 2).

Essential oil from the leaves of *E. sulcata* also did not interfere with moulting (inhibiting moulting) of surviving insects to 5th instar nymphs as we observed with *D. peruvianus*. It was observed that live insects arrive to 5th instar and thereafter to adulthood, but some insects died during development until reaching adult stage as showed in Table 2. The number of adults was affected because of the high number of dead insects in the fourth stage (Fig. 2). About the live insects, 96.66 ± 5.77% of untreated insects, reached adult stage, while 3.33 ± 5.77% ($p < 0.0001$), 3.33 ± 5.77% ($p < 0.0001$), 6.67 ± 11.55% ($p < 0.001$), 30 ± 17.32% ($p < 0.0001$), 33.33 ± 15.33% ($p < 0.0001$), and 53.33 ± 5.77% ($p < 0.001$) reached adult stage, when treated with pure essential oil and 1 µl of its dilutions (500, 250, 125, 62.5, 31.2 mg/ml), respectively. On the control acetone group, 93.33 ± 5.77% of insects reached adult stage (Table 2).

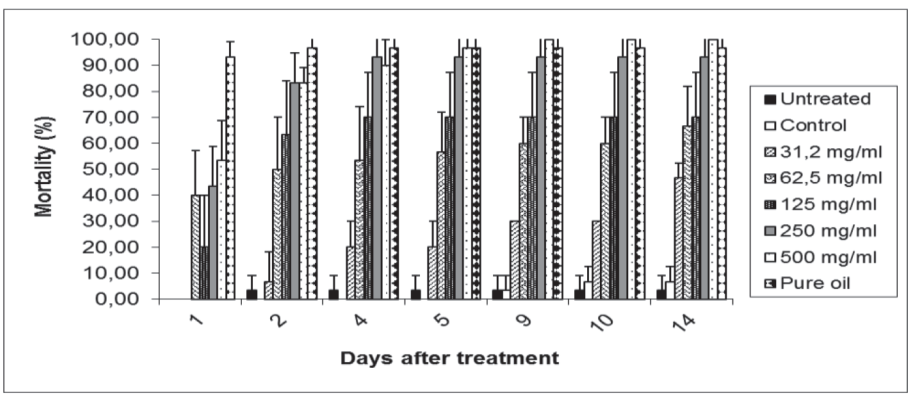

Figure 2 – Analysis of survival in adult insects after topical treatment of *Oncopeltus fasciatus* with essential oil of *Eugenia sulcata* leaves

Table 2
Analysis of survival in adult insects after topical treatment of *Oncopeltus fasciatus* with essential oil of *Eugenia sulcata* leaves

Groups	% survival adult insects ± SD
	10 DAT
Untreated	96.66 ± 5.77 ns
Control acetone	93.33 ± 5.77 ns
Pure essential oil	3.33 ± 5.77[a]
500.0 mg/ml	3.33 ± 5.77[a]
250.0 mg/ml	6.67 ± 11.55[b]
125.0 mg/ml	30.00 ± 17.32[a]
62.5 mg/ml	33.33 ± 15.33[a]
31.2 mg/ml	53.33 ± 5.77[b]

DAT, Days after treatment; SD, Standard deviation; ns, No significance: $p > 0.05$; Significance $^{a}p < 0.0001$; $^{b}p < 0.001$.
Percentage values of total insect (n = 10) was calculated in each group (in triplicates) from the beginning of experiment. Moulting period was scored with the sum of all insects from each triplicate and the range to 14th day after the topical treatments.

Discussion

Conventional pesticides show toxicity to the environment and harmful effects on human health. In this context, there is a growing interest in botanical insecticides due to their minimal costs and lack of ecological side effects (Khater, 2012),which makes them desirable alternatives to synthetic chemical insecticides for controlling pests. They are best suited for use in organic food production in industrialized countries but can play a much greater role in developing countries as a new class of ecofriendly products for pest control.

Essential oil products have recently emerged as one of the most important botanical insecticides with low impact on the field (Khater, 2012). Some essential oils have specific modes of action that make them good synergists. Terpenes, for example, contained in essential oils are neurotoxic to insects (Regnault-Roger, 1997). Moreover, several essential oils inhibit acetylcholinesterase activity in insects (Ryan and Byme, 1988) and their components are also antagonists of octopamine, a neurotransmitter in insects-receptors of *Periplaneta americana* and *Drosophila* sp. (Enan, 2005).

Examples of the use of essential oils include that from a Myrtaceae species (*Eucalyptus saligna*), which was shown to be active against grain storage pest insects, such as the maize grain weevil, *Sitophilus zeamais* (Coleoptera: Curculionidae) and the red flour beetle, *Tribolium confusum* (Coleoptera: Tenebrionidae) (Tapondjoua et al., 2005). Sen-Sung (2003) also showed bioactivity of fourteen essential oils against fourth-instar *A. aegypti* larvae in 24 h. Essential oils of *Vitex trifolia* L. and *Vitex agnus-castus* L.were also evaluated against larvae of *Spilosoma oblique* (Lepdoptera: Arctiidae), and they were reported to increase larval mortality and adult deformity, and reduce adult eclosion, female fecundity and egg fertility (Shishir et al., 2008).

Extraction and chemical analysis of essencial oil of *E. sulcata* indicated that β-caryophyllene was the main constituent, corresponding to 24.6% of total relative composition of the oil and the monoterpenes α-pinene and β-pinene were also found in great quantities, corresponding to 17.2% and 10.9%, respectively (Lima et al., 2012). The presence of many volatile substances in complex mixtures, may improve their efficacy as insecticides. Mixtures of plant compounds reduce the evolution of tolerance to natural insecticides, compared to a single compound, as exemplified with the green peach aphid, *Myzus persicae* (Feng and Isman, 1995; Isman, 2006). This was also observed for blends of β-caryophylene with other volatile substances from essential oils, which were able to repel *Trigonotylus caelestialium*, a rice leaf bug (Hori and Enya, 2013). The monoterpenes α-pinene and β-pinene, which together make up 28.1% of the essential oil used in the present study, also are recognised as substances from plant origin with insecticidal activity (Viegas-Júnior, 2003).

The present observation indicates that *E. sulcata* essential oil is capable of significantly increasing the mortality of both *O. fasciatus* and *D. peruvianus* nymphs and adults by 14 and 10 days, respectively, after treatment. No feeding deterrent effect was detected for any experimental treatment in this study, so that feeding interference cannot serve as a basic explanation for the high mortalities recorded (Fernandes et al., 2013). Details of the mode of action of the oil are largely unknown although a previous study performed by our group indicated that theessential oil from the leaves of *E. sulcata* inhibits acetylcholinesterase (Lima et al., 2012). The inhibition of this enzyme is recognised as one mode of action of several insectides, including those from natural origin (López et al. 2010). The neuroendocrine system of insects is a target for insecticide action and has been studied for almost a century (Kopec, 1917; Wigglesworth, 1934), however, very little information on the endocrinology of either *O. fasciatus* or *D. peruvianus* is available (Dorn and Romer, 1976, Dorn et al., 1976). Nevertheless, knowledge of the modes of action of available insecticides may help determine the chemical properties of novel compounds that may be suitable for modification of insect development and behavior at doses that can be safely and economically used in agriculture (Haynes, 1988).

Observations of the effects of *E. sulcata* essential oil on *D. peruvianus* and *O. fasciatus* indicate that its chemical components are promising candidates for insecticidal activity studies and for ecological control use of plague insect populations in agriculture. The current view of the modes of action of plant metabolites is still rather linear, whereas, in reality, it is likely that there are many compounds present in the oil and that the mechanisms involved may be forming a network in which pathways and molecules interact in a flexible and dynamic way (Rattan 2010; Garcia et al. 2012, Fernandes et al. 2013).

The toxic effects of the essential oil from *E. sulcata* leaves may be attributable to this complex network modes of action so that this oil could be a promising tool for integrated pest control programs against phytophagous hemipteran species which would be slow to develop resistance to this oil.

Authors' contributions

MSG drafted the paper, performed statistical analysis and contributed to biological studies in insects; BGL, CPF and LACT contributed in collecting plant sample and running the laboratory work with essencial oil; AFRO and DDN contributed to biological studies in insects; MGS contributed in plant identification and herbarium confection, CBM contributed to biological studies in insects and statistical analysis and interpretation of data, LR and DF designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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