Potential repellent activity of the essential oil of *Ruta chalepensis* (Linnaeus) from Chile against *Aegorhinus superciliosus* (Guérin) (Coleoptera: Curculionidae).

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Abstract

The objective of this study was to evaluate the repellent effect of the essential oil of the rue (*Ruta chalepensis*) against the weevil *Aegorhinus superciliosus*, an important pest of fruit crops in Chile. Rue essential oil was obtained by steam distillation, and its components were identified by GC-MS. Their effect on adult *A. superciliosus* insects was evaluated using four-arm olfactometric bioassays. The extraction process had a yield of 0.3% on a dry weight basis, and a chromatographic analysis showed the presence of nine compounds, which represented 89.3% of the total components. The major compounds were 2-nonanone (41.7%) and 2-undecanone (40.1%). Behavioral bioassays showed that the rue essential oil elicited a repellent effect against male and female *A. superciliosus* ($p \leq 0.05$) at a concentration of 1.92×10^7 ng/cm². However, at a lower concentration of the oil (285.7 ng/cm²), only females were repelled ($p \leq 0.05$). The repellency observed against *A. superciliosus* could be attributed to high concentrations of both ketones, suggesting that rue essential oil can be considered as a potential repellent that could reduce the infestation of this weevil. The role of the compounds identified and the repellent activity of this evergreen shrub are discussed.

Keywords: Essential oil, *Ruta chalepensis*, *Aegorhinus superciliosus*

1. Introduction

Ruta chalepensis (Rue) is an aromatic evergreen shrub that belongs to the family *Rutaceae*. It is native to the Mediterranean and is currently distributed worldwide (Akkari *et al.*, 2015). In Chile, it is traditionally cultivated for its pharmacological uses; infusions of its fresh leaves

are widely used as treatment for gastric disorders, headache and rheumatism, as well as for their diuretic, anti-inflammatory and anti-spasmodic properties. Analysis of the chemical composition of *R. chalepensis* extracts indicates that the leaves and stems contain alkaloids, phenols, flavonoids,

amino acids, saponins and furocoumarins, some of which are responsible for the reported activities (Kacem *et al.*, 2015). Furthermore, rue essential oil is a valuable source of active metabolites used in different industries, including cosmetics, perfumes and phytotherapy. Ketones, acyclic alkenes, monoterpenes hydrocarbons, sesquiterpenes, esters and aldehydes have been identified as the main chemical groups present in the essential oil, and the ketone 2-undecanone is a characteristic compound of the *Ruta* species (Haddouchi *et al.*, 2013; Ferhat *et al.*, 2014). These compounds are produced during secondary metabolism in the plants, and their synthesis and accumulation might vary by species (Conti *et al.*, 2013). In addition, both intrinsic and environmental factors influence this process (Ferhat *et al.*, 2014; Da Silva *et al.*, 2014). Recent studies have revealed several biological properties of rue essential oil. Insecticidal activity has been the focus of great interest for the potential use of this oil as an alternative botanical pesticide (Conti *et al.*, 2013). In fact, insecticidal effects against ticks, mosquitoes, curculionids pest of stored grain and moths (Bissinger and Roe, 2010; Majdoub *et al.*, 2014; Akkari *et al.*, 2015) and a repellent effect on *Cydia pomonella* Linnaeus (Lepidoptera: Tortricidae) and *Aedes aegypti* Linnaeus (Diptera: Culicidae) (Landolt *et al.*, 1999; Tabanca *et al.*, 2012) have been reported. Therefore, the bioactivity of rue essential oil would be useful for controlling pests of economic importance, such as the raspberry weevil, in our country.

In Chile, *Aegorhinus superciliosus* or the raspberry weevil is a native insect and one of the most important pests of fruit crops, such as the European hazelnut (*Corylus avellana*) (Fagales: Betulaceae), blueberries (*Vaccinium corymbosum*) (Ericales: Ericaceae) and raspberries (*Rubus idaeus*) (Rosales: Rosaceae) (Aguilera *et al.*, 2011). This polyphagous

insect begins to colonize the host plant when female adults oviposit near the neck of a tree. Neonate larvae move to the radical zone to feed and continue their development within this zone, while the adult weevil feeds on seasonal leaves and shoots. Currently, the widespread use of synthetic insecticides, especially organophosphates and carbamates, has not been able to prevent the damage caused by this pest. Because the application of insecticides is associated with many problems, such as high cost, residues on harvested fruit, environmental degradation and resistance development (Parra *et al.*, 2009), there is growing interest in the search for new control alternatives. In this context, natural products as well as essential oils can be alternatives for pest control because they contain bioactive substances that have low toxicity and a lesser impact on human health and the environment (Regnault-Roger *et al.*, 2012). Currently, no reports about the repellent effect of the rue essential oil in this curculionid exist. However, there is evidence that show the insecticidal potential of the essential oil of *Drimys winteri* on adult *A. superciliosus* (Rebolledo *et al.*, 2012), indicating that essential oils can be effective alternatives for the control of these insects. Therefore, the main objective of this study was to evaluate the repellent efficacy of the *R. chalepensis* essential oil against *A. superciliosus*.

2. Materials and Methods

2.1. Plant material and essential oil isolation procedure

The aerial parts of the rue plant were collected during the flowering stage (October 2012) from an experimental field located at the commune of Puerto Montt (41°28' South, 72°57' West) Región de Los Lagos, Chile. The plant identity was confirmed

by a comparison of macroscopic and microscopic morphological characteristics to Flora de Chile and specimens in the Herbarium of the Universidad de Concepción (CONC), Chile. The extraction process for the rue essential oil followed the methodology described by Meccia *et al.* (2009). A sample of 1298 g containing rue leaves, stems and flowers was cut into small pieces and air-dried at room temperature for six days and then oven-dried for 24 h at 20 °C. A 250 g dry matter sub-sample was subjected to steam distillation in a Clevenger-type apparatus with 500 mL distilled water for 4 h. The oil was dried over sodium sulphate and stored at 4 °C in an amber bottle prior to chromatographic analysis.

2.2. Analysis of volatile compounds by GC-MS

Volatile compounds (1 µL) of *R. chalepensis* essential oil dissolved in dichloromethane (HPLC grade, Sigma Aldrich, Steinheim, Germany) were injected at a concentration of 1 µg/µL into a gas chromatograph (Focus GC; Thermo Electron Corporation) coupled to a mass spectrometer (DSQ; Thermo Electron Corporation). The capillary column was BP-1 (30 m x 0.22 mm x 0.25 µm) and used helium as the carrier gas at a flow rate of 1.5 mL/min. The injector and interface were operated at 250 °C by setting the detector temperature at 200 °C, with an electron impact ionization of 70 eV. The initial oven temperature was programmed to 40 °C for 5 min and increased (5 °C/min) until 280 °C was reached. Mass spectroscopy was performed in a mass range from 30 to 350 *m/z*. Component identification was performed by searching a library (Mass Spectral Library Version 2.0) of mass spectra using a matching algorithm with a reverse search technique and by the injection of an alkene series (C₉-C₂₆) that was used as a reference for calculating the Kovats indices (KI). Experimental KI

were compared with the theoretical KI of compounds reported in the NIST database (NIST ver. 2.0, Thermo), as described in Tampe *et al.* (2015).

2.3. Insects

Aegorhinus superciliosus adults were manually collected between November 2012 and February 2013 from a blueberry plantation in the commune of Collipulli (37°50' South, 72°08' West), La Araucanía, Chile. Both sexes of *A. superciliosus* were collected at least one month before being used in the olfactometric bioassays. During that time period, the insects were acclimated and maintained under laboratory conditions. They were fed blueberry leaves and shoots and distilled water, and the photoperiod was a 16 h photophase at 20°C. Twenty-four hours before each bioassay, the insects were separately maintained in individual Petri dishes (5 cm i.d and 2 cm height) according their sex and deprived of food. The methodology used to identify the sex of the insects, consisted of examining the femur length of the third pair of legs; if this length exceeds the posterior suture of the fourth sternite, the insect is male, and if it does not, the insect is female (Reyes, 1993). Then, the insects were observed for 10 minutes, and those that were active and walking were selected for the olfactometric assays. A different individual was used in each separate experimental replicate (Parra *et al.*, 2009).

2.4. Olfactometric bioassay

The olfactometric bioassays were performed in a four-arm olfactometer (40 by 40 by 2.5 cm), which was described by Parra *et al.* (2009). The behavioral response of *A. superciliosus* to rue essential oil was studied according to the methodology described in Tampe *et al.* (2015), which consisted of an observation

of the movement of each insect into the olfactometer during a 20 min period. In addition, EthoVision 3.1 software was used to determine the time the insect spent in each section of the olfactometer, which was divided into 5 areas: a central square zone where the vacuum bomb, with an air flow (800 mL/min) that carried the volatile stimuli into the olfactometer, was connected and four zones corresponding to the arms, two of which were enriched with air containing volatile components released from the essential oil (S), and the other two contained dichloromethane (HPLC grade, Sigma Aldrich, Steinheim, Germany) as a control (C). Bioassays were performed by applying 1 μ L of rue essential oil on Whatman N° 1 filter paper (0.5 cm wide by 3.5 cm long) that was placed into glass tubes (7 cm long $\times$ 1.5 cm i.d.) in two opposite arms of the olfactometer (S), while the two opposite arms of the olfactometer contained only paper (C). The dose used was 1.92×10^7 ng/cm². To evaluate the diluted oil, 50 μ L of the oil diluted in CH₂Cl₂ was applied to Whatman N° 1 paper filters in two opposite arm of the olfactometer (S) under the same conditions as in the previous bioassay, while the other two arms contained only dichloromethane (C). The dose used was 285.7 ng/cm² because it was previously determined as an effective repellent in behavioral bioassays in *A. nodipennis* (Tampe *et al.*, 2015). Twenty repetitions were performed for each sex.

2.5. Statistical analysis

A statistical analysis was performed using Stats Direct software, version 2.7.2. The data obtained in the bioassays were expressed as the average time spent

in each arm of the olfactometer $\pm$ standard error and were compared by using a non-parametric Friedman test ($p \leq 0.05$) followed by a Conover-Inman test.

3. Results

Steam distillation of the aerial parts of *R. chalepensis* yielded 0.3% (mL/g) of a yellowish color oil and strong odor. In the GC-MS analysis of the sample of rue essential oil, nine compounds were identified, and among them were ketones and esters (Table 1), representing 89.3% of the total components. The main constituents were the two aliphatic ketones 2-nonanone (41.7%) and 2-undecanone (40.1%). In addition, 7 unidentified compounds were present in the sample, representing 10.7% of the total components. The mass spectral data are shown in Table 2.

The olfactometric response of *A. superciliosus* toward rue essential oil indicated that both females and males were repelled by the essential oil ($p < 0.0001$). The average time spent by the females in the stimulus source was significantly less (1.69 ± 0.62 min) than in the control (16.47 ± 0.7 min; $F = 46.11$; $df = 2$; $p < 0.0001$) (Figure 1A). Similar behavior was observed in males; where the male insects spent less time in the stimulus (2.41 ± 1.0 min) than in the control (16.48 ± 1.0 min; $F = 39.91$; $df = 2$; $p < 0.0001$) (Figure 1B). In bioassays using diluted rue essential oil (285.7 ng/cm²; Figure 1C and 1D), males did not show a behavioral difference between the stimulus and the control ($p > 0.05$), while females were repelled by the tested dose of oil ($F = 71.47$; $df = 2$; $p = 0.0105$) remaining for an average of 11.21 ± 0.7 min in the control compared to 7.22 ± 0.6 min in the stimulus.

Figure 1. Average time spent (min) ($\pm$ SE) of *Aegorhinus superciliosus* in rue essential oil (1.92×10^7 ng/cm²) (Figure A and B) and in the dilution of rue essential oil (285.7 ng/cm²) (Figure C and D) in the olfactometer test. Different letters indicate significant difference ($p \leq 0.05$) based on the non-parametric Friedman test followed by Conover-Inman test. S= Stimulus, C= Control and DZ= Decision zone. N=20 per sex.

Table 1. Percentage of compounds identified in the *Ruta chalepensis* essential oil.

Rt	Compound	Identification	Percentage (%)
11.08	2-Octanone	MS, KI	0.51
14.34	2-Nonanone	MS, KI	41.71
15.84	unknown 1	MS	0.5
16.04	unknown 2	MS	2.76
16.80	unknown 3	MS	0.73
17.27	2-Decanone	MS, KI	2.18
18.68	unknown 4	MS	5.63
20.2	2-Undecanone	MS, KI	40.11
22.07	2-Dodecanone	MS, KI	0.97
25.41	2-Tridecanone	MS, KI	0.91
28.75	unknown 5	MS	0.39
35.84	unknown 6	MS	0.24
36.12	Ethyl palmitate	MS, KI	1.97
39.06	unknown 7	MS	0.42
39.33	Ethyl oleate	MS, KI	0.22
39.77	Ethyl stearate	MS, KI	0.77

Rt= Retention time, MS= mass spectrum, KI= Kovats indices (KI) was performed by injecting an alkenes series (C₉-C₂₆). The experimental KIs were compared with theoretical KI compounds reported in the database “NIST”

Table 2. Mass spectral data of the unknown volatile compounds from *Ruta chalepensis*.

Rt	Compound	(%)	<i>m/z</i>
15.84	unknown 1	0.5	32(100), 79(30.2), 43(19.9), 94(13.9), 41(10), 51(10.0), 77(9.9), 39(7.7), 105(6.3), 78(5.4), 40(5.3), 91(4.2), 93(4.0), 31(3.5), 70(3.4).
16.04	unknown 2	2.76	79(100), 94(49.3), 77(28.0), 51(23.7), 32(18.5), 39(16.6), 41(15.7), 93(12.3), 105(10.9), 91(8.8), 106(7.7), 53(7.4), 80(6.0), 147(5.8), 120(5.4).
16.80	unknown 3	0.73	128(100), 32(72.3), 51(34.2), 127(16.0), 50(14.8), 129(11.0), 126(9.5), 102(7.0), 101(6.0), 75(5.3), 64(4.6), 74(3.8), 40(3.79), 77(3.6), 76(3.4).
18.68	unknown 4	5.63	43(100), 41(16.2), 70(12.6), 55(11.5), 126(11.4), 69(10.8), 97(10.0), 87(9.4), 56(8.7), 42(7.1), 71(6.0), 98(4.6), 57(3.7), 84(3.7), 39(3.7).
28.75	unknown 5	0.39	157(100), 115(53.1), 43(41.6), 129(21.1), 203(19.6), 42(17.4), 111(13.7), 130(10.4), 31(9.9), 69(8.1), 158(7.6), 47(7.3), 32(6.7), 45(6.6), 56(5.6).
35.84	unknown 6	0.24	55(100), 41(94.2), 69(63.2), 97(53.8), 96(40.1), 70(36.7), 98(35.3), 101(33.9), 95(31.2), 83(30.4), 67(29.1), 73(24.5), 81(23.9), 68(23.6), 43(23.4).
39.06	unknown 7	0.42	32(100), 67(17.6), 41(15.4), 95(12.4), 55(11.3), 81(10.2), 69(9.5), 43(9.3), 96(9.2), 68(8.6), 54(6.3), 40(5.8), 109(5.7), 79(5.6), 97(5.5).

Rt = Retention time, *m/z* = mass-to-charge ratio

4. Discussion

Steam distillation usually produces yields less than 1% (Regnault-Roger *et al.*, 2012). Our essential oil yield was 0.3% (v/w), which is in line with a report by Dob *et al.* (2008), who indicated that the essential oil of *R. chalepensis* obtained from Algeria

by hydrodistillation had a yield of a 0.27%, based on sample dry weight. On the other hand, the yield of our sample was lower than that reported by Tounsi *et al.* (2011), who showed an increasing trend (0.39% to 2.46%) of essential oil obtained from the leaves, stems, flowers and fruits of *R. chalepensis*. Similarly, Mejri *et al.* (2010) showed a higher yield (5.51%)

of *R. chalepensis* essential oil from plants growing in Tunisia and indicated that the drying process had a significant effect on the proportions of main components. The differences in the oil yield might depend on the developmental stage of the plant itself or the different organs used. Some examples have demonstrated that the net essential oil content has been associated with the early growth period in a plant or with senescence (Sangwan *et al.*, 2001). Our plant sample could have been extracted during a period of low accumulation of oil, which would explain the observed differences. Moreover, our essential oil was obtained from pooled organs (leaves, stems and flowers), but the yield was lower than in other studies that used specific *R. chalepensis* organs (Tounsi *et al.*, 2011). In addition, the methods employed to obtain the oil (hydro and steam distillation), together with the diverse weather conditions in the growth habitat, could influence the obtained yield (Mejri *et al.*, 2010; Tounsi *et al.*, 2011; Regnault-Roger *et al.*, 2012).

Interestingly, this essential oil contained a 2-ketone series from C₈ to C₁₃ (Table 1). The essential oils from the genus *Ruta* are known for having two methyl-2-ketones (2-nonanone and 2-undecanone) in their chemical profile; reaching 80% of the oil composition (Haddouchi *et al.*, 2013), which is consistent with our results (81.8%). Similarly, the ketone-type compounds identified as main constituents in our study were consistent with those reported by other authors in other species from the same genus (Dob *et al.*, 2008; Da Silva *et al.*, 2014), indicating that the presence of 2-undecanone does not change with respect to the geographical area where plant grows, but its proportions vary, ranging from 28.2% to 67.8%. The proportions of 2-nonanone range from 5.2% to 53.1%. The nature and proportions of other constituents of our essential oil were not the same as in other reports. We identified nine compounds, with ketones and esters among them (Table 1).

These results disagree with the report by Mejri *et al.* (2010; 2012), who found oxygenated compounds in aerial parts of *R. chalepensis* essential oil; among them were alcohols, monoterpenes and acetates. Moreover, Tounsi *et al.* (2011) reported the presence of monoterpenes and fatty acids from different organs of cultivated and wild rue plants. According to Wang *et al.* (2005), the proportion of water used and the extraction time influences the content of oxygenated compounds in the essential oil because the water affects the oxidation or hydrolysis of these compounds. Similarly, Stashenko *et al.* (2000) and Mejri *et al.* (2012) reported that the amount of oxygenated compounds increases with the duration of hydro-distillation. However, our distillation time was higher than both of these studies (240 compared to 210/120 min), and we did not observe the presence of these compounds. Other authors have indicated that the proportion of terpenes is influenced by the circadian rhythm, plant stage and environmental temperature. Therefore, the physiological expression of the secondary metabolism of a plant could present constant changes in some of its metabolites according to the biotic and abiotic factors to which it is subjected (Regnault-Roger *et al.*, 2012).

The behavioral response produced by the rue essential oil on both *A. superciliosus* sexes (Figure 1A; 1B) indicates its potential value as a natural repellent. However, this effect was absent for males when the concentration of the oil was lowered to 285.7 ng/cm² (Figure 1D). These results were not consistent with the reported by Tampe *et al.* (2015), who determined this concentration as effective in weevil other of the same genus *Aegorhinus nodipennis* (Hope) using the *Achillea millefolium* L. essential oil and its main constituent, thujone. Behaviorally, both males and females of *A. superciliosus* responded differently when subjected to volatile stimuli. Apparently, females are more sensitive than males because the

females must find a suitable host for themselves and their offspring. Similar observations were also reported by Palma *et al.* (2012), who indicated that in behavioral assays, a stronger response to the volatile compounds of their host plant, red clover, was observed in *Hylastinus obscurus* (Coleoptera: Curculionidae) females than in males. Moreover, in agreement with our observations, Conti *et al.* (2013) reported that *R. chalepensis* essential oil was mainly composed of 2-nonanone (37.4%) and 2-undecanone (20.5%) and was an effective repellent against the hematophagous mosquito *Aedes albopictus* Linnaeus (Diptera: Culicidae) at an RD_{50} of 0.000215 $\mu\text{L}/\text{cm}^2$ for skin and at an RD_{90} of 0.007613 $\mu\text{L}/\text{cm}^2$. Similarly, *R. graveolens* essential oil and its main compound, 2-undecanone ($43.2\% \pm 0.8$) repelled *A. aegypti* L. at a dose of 0.187 mg/cm^2 for the oil and 0.109 mg/cm^2 for 2-undecanone (Tabanca *et al.*, 2012), which indicated that *A. superciliosus* females are more susceptible to this essential oil. Other authors have determined that methyl ketones ($C_7 - C_{15}$) protected against the malaria mosquito, *Anopheles gambiae* Giles (Diptera: Culicidae) (Innocent *et al.*, 2008) and that the repellent BioUD®, whose active ingredient is 2-undecanone, was at least 2-4 times more repellent than DEET (*N,N*-Diethyl-meta-toluamide) against three species of ixodid ticks (Bissinger *et al.*, 2009; Bissinger and Roe, 2010). On the other hand, 2-nonanone and 2-undecanone was shown to be attractive to the foreign grain beetle, *Ahasverus advena* (Waltl) (Coleoptera: Cucujidae) at a concentration of 59 and 131 $\text{ng}/\mu\text{L}$, respectively (Wakefield *et al.*, 2005) and to both sexes of the olive bark beetle *Phloeotribus scarabaeoides* (Bernard) (Coleoptera: Scolytidae) using 10 μL of these ketones in an olfactometer bioassay (Szauman-Szumski *et al.*, 1998). Likewise, 2-nonanone has been reported by eliciting an attractant behavioral response in the same weevil, *A. superciliosus* at concentrations of 10 and 100 $\mu\text{g}/\text{mL}$ (Parra *et al.*, 2009).

Currently, the modes of action and molecular targets of the ketones in curculionids are not well understood. However, there is evidence that the ketone, 2-undecanone can activate and inhibit the odorant receptors (ORs) of *A. aegypti* L. (Diptera: Culicidae) (Bohbot and Dickens, 2010). The ORs are located in the dendritic membrane of the olfactory sensory neurons (OSNs) of the insects. They are responsible for triggering olfactory transduction by changing the action potentials as a message sent to the brain (Kaupp, 2010). The insect repellent 2-undecanone activates and inhibits the AaOR8 and AaOR2 receptors respectively; *i.e.*, this compound can act as an olfactory agonist or antagonist in *A. aegypti*, modulating receptor activity that is behaviorally expressed by different insect responses (Bohbot and Dickens, 2010). This finding suggests that the repellent effect produced by rue oil in both sexes of *A. superciliosus* could be attributed to 2-undecanone. The potential use of this substance can be explored in the future for developing biodegradable alternatives for synthetic pesticides against raspberry weevils.

5. Conclusions

In conclusion, the chemical analysis of the essential oil obtained from the aerial part of *Ruta chalepensis* did not show great differences to others studies reported in the literature. The ketones 2-nonanone and 2-undecanone were the main compounds identified in the oil and did not vary much with respect the geographic area where the plants grew. The repellency observed against *Aegorhinus superciliosus* can be attributed to high concentrations of both ketones. Our results suggest that rue essential oil can be considered as a potential repellent that might reduce the infestation of this weevil. Future studies should aim to (1) evaluate the effects of 2-nonanone and 2-undecanone in behavioral bioassays, (2) determine the electrophysiological

response of *A. superciliosus* to these compounds and (3) evaluate the effectiveness of both the essential oil and the two ketones under field conditions.

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