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## Research Article

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**Posted Date:** May 18th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-523020/v1>

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**Olfactory response of the green lacewing *Chrysoperla externa* to volatile organic compounds of *Eucalyptus urograndis***

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**Abstract** The evaluation of the direct effects of the relationship between plants and predators without considering the participation of herbivores can provide vital information for the study of ecological interactions and integrated pest management. In this context, the present work studied the behavioral responses of *Chrysoperla externa* (Neuroptera: Chrysopidae) larvae to the volatile organic compounds of young and mature, undamaged and damaged leaves of *Eucalyptus urograndis* (Myrtaceae), and investigate the chemical composition of leaf essential oils and their effects on the green lacewing. The responses of the *C. externa* larvae to the odors emitted by leaves were evaluated by an experimental behavior test using a Y-tube olfactometer. The essential oil was extracted by hydrodistillation of the young and mature leaves with and without damage. The larvae respond attractively to the volatiles emitted without the participation of herbivores, and it selected preferentially odors emitted by young leaves with simulated herbivory. The chemical composition was analyzed using gas chromatography coupled with mass spectrometry. This research identified 32 compounds; some of them had not been identified in other studies. Young leaves had a higher content of essential oil compared to mature leaves. Among the compounds identified, eucalyptol,  $\alpha$ -Terpineol, Aromadendrene, and  $\alpha$ -Terpinyl acetate are the major compounds. An inversion in the content of eucalyptol (which decreases) and  $\alpha$ -terpinyl acetate (which increases) is observed when young and mature leaves are damage. Thus, this work contributed with basic data on the potential use of eucalyptus forests as maintainers of natural chrysopids populations.

**Keywords** Chemical composition, eucalyptol,  $\alpha$ -Terpinyl acetate, olfactometer, plant-predator interactions, simulated herbivory

## Introduction

*Eucalyptus* is a genus that originates from Australia, Tasmania, and other islands in Oceania, having about 730 species, of which only 20 are currently used for commercial purposes in the world (Júnior et al., 2014). Data from the Brazilian Tree Industry (IBÁ) show that the reforestation area corresponds to 7.84 million hectares with eucalyptus, pine, and other forest species (acacia, rubber, paricá, and teak) (IBÁ, 2019). These trees are used for cellulose and paper (35%), steel and charcoal (13%), wood panels and laminate flooring (6%), and other purposes (IBÁ, 2019; Santarosa et al., 2014). For this reason, the forestry sector has great economic importance in Brazil. In 2018, this sector was responsible for R\$ 86.6 billion of gross domestic product (GDP), representing 1.3% of all wealth generated in the country and 6.9% of the industrial sector. This sector is also responsible for generating R\$ 12.8 billion in federal, state, and municipal taxes, which corresponds to 0.9% of the total revenue of the country and for the direct employability of 513,000 people and 3.8 million employability direct and indirect (IBÁ, 2019).

*Eucalyptus urograndis* is one of the most used clone species in Brazil, a hybrid resulting from the cross between *E. grandis* and *E. urophylla* (Paludzyszyn-Filho et al., 2004). This combination resulted in vigorous trees with the wood of greater density and with great resistance to cancer disease caused by the fungus *Cryphonectria cubensis* (Bruner) Hodges, 1980 (Diaporthales: Cryphonectriaceae) (Paludzyszyn-Filho et al. 2004; Valeri et al. 2001). However, several insects have adapted to *Eucalyptus* plantations, probably due to the similarity with other Brazilian native plants and homogeneity of the planting that is a constant source of food (Machado et al. 2016; Queiroz et al. 2014). The adaptation occurred for several insect herbivores species, such as leaf-cutting ants (*Atta* spp. and *Acromyrmex* spp.), termites of the genera *Coptotermes* spp. and *Heterotermes* spp., and the defoliating caterpillars (*Eacles imperialis magnifica*) (Walker, 1856) (Lepidoptera: Saturniidae); as well as introduced species, such as the bronze bug, *Thaumastocoris peregrinus* (Carpintero & Dellapé, 2006) (Hemiptera: Thaumastocoridae), the gall wasps, *Epichrysocharis burwelii* (Schauff) (Hymenoptera: Eulophidae) and *Leptocybe invasa* (Fisher & La Salle) (Hymenoptera: Eulophidae), and the psyllid, *Ctenarytaina spatulate* (Taylor, 1997) (Hemiptera) (Queiroz et al., 2014).

The Chrysopidae species is among the natural predators used in the biological control of insects and pests in *Eucalyptus* forests; it is the second-largest family of Neuroptera with 75 genera, 11 subgenera, and 1,200 species (Brooks & Barnard, 1990). The chrysopids, also called green lacewings, are known as trash-carrier due to the behavior that larvae of many species carry debris on their backs, which gives them protection against natural enemies by the physical barrier and by camouflage. The debris consists of exoskeletons from their prey, fibers of plant or animal origin, and other particles they encounter during their movement, being kept attached to the body

thanks to the numerous long, smooth, or serrated bristles, with a straight or hook-shaped tip, existing on the dorsal surface and the lateral tubercles of his chest and abdomen (Albuquerque, 2009). Within green lacewing species, *Chrysoperla externa* (Hagen, 1861) is a predator with wide geographical distribution and a variety of habitats, considered one of the most promising species for biological pest control (Albuquerque et al., 1994; Resende et al., 2017). Besides, the chrysopid posture is readily recognized and easily differentiated from other insects because the eggs are pediceled (Soares et al., 2007). The larvae of *C. externa* have great reproductive and locomotion capacity, tolerance to insecticides, and they can feed on a wide variety of arthropods, including mites and small phytophagous insects, such as aphids (Soares et al., 2007). The knowledge about the life cycle and its assertiveness in the search for prey, adaptation to different climatic conditions, combined with the development of diets and mass creation in the laboratory, reflected in the increased interest of *C. externa* and the Neuroptera group in general for application in biological control programs (Duelli, 2001; Souza & Carvalho, 2002; Carvalho & Souza, 2009; Salamanca et al., 2010; Salamanca, 2015).

Thus, to understand the interactions between predatory natural enemies and forest tree species, this study aimed to verify whether the volatiles emitted by young and mature, damaged, and undamaged leaves of *E. urograndis* act attractants of chrysopid larvae. The hypothesis is that there is a preference for chrysopid larvae for the volatiles of young leaves subject to injury, considering that they have a higher production of volatile organic compounds, attracting the predator. Also, was evaluated the essential oil chemical composition of *E. urograndis* leaves. The hypothesis is that, with the injury, there will be a change in the essential oil composition of young and mature leaves of *E. urograndis*. With the observations obtained from the experimental analyzes, it will be possible to list the compounds that are attractors or repellents of *C. externa* individuals, resulting in an essential understanding of the behavior of this natural predator used in the biological control of eucalyptus forests.

## **Methods and Materials**

### **Plant culture**

*Eucalyptus urograndis* clones (Urograndis I144 type) were purchased from a plant nursery, "Viveiro Valor Verde" (Araguari, Minas Gerais, Brazil; 18°39'29.3"S and 48°09'09.4"W). The experiments were carried out with *E. urograndis* seedlings with 70 days old, 30 cm high, and containing from 10 to 12 expanded leaves.

### **Insect culture**

*Chrysoperla externa* larvae were obtained from the ALB Agroambiental biofactory (Uberlândia, Minas Gerais, Brazil). The rearing was carried out according to a methodology modified by Macedo & Soares (2000). In this procedure, the larvae were obtained from adults of *C. externa*, which were collected in the field, sent to the biofactory, and placed in cages. The laboratory environment was maintained under controlled conditions of temperature ( $25\pm 2$  °C), humidity ( $70\pm 10$  %), and using a photoperiod of 14:10 h (light:dark). Plastic cages were prepared from PVC pipe (segments of 23 cm x 23 cm), sealing at the top and bottom with organza fabric. The cage is lined internally with a sheet of A4 paper to allow the removal of the eggs. In each cage were inserted about 12 adult couples of *C. externa* and fed in the upper part through cotton containing an aqueous solution of the yeast and honey. A part of the collected eggs was reserved for the re-start of the rearing cycle of *C. externa*. The other part of the eggs was transferred to a plastic container containing *Anagasta kuehniella* eggs (Zeller, 1879) (Lepidoptera: Pyralidae) to feed the larvae obtained from *C. externa* eggs. The experiments described below were carried out with larvae of *C. externa* at  $7\pm 2$  days old.

### **Simulated herbivory treatment**

The simulation of herbivory on *E. urograndis* leaves was done by artificial damage technic through removing 6 mm leaf discs using a hole punch (P202 model, Tilibra). The experiments were divided into three groups: (1) seedling without damage on leaves; (2) seedling with young leaves with damage: four to five holes in four leaves located at the top of the seedling; and (3) seedling with mature leaves with damage: four to five holes in four leaves located at the bottom of the seedling. A reduction of 10 to 15 % of the area of each leaf was estimated, representing about 1.5 cm<sup>2</sup> of total area leaves. Holes were not made in the central rib of the leaves.

### **Behavioral evaluation using Y-tube olfactometer system**

The evaluation of the olfactory response of *C. externa* to volatile organic compounds in leaves of the *E. urograndis* was carried out using a modified model by Akol & Njagi (2003), Du et al. (1996), and Han & Chen (2002) (Fig. 1). The tubes with the plants were wrapped with aluminum foil and closed up to the height of the stem, avoiding contamination of the air with the volatile compounds from the plastic of the tube, or from the substrate used to grow the plants, or from the microorganisms present in the substrate (Pinto-Zevallos et al., 2013a,b). The Y-tube olfactometer used presents arms with 2 cm internal diameter and 17 cm long at 120° angle. The airflow in the Y-tube was generated by a vacuum pump (Big Air, A320 model) which went to the activated carbon filter (Planeta Água brand, Fit 200 model) for purification and removal of any impurities. Then, the air passed through a glass

chamber (8.5 cm x 23 cm), where the plants were placed. The entire system was interconnected by PTFE tubes. An airflow of 1.5 L min<sup>-1</sup> was used in the system and the flow was controlled through two flow meters (Key Instruments, LPM Air model).

According to the methodology of Resende et al. (2015), a previous study of the displacement of the air inside the Y-tube was carried out using water and dry ice. Our results showed there is no mixing of the air between the different arms. Each bioassay used a larva inserted in the base of the olfactometer. The choice was considered when the larva entered more than 1/3 of the Y-tube arm and remained for 15 seconds. The maximum time established for the bioassay was 10 minutes. Only larvae that chose according to these criteria were considered in the statistical analysis. Four hundred nine bioassays were performed because 280 larvae were used according to the established criteria, and 129 were not considered in the data because they did not respond to the established criteria.

To avoid any positional effects, Y-tube was horizontal turned 180° (clockwise direction) after each tested larva, and the odors presenting side were changed every three larvae. The Y-tube was changed after six larvae, and every 12 larvae, plants were changed since the production of volatiles can vary between individuals (Pareja et al., 2009). After 12 tests, the Y-tube and the glass chambers were washed with detergent (neutral soap), water, 70% ethanol and placed in an oven at 100 °C for 60 minutes. The tests were carried out from 7 am to 5 pm under constant laboratory conditions regarding luminosity (fluorescent light) and temperature (25 °C).

The behavior response of *C. external* larvae to the *E. urograndis* on the olfactory system was evaluated using 40 larvae in each of the seven combinations as described following (1) clean air in both arms of the olfactometer; (2) plant without damage *versus* clean air; (3) young leaves with damage *versus* clean air; (4) mature leaves with damage *versus* clean air; (5) young leaves with damage *versus* plant without damage; (6) mature leaves with damage *versus* plant without damage; and (7) young leaves with damage *versus* mature leaves with damage.

### **Data analysis**

The absolute frequency and percentage were used for categorical variables in the description of the results of the olfactometry analysis. The absolute frequency ( $f_i$ ) is the number of times that a specific variable presents a certain value/category. The percentage ( $p_i$ ) is the result of the ratio between the absolute frequency and the sample size, multiplied by 100. To check if there were significant differences in the percentage of choice between the alternatives of each olfactometry test, the chi-square test ( $\chi^2$ ) was applied, considering the expected proportion of

0.50 (50%) in each of the arms of the Y-tube olfactometer. The analyzes were performed in the statistical environment R Development Core Team (version 3.3.1) with a significance level of 5% (Aboubakar Souna et al., 2019).

### **Essential oil extraction**

Essential oil (EO) was extracted from the leaves of *E. urograndis*. These leaves were separated into four groups: young leaves without damage, young leaves with damage, mature leaves without damage, and mature leaves with damage. All leaves were damaged moments before the oil was extracted. Initially, the moisture content of each group of leaves was measurement by the gravimetric method using an infrared moisture determination balance (Quimis, model Kett FD-600). The method was done with 1.0 g of leaves at a temperature of  $105 \pm 5$  °C for 15 min. The extraction of the EO from each group of leaves was carried out in a Clevenger apparatus, by hydrodistillation, under reflux for 4 hours with 50 g of fresh leaves (Silva et al., 2020). The extraction was carried out in triplicate. The EO was extracted from water with dichloromethane (Vetec, RJ, Brazil) (3 x 10 mL). The solvent was removed by evaporation in a heating plate at 35 °C. Essential oils obtained were stored in glass bottles, sealed, and kept in a refrigerated environment in the absence of light.

### **Essential oil chemical composition**

The composition of EOs was identified by a gas chromatograph coupled to a mass spectrometer (GC-MS) (Shimadzu, QP2010 model) using a DB-5 capillary column (J&W, 30 m × 0.25 mm × 0.25 m). The samples of EOs were solubilized in dichloromethane at 5 mg mL<sup>-1</sup>. The conditions used were: helium as a carrier gas with a constant flow of 1.02 mL min<sup>-1</sup>; injector temperature of 240 °C; detector temperature of 220 °C; splitless mode of injection (1:10); oven temperature programmed from 60 °C and increase to 246 °C at 3 °C min<sup>-1</sup>, maintained for 38 min; the ionizing potential of 70 eV; a range of *m/z* 40-650. The compound identification of the EO was based on the similarity index (SI) obtained by the software (LabSolution-GC-MS Solution) with the mass spectral commercial libraries Nist27, Nist147, Wiley7, Wiley229, and Shim2205 libraries. Arithmetic index (AI) was also used to identify compounds about standard alkanes (Adams 2007). The AI was calculated using the equation:  $AI(x) = 100 C(Pz) + 100 [(RT(x) - RT(Pz)) / (RT(Pz + 1) - RT(Pz))]$ , in which RT is the retention time in min, x is an unknown compound, C is the carbon number of the alkane Pz that runs before x, and Pz + 1 is the alkane that runs after x. The AI obtained was compared with AIs of the Webbook-NIST Standard Reference Data (Wallace, 2018) and the Adams book (Adams, 2007).

## Results

### Olfactometry tests

The results obtained with the choices of *C. externa* larvae in each olfactometry test performed with *E. urograndis* leaves are shown in Fig. 2. The results of the Chi-square test are listed in Table 1. In the treatment in which clean air was offered on both sides of the Y-tube olfactometer, the larvae randomly chose both the left and right sides, and there was no significant difference ( $\chi^2 = 0.63$ ;  $p = 0.429$ ). When opposed to clean air, the larvae preferred the volatile compounds of the plants without damage ( $\chi^2_1 = 7.23$ ;  $p = 0.007$ ) and the plants with young leaves with damage ( $\chi^2 = 11.03$ ;  $p = 0.001$ ). The frequency of choice of the larvae on one side of the Y-tube was more than 70 % in these tests. However, the seedlings with mature leaves damage, although chosen more frequently about clean air (57.5 versus 42.5 %), did not show any significant difference ( $\chi^2 = 0.63$ ;  $p = 0.429$ ). There was also no significant predominance of the preference of larvae for the plants with young leaves with damage ( $\chi^2 = 3.02$ ;  $p = 0.082$ ) or mature leaves with damage ( $\chi^2 = 2.03$ ;  $p = 0.155$ ) when opposed to the plant without damage. Therefore, although not significant, the percentage of choice of larvae for the plants without damage (62.5 %) was higher than the plants with mature leaves with damage (37.5 %). There was also a predominance of choice of the larvae for the plants with young leaves with damage (80 %) in comparison to the plants with mature leaves with damage (20 %), showing a significant difference ( $\chi^2 = 13.23$ ;  $p < 0.001$ ).

### Essential oil extraction

The extraction yield and the amount of EO of *E. urograndis* leave obtained by hydrodistillation are shown in Table 2. The yield obtained in this study ranged from 0.29 % for mature leaves up to 0.50 % for young leaves.

### Essential oil chemical composition

The chemical composition of EOs from each group of leaves of the *E. urograndis* was determined by CG-MS. The chromatogram profiles (Fig. S1) showed 42 peaks which are was possible to identify 32 compounds. The AI of the identified compounds of EO is showed in Table S1. Table 3 shows the percentage of the identified compounds by total ion chromatogram. Figure 3 shows the structures of the compounds identified, and 69 % of them are monoterpenes oxygenated, and 22 % are oxygenated sesquiterpenes. Eucalyptol, Linalool, Borneol,  $\alpha$ -Terpineol, Neral, Carvone, Geraniol, and  $\alpha$ -Terpinyl acetate are the major oxygenated monoterpenes. Alpha-

copaene, Aromadendrene, Spathulenol, Caryophyllene oxide, Globulol, and Viridiflorol are the major sesquiterpenes.

According to Table 3, compounds **1**, **11**, **21**, and **36** presented the highest concentrations in all EO's. There was a higher concentration of Spathulenol (**36**) (12.20 %) in the EO of young leaves with damage than Eucalyptol (**1**) (10.36 %) and  $\alpha$ -Terpineol (**11**) (11.9 %). Essential oils of the mature leaves with damage presented 12.37 % of Spathulenol (**36**), a result higher than that of Eucalyptol (7.59 %) (**1**). However, the chromatogram analysis indicated that this peak is a mixture of Spathulenol with Caryophyllene oxide and Globulol; they are all oxygenated sesquiterpenes. The sum of these compounds is representative, as it amounts to almost 50% of the mass of EO.

Table 4 shows the percentage variation of the main compounds in the EOs of young and mature leaves with damage from *E. urograndis*. Compounds **28** and **42**, although having a low concentration of TIC, are among the sixteen main compounds identified as they make up about 80 % of the composition of the EOs of *E. urograndis*. These two compounds showed a significant change in their percentage of composition in the EO from the damaged leaf. It was observed that the EOs of young and mature leaves with damage showed an increase in the concentration of these compounds concerning leaves without damage. Essential oils of damaged leaves demonstrated a rise in both productions of  $\alpha$ -Terpineol (**11**) (9.6 and 19.5 % for young and mature leaves, respectively) and  $\alpha$ -Terpinyl acetate (**21**) (47.9 and 25 % for young and mature leaves, respectively).

In contrast, there was observed a significant drop in Eucalyptol (**1**) production (-63.2 and -62.7 % or young and mature leaves, respectively) in the EOs from leaves with damage. Essential oils of damaged young leaves resulted in an increase of 50.0 % in Aromadendrene (**28**) and 39.7 % in 5-Hydroxy-isobornyl-isobutanoate (**42**). Essential oils of mature damaged leaves resulted in a rise of 44.9 % in Aromadendrene (**28**) and a reduction of 24.8 % in 5-Hydroxy-isobornyl-isobutanoate (**42**).

## Discussion

### Herbivory simulation and olfactometry tests

In all analyzes carried out, although the sources of odor and the arms of the olfactometer were inverted to avoid any bias, an additional test was carried out with the provision of clean air in both arms. The result of this test demonstrated that there was no defect on either side of the olfactometer, proving the efficiency of the system and corroborating the results of other studies that used a similar methodology. Tests of this nature are commonly performed in experiments with an olfactometer system to ensure that no bias occurs, as an example, Blassioli-

Moraes et al. (2005) evaluated the response of the parasitoid *Telenomus podisi* (Ashmead) (Hymenoptera: Scelionidae) to the volatiles of soybean seeds *Glycine max* (L.) Merrill and nymphs of *Euschistus heros* (F.) (Heteroptera: Pentatomidae).

Our results demonstrated that *C. externa* larvae have a behavior of choice when in contact with volatile organic compounds of *E. urograndis*, corroborating other studies that used *Chrysoperla* species as a model. For instance, Salamanca et al. (2015) demonstrated the attraction of adults of *C. externa* to the volatiles of the *Coriandrum sativum* L. (Apiaceae) when isolated and in the presence of flowers of *Rosa hybrida* L. (Rosaceae). Resende (2012) found that unmated and mated adults of *C. externa* had different choices to the volatile coriander and fennel *Foeniculum vulgare* Mill. According to this author, unmated adults (males and females) were attracted to coriander, whereas mated ones were attracted to fennel. In a study by Zhu et al. (2005), *Chrysoperla carnea* and *Chrysopa oculata* Say (Neuroptera: Chrysopidae) had different choices when in contact with volatiles of the alfalfa (*Medicago sativa* L.) and pheromones from the aphid *Acyrtosiphon pisum* Harris (Hemiptera: Aphididae). In this study, *C. carnea* was attracted by the volatiles of alfalfa and *C. oculata* by the volatiles of the aphid. Reddy (2002) identified the preference of adults of *C. carnea* to the volatile leaves of eggplant (*Solanum melongena* L., Solanaceae), okra (*Abelmoschus esculents* L., Malvaceae), and pepper (*Capsicum annum* L., Solanaceae) subject to artificial mechanical damage.

In our study, only the insect predator and the plant were used to identify the role of volatiles in the plant-predator interactions. The results found here corroborate with other studies mentioned above since the larvae of *C. externa* made choices according to the different types of volatiles of the *E. urograndis*, without the participation of prey and other elements. According to Hogervorst et al. (2008), Limburg & Rosenheim (2001), and Takabayashi & Shiojiri (2019), predators respond to the chemical clues emitted by plants both for locating their prey and for the consumption of elements produced by plants to supplement their diet, like “honeydew” and extrafloral nectar. Ananthakrishnan (1992) reports that a natural enemy can recognize the factors of the host plant and that the decision to choose foraging in the plant is made regardless of the presence or absence of its prey or host.

The olfactometry tests indicated a variation between the volatiles emitted by young and mature leaves, corroborating with Boege & Marquis (2005). These authors suggest an inverse relationship between age and the production of indirect defenses by plants, resulting in changes in the interactions between plants, herbivores, and their natural enemies throughout the development of plant structures. Several other studies corroborate the difference between volatile organic compounds according to the ontogenetic stage of the leaves (Bracho-Nunez et al., 2011; Cole, 1980; Li et al., 1996; Maatallah et al., 2020). The damage caused on the young and mature leaves

triggered different responses of the larvae of *C. externa*, with preference to the odors emitted by young leaves. Other studies also report the preference of insects for odors of young leaves due to aspects related to oviposition and nutrition (Nahrung & Allen, 2003; Tanaka & Nakasuji, 2002).

### **Essential oil extraction**

About 17,500 aromatic species of higher plants, mainly from the Myrtaceae, Lauraceae, Lamiaceae, and Asteraceae families, produce essential oils (Regnault-Roger et al., 2012). Among the species of the Myrtaceae family, the genus *Eucalyptus* has more than 200 species with essential oil production in its leaves (Pino et al., 2002; Vitti & Brito, 2003) but less than 20 are exploited industrially (Lu et al., 2016). One of them is *E. urograndis* and is not included in the list of the main species of eucalyptus used to produce essential oil, probably because they do not have sufficient extraction yield to justify the commercial exploitation of their essential oil. Pereira (2010) collected leaves at random points in the crown of adult individuals of *E. urograndis* and found a yield of 1.56 %. The result found by this author was expressive when compared to the three species with the highest yield in Brazil, *E. citriodora* Hook, *E. globulus* Labill, and *E. staigeriana* F.Muell, with 1-1.6, 1.7-2, and 1.4 %, respectively (Vitti & Brito, 2003). However, other studies have yield results similar to those found in this study for the mature leaves. Goldbeck et al. (2014) used leaves from the canopy of *E. urograndis* individuals of 19 months old and found a 0.29 % yield for the essential oil extraction. Bonora (2016) evaluated mature leaves of *E. urograndis* and found a 0.20 % yield.

In our study, the yield found for the essential oil of mature leaves of *E. urograndis* is not high compared to other eucalyptus species. In contrast, the essential oil yield of young leaves was considered moderate, according to other studies. Silva et al. (2006) evaluated the yield of 11 *Eucalyptus* species, with *E. citriodora*, *E. viminalis* Labill, and *E. globulus* achieving the best results, 1.70, 1.56, and 1.07 %; the worst results were found for *E. pellita* F. Muell and *E. cloeziana* F. Muell, being 0.00 % and 0.12 %, respectively. In an evaluation of essential oils from 12 *Eucalyptus* species, Bonora (2016) found higher yields in *E. staigeriana* F. Muell and *E. citriodora*, with 1.6 and 1.3 %, respectively, and lower yields in *Corymbia ptychocarpa* F. Muell and *E. saligna* Smith, with 0.021 and 0.11 %, respectively.

Regarding the influence of the age of the *E. urograndis* leaves on the production of essential oil, there is still no clear trend, and further studies are needed to clarify the theme (Vitti and Brito 2003). In this sense, the results of this study have a significant contribution, since variation in yield and greater production of essential oil

was found in young leaves, corroborating with studies by Silvestre et al. (1997) and Li et al. (1994), who found the higher yield of essential oil in young leaves of *E. globulus* and *E. nitens* H. Deane & Maiden, respectively.

### **Essential oil chemical composition**

In our study, 32 compounds were identified by GC-MS analysis, which is higher than other studies, such as Goldbeck et al. (2014), Bonora (2016), Araújo et al. (2010), and Pereira (2010), who identified 21, 17, 10 and 10 compounds, respectively. This variation in the identification between the studies is due to the differences in the extraction methodology as well as in the plants used in the analysis because according to Darrow & Bowers (1997), despite the existence of genetic control, the variations of season, day, intra-plant, inter-plant, and intraspecific can influence the total content and proportions of plant secondary metabolite compounds.

Araújo et al. (2010) and Goldbeck et al. (2014) found a predominance of oxygenated monoterpenes and oxygenated sesquiterpenes in their essential oil studies *E. urograndis* leaves, corroborating the results found in this study. Vitti & Brito (2003) also report the predominance of terpenic compounds - mono and sesquiterpenes in essential oils. According to Harbone (1991), these compounds are related to plant metabolism functions, which can be found in hormones, membrane structures, and according to Andrew et al. (2013), Lawler et al. (1999), and Marsh et al. (2006), they are important in direct and indirect interactions with herbivores and other organisms.

The predominance of Eucalyptol,  $\alpha$ -Terpineol, and  $\alpha$ -Terpinyl acetate found in this study agrees with the results of Bonora (2016). This study was carried out with mature leaves of *E. urograndis*, and the compounds previously mentioned had a higher concentration (Eucalyptol,  $\alpha$ -Terpineol, and  $\alpha$ -Terpinyl acetate with 17.7, 17.8, and 15.6 %, respectively). This variation between compounds is also corroborated by Dellacassa & Moyna (1992), who clarifies that the existence of qualitative variations among individuals of the same species is common due to genetic, environmental, type of leaves selected and differences in extraction techniques and equipment used in the analysis of essential oils.

The olfactometry tests demonstrated that the leaves of *E. urograndis* responded to simulated herbivory by changing the concentration of the compounds in its essential oil, corroborating with Turlings et al. (1990). Also, such response influenced the behavior of *C. externa*, following Turlings & Wackers (2004) when stating that behavioral observations and chemical analyses strongly suggest that the induced volatiles of plants plays a fundamental role in the location of the host or prey by natural enemies.

The results of this research demonstrate a preference for *C. externa* over the odor of young leaves with damage of *E. urograndis* (Table 1). Considering only the variation of the compounds Eucalyptol,  $\alpha$ -Terpineol and

$\alpha$ -Terpinyl acetate after the damage in young and mature leaves shown in Table 4, there is an inversion in the concentration between Eucalyptol (which decreases) and  $\alpha$ -Terpinyl acetate (which increases) with the damage caused to the leaf, whether young or mature. Besides, observing Table 2, the essential oil content of young leaves is higher than the mature leaves. From this, an important observation can be highlighted, the content of volatile compounds may have influenced the attraction of *C. externa* since when there are options between clean air and volatiles, the *C. externa* larvae prefer the volatiles. The higher the volatile content, the greater the attraction. Therefore, in the test of choice between young and mature leaves with damage, the *C. externa* larvae prefer young leaves with damage. Also, another important observation must be highlighted, the inversion observed in the content of Eucalyptol (which decreases) and  $\alpha$ -Terpinyl acetate (which increases) may be due to the damage caused to the leaves since this was observed in young and mature leaves. This damage may have influenced the biosynthesis of  $\alpha$ -Terpinyl acetate, a more volatile compound than Eucalyptol. From the observation of the larva choice (Fig. 1) between young leaves with damage and young and mature leaves without damage, it is verified the preference of the larvae for the volatiles emitted by the young leaves with damage. This shows that something different may be present in the volatiles that acted as major attractors of *C. externa* larvae. The inversion is in the content between Eucalyptol (which decreases), and  $\alpha$ -Terpinyl acetate (which increases) may justify this behavior of *C. externa*. These results contribute to identifying the potential use of eucalyptus forests as natural maintainers of *C. externa* populations.

**Acknowledgments** To the Graduate Program in Ecology and Conservation of Natural Resources of the Federal University of Uberlandia that allowed this study to be carried out. To the Laboratory of Ecology and Biodiversity (LEBIO) of the Federal University of Sergipe and the Laboratory of the Nucleus for Research in Natural Products (NuPPE), the Institute of Chemistry from the Federal University of Uberlandia that provided the adequate structure for the realization of research.

**Funding** This research was supported by grants from FAPEMIG, grant numbers APQ-02342-18, APQ-01392-14 and APQ-02481-14 awarded to R.M.F.S., A.O. and M.P., respectively, and federal funding agency Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), grant number 449846/2014-8, awarded to M.P. and grant number to 312752/2018-0, awarded to J.C.S. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. The authors thank Ricardo

Reis Soares for GC-MS equipment, and to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for scholarships.

**Conflicts of interest/Competing interests** The authors have no conflicts of interest to declare relevant to the content of this article.

**Ethics approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** The authors consent to the publication of the article and its data in this journal.

**Availability of data and material** Data are not openly accessible.

**Code availability** Not applicable.

**Author contributions** David J. V. Borges: Methodology, CG-MS analysis, Writing - original draft. Rafael A. C. Souza: Methodology, CG-MS analysis, Writing - original draft. Alberto de Oliveira: Funding acquisition, Supervision, Conceptualization, Writing - review & editing. Raquel M. F. Sousa: Funding acquisition, Supervision, Conceptualization, Writing - review & editing. Jean C. Santos: Funding acquisition, Supervision, Conceptualization, Writing - review & editing.

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## Tables

**Table 1.** Difference tests of the percentage of choice for the treatment of the olfactometer. \* $p < 0.05$ .

Olfactometer treatment		Chi-square test	
Left	Right	$\chi^2$	<i>p-value</i>
Clean air	Clean air	0.63	0.429
Young and mature leaves without damage	Clean air	7.23	0.007*
Young leaves with damage	Clean air	11.03	0.001*
Mature leaves with damage	Clean air	0.63	0.429
Young leaves with damage	Leaves young and mature without damage	3.02	0.082
Mature leaves with damage	Leaves young and mature without damage	2.03	0.155
Young leaves with damage	Mature leaves with damage	13.23	< 0.001*

**Table 2.** Essential oil (EO) content from *E. urograndis* leaves by hydrodistillation method.

<b>Samples</b>	<b>Mass (g)</b>	<b>Moisture content (%)</b>	<b>EO (mg)</b>	<b>Yield (%)</b>
Young leaves without damage	50.0±0.2	60.7±2.42	99.5±0.3	0.50±0.02
Young leaves with damage	50.0±0.2	60.7±2.42	100.4±0.6	0.50±0.03
Mature leaves without damage	50.0±0.2	55.37±3.16	82.0±0.1	0.37±0.05
Mature leaves with damage	50.0±0.2	55.37±3.16	65.4±0.7	0.29±0.03

**Table 3** Chemical composition and total ion chromatogram (%) of the essential oils from *E. urograndis* leaves.

Peak	Compound	TIC (%)			
		Young leaves without damage	Young leaves with damage	Mature leaves without damage	Mature leaves with damage
01	Eucalyptol	28.16	10.36	20.33	7.59
02	Linalool oxide <cis-> (furanoid)	0.25	0.20	0.19	0.13
03	Linalool oxide <trans-> (furanoid)	0.29	0.19	0.22	0.16
04	Linalool	0.60	0.57	0.57	0.49
05	NI	0.40	0.06	0.1	0.05
06	Fenchol<endo->	0.63	0.54	0.69	0.56
07	Campholenal<alpha->	0.26	0.06	0.18	0.16
08	Borneol	1.31	1.80	2.08	2.38
09	Terpinen-4-ol	0.99	1.18	1.43	1.38
10	$\rho$ -Cymen-8-ol	1.17	0.85	0.55	0.50
11	$\alpha$ -Terpineol	10.85	11.90	11.49	13.73
12	NI	1.48	0.55	0.58	0.48
13	2-hydroxy-1,8-cineole	3.08	1.68	1.59	1.46
14	Neral	0.48	0.27	0.27	0.23
	Carvone*				
15	Geraniol	0.55	0.76	0.90	1.04
16	Geranial	1.14	0.55	0.52	0.58
17	NI	1.48	1.02	0.94	0.92
18	NI	1.52	0.74	1.12	1.13
19	Verbenyl acetate<trans->	0.18	0.21	0.17	0.19
20	Exo-2-Hydroxycineole acetate	0.53	0.61	0.48	0.56
21	$\alpha$ -Terpinyl acetate	14.24	21.06	17.62	21.96
22	NI	0.35	0.33	0.34	0.37
23	Alpha-copaene	0.27	0.40	0.30	0.38
24	Geranyl acetate	1.67	2.26	1.65	1.89
25	NI	0.82	0.33	0.32	0.48
26	Carvone hydrate	1.27	1.53	1.49	1.55
27	NI	1.02	1.15	1.20	0.91
28	Aromadendrene	0.70	1.05	0.49	0.71
29	NI	1.10	1.11	1.08	1.38
30	NI	1.70	2.02	2.40	2.61
31	NI	0.59	0.73	0.59	0.65
32	Geranyl isobutyrate	2.26	3.60	3.22	4.44
33	NI	1.13	1.59	1.25	1.27

34	Flavesone	0.39	0.71	0.49	0.67
35	8-acetoxy-Carvotanacetone	2.84	3.99	3.49	3.61
	Spathulenol **				
36	Caryophyllene oxide **	7.25	12.20	10.75	12.37
	Globulol **				
37	Viridiflorol	0.90	1.55	2.14	2.82
38	NI	0.49	0.83	0.77	0.94
39	Humulene epoxide II	0.28	0.48	0.35	0.55
40	Isoleptospermone	1.58	2.64	2.39	3.21
41	NI	2.52	4.64	1.88	2.43
42	5-Hydroxy-isobornyl-isobutanoate	1.16	1.62	1.29	0.97
	<b>Total identified (%)</b>	<b>85.28</b>	<b>84.82</b>	<b>87.33</b>	<b>86.27</b>

NI: not identified. \*Carvone mixed with Neral. \*\* Caryophyllene oxide and Globulol mixed with Spathulenol.

**Table 4** Variation of the percentage of some compounds in the essential oils of *E. urograndis* according to the damage.

Peak	Compounds	TIC (%) Average					
		Young leaves without damage	Young leaves with damage	Difference (%)	Mature leaves without damage	Mature leaves with damage	Difference (%)
01	Eucalyptol	28.16	10.36	-63.2	20.33	7.59	-62.7
11	$\alpha$ -Terpineol	10.85	11.90	9.6	11.49	13.73	19.5
21	$\alpha$ -Terpinyl acetate	14.24	21.06	47.9	17.60	22.00	25.0
28	Aromadendrene	0.70	1.05	50.0	0.49	0.71	44.9
42	5-Hydroxy-isobornyl isobutanoate	1.16	1.62	39.7	1.29	0.97	-24.8

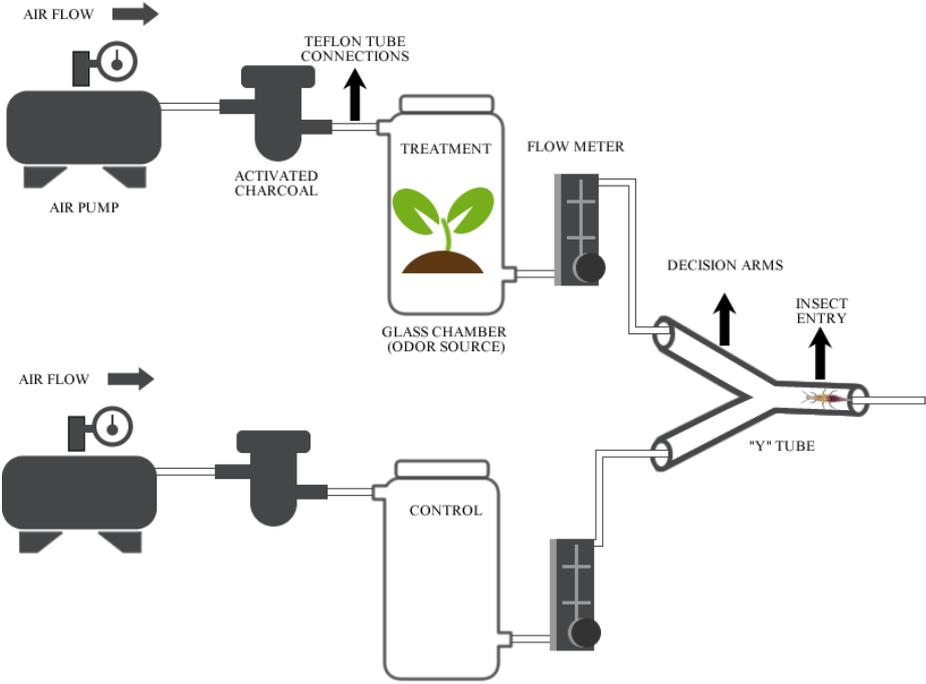
## Figure legends

**Fig. 1** Schematic diagram of the Y-tube olfactometer system.

**Fig. 2** Frequency distribution of the *C. externa* larva choice (n = number of larvae that chose one of the arms) (ns: not significant; \* $p < 0.05$ ).

**Fig. 3** Structures of the compounds identified in the essential oils from *E. urograndis* leaves.

Figure 1



**Figure 2**

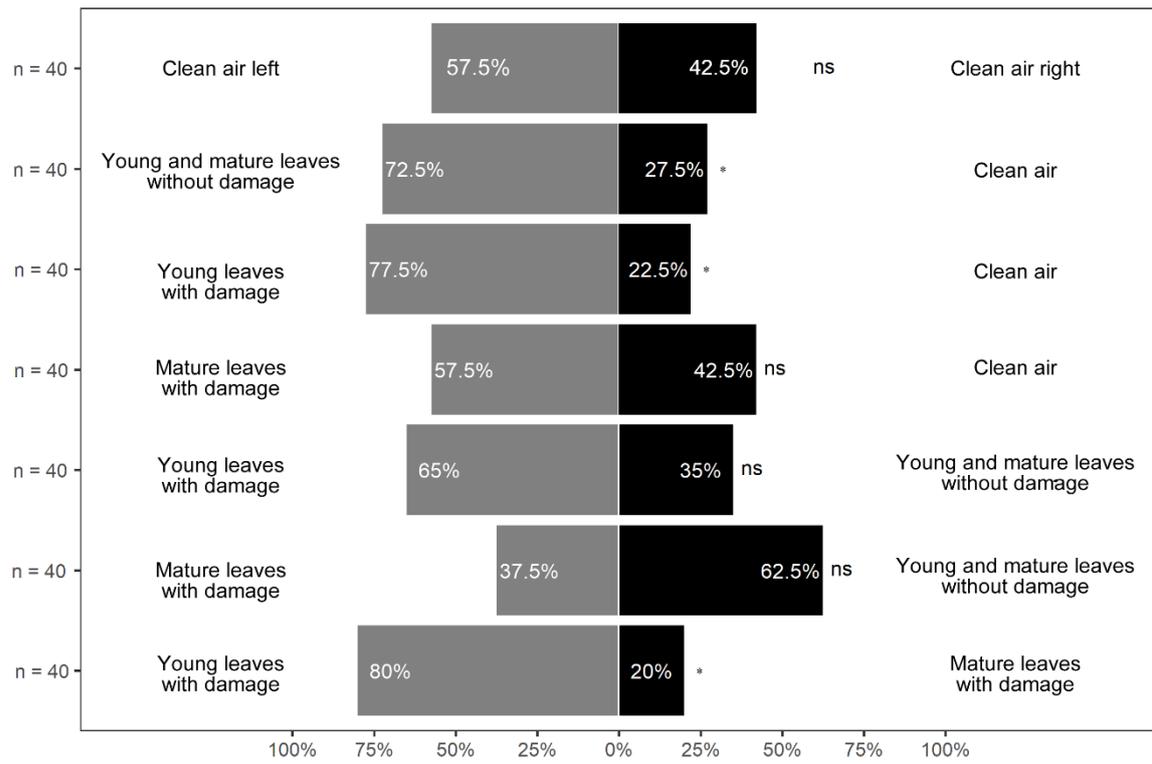
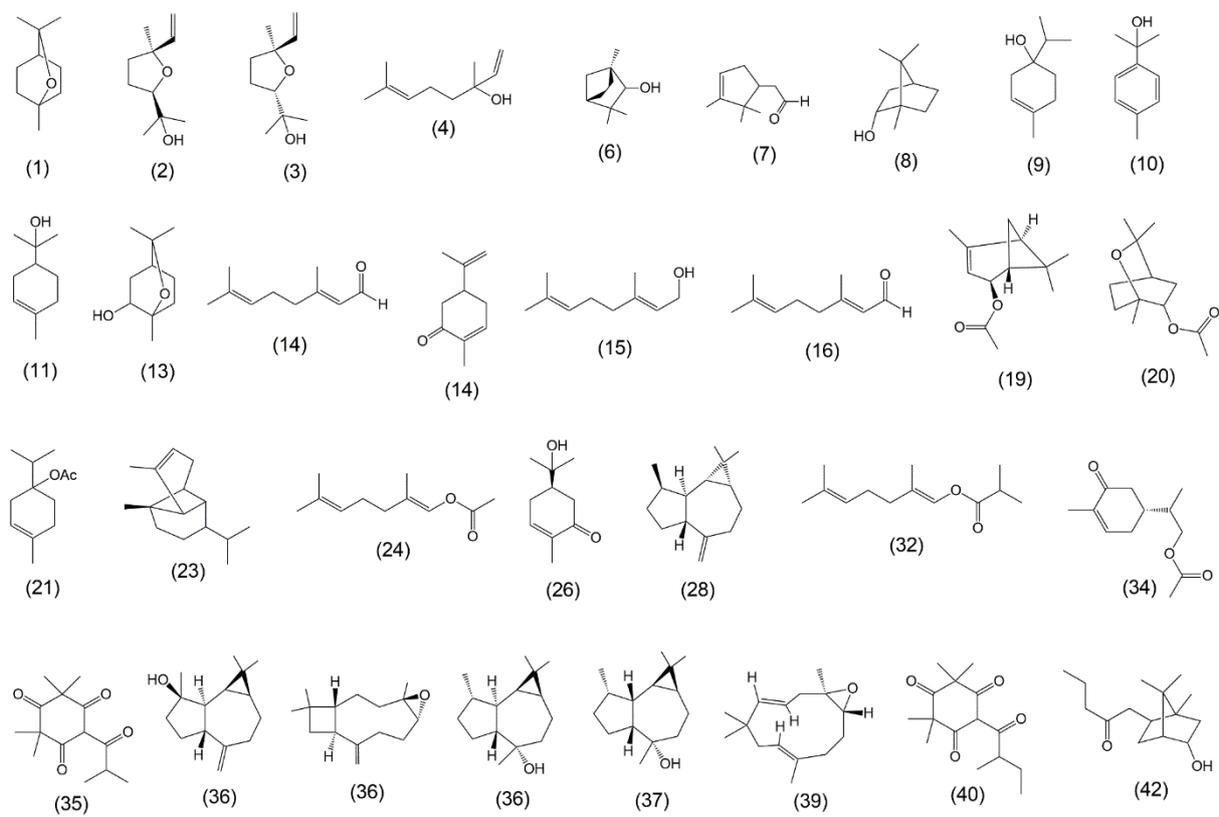
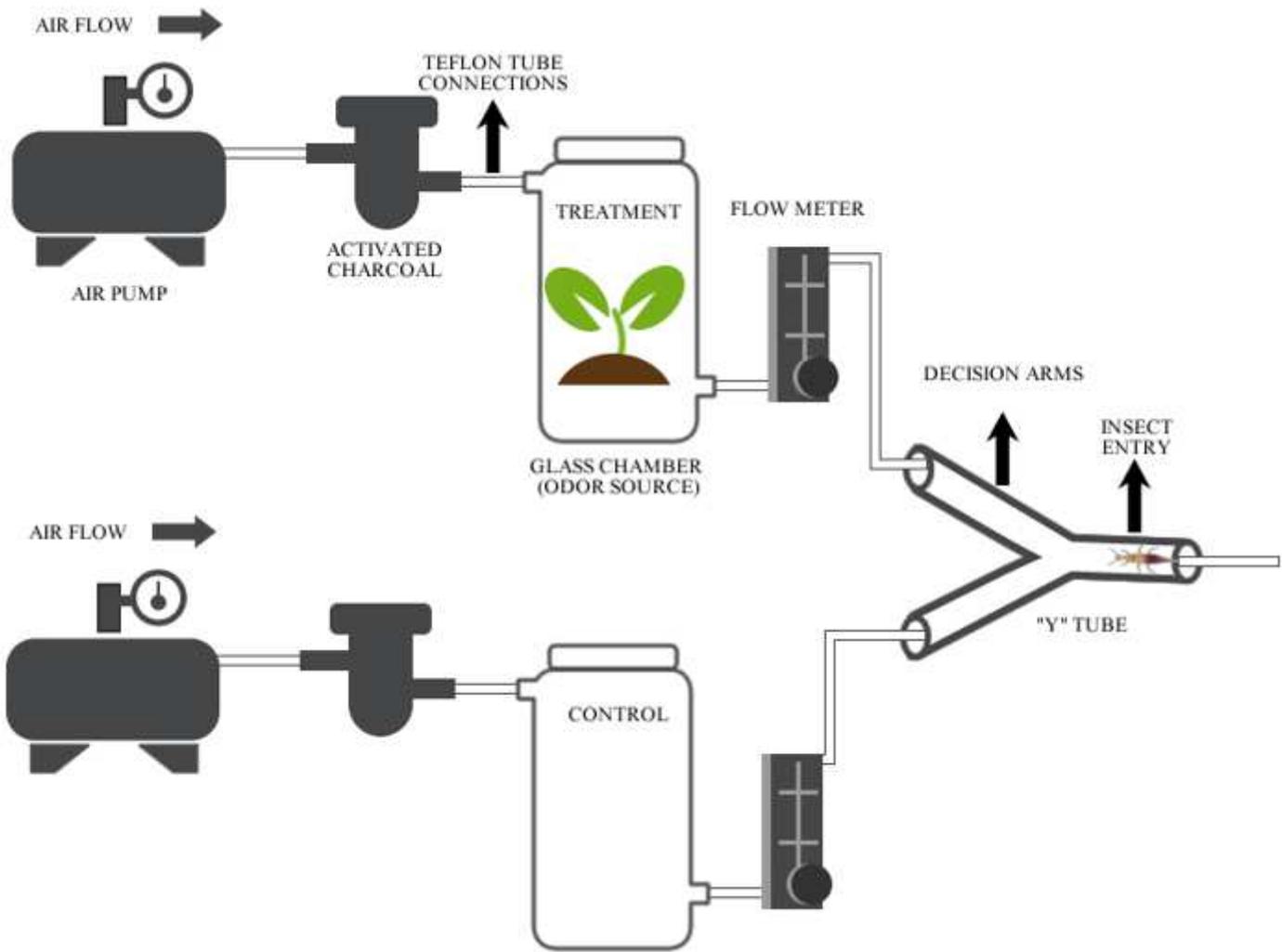


Figure 3

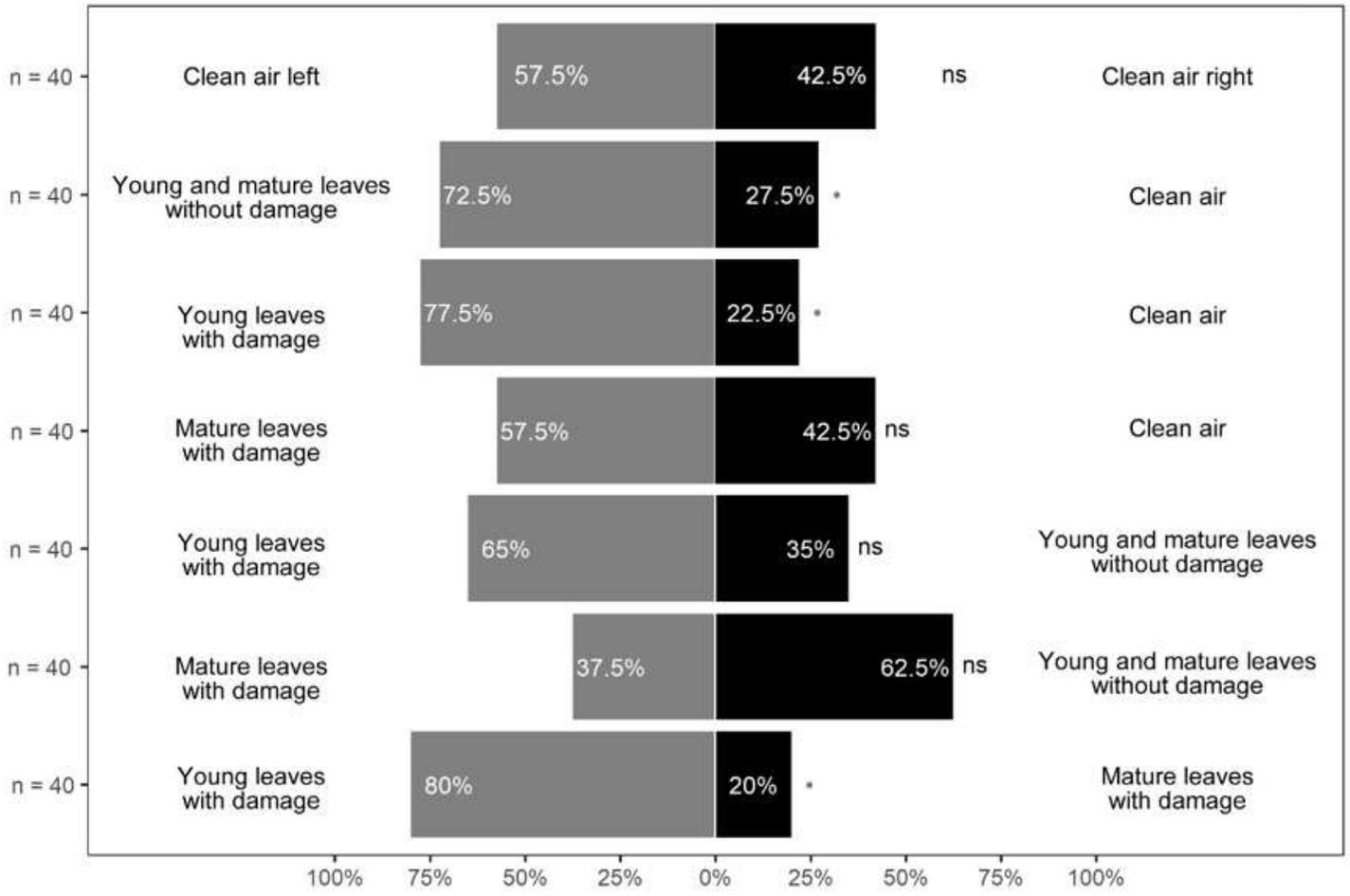


# Figures



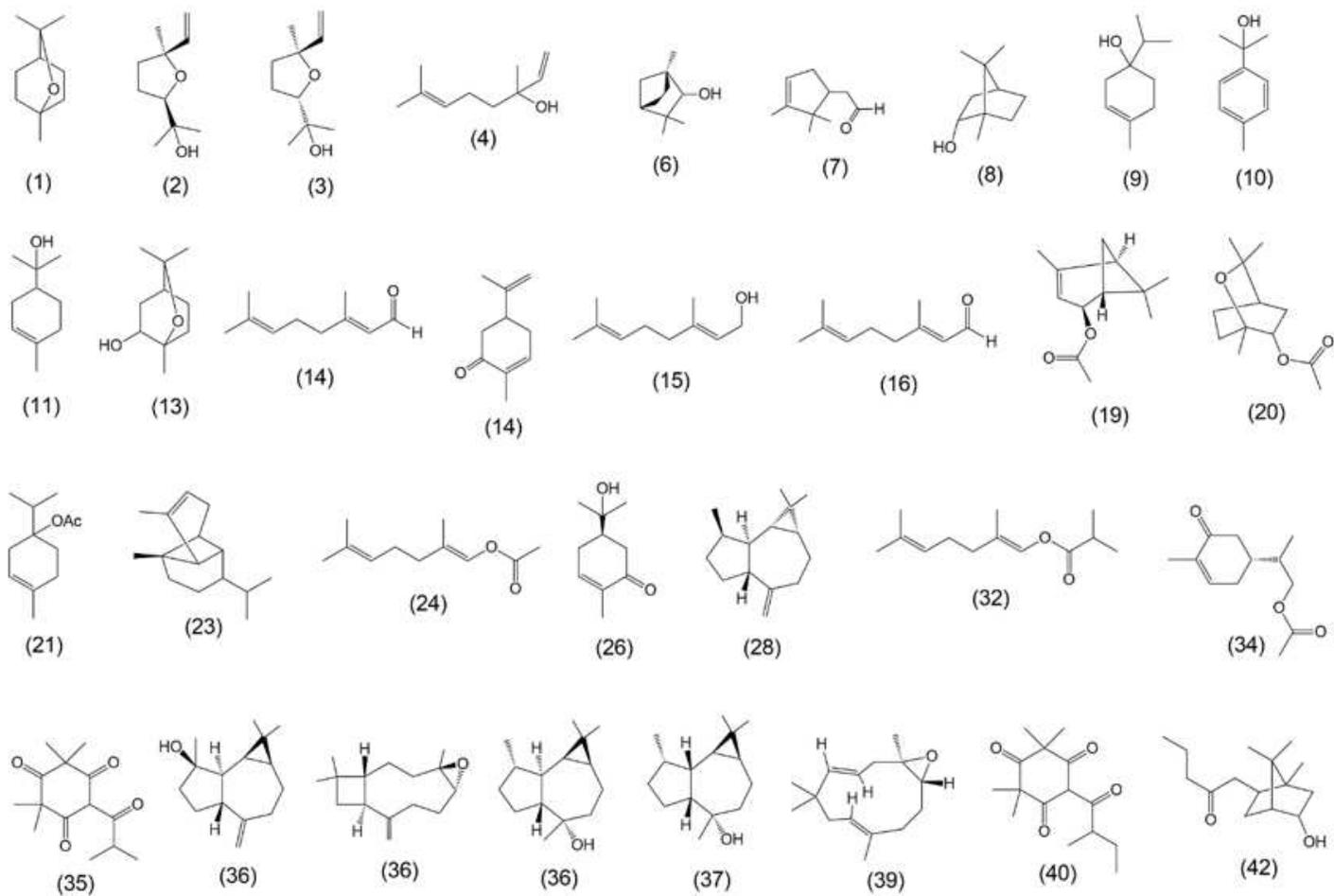
**Figure 1**

Schematic diagram of the Y-tube olfactometer system.



**Figure 2**

Frequency distribution of the *C. externa* larva choice ( $n$  = number of larvae that chose one of the arms) (ns: not significant;  $*p < 0.05$ ).



**Figure 3**

Structures of the compounds identified in the essential oils from *E. urograndis* leaves.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementarymaterial2a.opcaofigmodif12052021.docx](#)