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Antifungal activity of the essential oil obtained from *Cryptocarya alba* against infection in honey bees by *Nosema ceranae*

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Abbreviations: GC-MS, Gas chromatography–mass spectrometry; EO, essential oil; INDAP, Instituto de Desarrollo Agropecuario; SAG, Servicio Agrícola y Ganadero; OECD, Organization for Economic Co-operation and Development; OIE, Office International des Epizooties; LD_{50}, median lethal dose; PCRq, real-time polymerase chain reaction; EI+, electron impact; RI, retention index; *N. ceranae, Nosema ceranae; N. apis, Nosema apis; A. mellifera, Apis mellifera*.

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ABSTRACT

The honeybee disease nosemosis type C is a serious problem since its causative agent, microsporidium *Nosema ceranae*, is widespread among adult honey bees. Some of the feasible alternative treatments that are used to control this disease are plant extracts. The aim of the present work was to evaluate the effects of essential oils of Chilean plant species, such as *Cryptocarya alba*, which is used against *N. ceranae*, and to identify and quantify the majority active compounds in the EO as well as their potential use for the control of nosemosis.

Essential oils were obtained using the stripping steam technique with Clevenger equipment and were subsequently analyzed by Gas chromatography–mass spectrometry. Mortality was recorded daily over at least 8 days as worker honeybees were exposed to a range of doses of EO dispersed in a sucrose solution. *C. alba* oil appears to be nontoxic to *A. mellifera* adults at the tested concentration (the same concentration inhibits the growth of *N. ceranae*), showing that this oil can be used for the treatment of nosemosis.

EO effectiveness was demonstrated against *N. ceranae* by calculating the percentage of decrease in infected bees from untreated infected groups vs infected groups treated with EO or the reference drug fumagillin. It was determined that a dose of 4 µg EO/bee was most effective in controlling *N. ceranae* development.

We determined innocuous doses of *C. alba* essential oil for honeybees. We demonstrated the antifungal activity of *C. alba* EO at 4 µg/bee against *N. ceranae* and compared it to its major monoterpenes, such as *β*- phellandrene (20 µg/bee), eucalyptol (20 µg/bee) and *α*- terpineol (20 µg/bee). The major compounds of *C. alba* EO, α-terpineol, eucalyptol and *β*-phellandrene, had significant effects against *Apis mellifera* infection by *N. ceranae*, but the antifungal effect of the complete essential oil on *N. ceranae* was larger than the effect of *α*-terpineol, eucalyptol or *β*- phellandrene separately, showing that *C. alba* oil may be a candidate for the treatment or prevention of nosemosis.

Key words: *Apis mellifera*, essential oil, *Nosema ceranae*, *α*- terpineol, eucalyptol, *β*- phellandrene

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INTRODUCTION

Due to the importance of honeybees, *Apis mellifera*, which are pollinators of many crops, large-scale losses of honeybee colonies in some countries in recent years have attracted the attention of both the scientific community and public (Neumann and Carreck, 2010). There is currently insufficient evidence to pinpoint an exact factor as the sole cause of this complex problem. Nevertheless, several possible contributing factors have been suggested to be responsible for colony losses, acting either alone or in combination (Neumann and Carreck, 2010).

Nosemosis is among the most prevalent diseases that affect honeybees today, and it is caused by two distinct species of single-cellular fungal microsporidian parasites, *Nosema apis* and *Nosema ceranae*. Currently, the only effective treatment for *N. apis* and *N. ceranae* infection is fumagillin (Higes et al., 2011). However, prolonged fumagillin usage in apiculture may contribute to these microsporidia developing resistance to this drug, and it has recently been reported that declining concentrations of this molecule may exacerbate *N. ceranae* infection rather than suppress it (Huang et al., 2013).

In recent years, *N. ceranae* has become one of the most prevalent honeybee pathogens worldwide (Higes et al., 2013). In some regions (especially warm areas), it is reported to be implicated in the general phenomenon of honeybee colony loss (Bekele et al., 2015). In colder areas, its responsibility in f widespread mortality has been ruled out (Hedtke et al., 2011). Despite these discrepancies, it is generally accepted that better colony development can be expected following treatments against *N. ceranae* in geographic areas with a high prevalence of microsporidium (Higes et al., 2013).

N. ceranae has been identified in South American countries, including Brazil, Uruguay and Argentina (Medici et al., 2011). In Chile, *N. apis* was first detected in 1978 through analyses carried out in the Servicio Agrícola y Ganadero (SAG) laboratories. Hinojosa and González (2004) note that according to their results, for six coastal and interior communes sampled from the VI Region, *N. apis* was recorded to have a prevalence of 60.0 ± 6.9% in the spring of 1999, 78.3 ± 5.8% in the spring of 2000, 14.3 ± 5% in the summer of 2000, and 23.0 ± 6% in the summer of 2001. Their results indicate that in this area, the highest incidence occurs in spring due to high fecal contamination within the colony in late winter.

Martínez et al., (2012) only identified the presence of *N. ceranae* in Chile in the Bío-Bío region, which accounts for 42.0% of the country's honey production. The study evaluated four provinces in the

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region, obtaining information from a total of 26 communes. Forty-nine percent showed the presence of *N*. *ceranae*. Rodriguez et al., (2012) reported the presence and coexistence of viruses and *N*. *ceranae* in the same apiaries from the Maule District.

Bravo et al. (2014) reported the presence of *N*. *ceranae* in the V Region of Valparaiso. This assessment was based on the report of INDAP-Agroapilculture 2011-2012, which described the death of 2,915 hives in the Valparaíso Region. This region has a total of 6,167 hives, which corresponds to 47.3% of the total hives in Chile.

C. *alba* is a tree that is native to Chile and grows from southern Coquimbo to the Los Lagos Region. It is a perennial tree with dense and dark foliage and reaches 15-20 m tall (Barreau, 2009). Its leaves contain 0.3% essential oil, the main components of which are α -pinene, 3-pinene, 3-terpinene, cymol, terpinen-4-ol, and 1,8-cineole (Avello et al., 2012). These components have been shown to have antifungal activity against *Fusarium oxysporum* and *Penicillium* sp. Studies of essential oils extracted from other species that contain terpinen-4-ol, 1,8-cineole or cymol as the main components have shown insecticidal activity against insect pests of stored products, such as *S*. *zeamais* (Kouninki et al., 2007). There are several newer studies, including an *in vitro* study on the antimicrobial activity of the Andiroba (*Carapa guaianensis*) and Copaíba (*Copaifera officinalis*) essential oils against *Paenibacillus* species, including *Paenibacillus larvae* (Santos et al., 2012), and an *in vitro* study on the activity of *Scutia buxifolia* samples against *Paenibacillus* species as evaluated by disk diffusion and microdilution methods (Boligon et al., 2013).

The aim of the present work was to evaluate the effects of EO of Chilean *C*. *alba* against *N*. *ceranae*, and its potential use for the control of the nosemosis as well as to identify and quantify the major active compounds in the EO.

MATERIALS AND METHODS

Plant material and extraction of *Cryptocarya alba* essential oil

Aerial parts of *Cryptocarya alba* (Molina) Looser (synonym *Peumus alba* Molina), Lauraceae at the beginning of the flowering stage were collected from Altos de Chicauma (33°00'-33°17'S and 70°53'-71°00'W), Santiago province, in the central zone of Chile, in September 2013. The species was

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identified by Dr. Carlos Valdovinos, and a voucher specimen was deposited at the Herbarium of the Facultad de Ciencias Químicas y Farmacéuticas de la Universidad de Chile (N° 22472 SQF). EO was extracted via water distillation in a Clevenger type apparatus. EO was dried over anhydrous sodium sulfate, stored at 4 °C and kept under nitrogen until further analysis (Avello et al., 2012).

Essential oil chromatographic analysis

GC-MS analysis was performed on a Varian Gas Chromatograph Series 431 (Agilent Technologies, Inc., Santa Clara, CA, USA) fitted with a DB-5 ms fused silica capillary column (30 x 0.25 mm; film thickness, 0.25 μm) using split/split-less injection and coupled to a Series 220 Mass Detector (Agilent Technologies, Inc.). The following conditions were used: injection volume: 0.8 μL with a split ratio of 1:80; carrier gas: helium at 1.5 mL/min in constant flow; injector temperature: 250˚C; and oven temperature: 50-260˚C at 2˚C/min. The mass spectra electron impact (EI+) mode was set at 70 ev with an ion source temperature of 260℃. Mass spectra were recorded within the range of 40-300 atomic mass units. Identification of the essential oil constituents was completed based on the following: the retention index (RI), determined with respect to a homologous series of n-alkanes (C_5-C_{28}; PolyScience, Niles, IL, USA) under the same experimental conditions; co-injection with standards (Sigma-Aldrich); identification using an MS library [NIST 05 and Wiley; NIST/ EPA/NIH Mass Spectral Library with Search Program (data version, NIST 11; software version 2.0g), available online: http://www.nist.gov/srd/nist1a.cfm]; and comparison with previously reported MS data (Adams, 2007).

The major compounds present in *C. alba* EO have previously been quantified through GC, with the aim of constructing a calibration curve with each pattern of different concentrations. The patterns used for comparison were from SIGMA ALDRICH®, USA (99.9% purity).

From the calibration curve with standards, the concentration of monoterpenes was determined in essential oil for β- phellandrene ($y = 10497x + 10838$ $R = 0.99813$ F cal < F tab), eucalyptol ($y = 5404.3x + 20345$ $R = 0.99538$ F cal < F tab) and α-terpineol ($y = 1413.9x + 4657.1$ $R = 0.99603$ F cal < F tab).

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Experimental honeybee

Frames of sealed broods obtained from a healthy colony of *A. mellifera* (*Nosema* free as confirmed by PCR) sited in an experimental apiary located in Buin were kept in an incubator at 34 ± 2 °C to provide newly emerged, *Nosema* free honeybees. The emergent worker bees were carefully removed, confined to cages in groups of 20, and kept in the incubator for five days. The bees were fed *ad libitum* with a solution of sucrose (50% w/w in water). In addition, 2% Promotor L (Calier Laboratory), a commercial mixture of amino acids and vitamins, was added as a dietary supplement in a plastic feeder.

Five days after eclosion, bees were starved for 2 h, and 20 bees per group were fed 10 µl of a 50% sucrose solution containing either EO or one of the major monoterpenes. Three replicate cages of 20 honeybees each were used in addition to one control cage (n = 20), in which the bees were fed 10 µL of a plain sucrose solution (Martín-Hernández et al., 2009).

Acute oral toxicity test of *C. alba* essential oil in honeybees

Adult worker honeybees were exposed to a range of EO doses dispersed in a sucrose solution. In the control group, bees were fed the same diet, free of EO. Mortality was recorded daily over at least 8 days and was compared between the control group and the two groups that received a reference toxicant (dimethoate at 0.1 and 0.35 µg/bee) in syrup, which was supplied *ad libitum* (50% sucrose water with 2% Promotor L) in a plastic feeder.

Each test group of bees was provided with a 50% sucrose solution in water containing 0.4 µg EO/bee, β- phellandrene, eucalyptol and α-terpineol at 20 µg/bee. Bees were kept in the dark in an experimental room at a temperature of 33 ± 1 °C. The relative humidity, normally approximately 50-70 %, was recorded throughout the test. Mortality was recorded 4 hours after the start of exposure and additionally at 24 h and 48 h (i.e., after the administered dose). If a prolonged observation period was required, further assessments were made at 24 hour intervals, up to a maximum of 8 days, provided that the control mortality did not exceed 10%. The amount of diet consumed per group was estimated as the volume consumed divided by the total number of individuals. Comparison of the rates of consumption of treated and untreated diet within the first six hours can provide information about the palatability of

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the treated diet. All abnormal behavioral effects observed during the testing period were recorded as indicated in the Organization for Economic Cooperation and Development guidelines (OECD 214, 1998; OIE, 2008).

Differences in survival after 8 days of observation were assessed by Kaplan–Meier analysis followed by the Logrank test. A p value of <0.05 was considered statistically significant. All statistical analyses were performed with the GraphPad Prism 5 software package.

Preparation and identification of *N. ceranae* spores

To obtain fresh *N. ceranae* spores, three naturally infected colonies of *A. mellifera* located in the Region of Valparaíso, Chile, were used. One-hundred flying bees were collected from each colony and processed separately according to Botías et al. (2012) and Martín-Hernández et al. (2007). In short, bees were killed by freezing, and the abdomen of each sample (bee) was pooled and macerated with a Stomacher 80 Biomaster (Seward, West Sussex, UK) provided with strainer bags (BA6040/STR, Seward) in 25 mL of PCRq water initially and, after recovery, in 10 mL of PCRq water. Centrifugation for 6 min at 800 rpm resulted in a pellet that was resuspended in 1 mL of PCRq water and stored at room temperature until use.

Assessment of spore species was performed by PCR according to Martín-Hernández et al., (2007, 2012). Briefly, aliquots of the macerates were shaken at 9500 rpm for 95 s with ceramic beads (MagNA Lyser Green Beads, Roche 03 358 941 001). DNA was extracted with a MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche 03 730 964 001) in a MagNA pure compact machine (Roche). PCR amplification was performed with the 218MITOC FOR/218MITOC REV and 321APIS FOR/ 321APIS REV primers, specific for *N. ceranae* and *N. apis*, respectively, with COI as an internal control. The PCR products were analyzed by the QIAxcel System (Qiagen, Hilden, Germany) using a QIAxcel DNA High Resolution Kit (Qiagen, No. 929002) to detect positive and negative reactions. Negative controls were included to detect and exclude possible contaminants.

Once the presence of *N. ceranae* spores and absence of *N. apis* spores were ascertained, the suspensions were mixed, purified by Percoll density gradient centrifugation (Martín-Hernández et al., 2009) and maintained at room temperature for 5 days or less. Finally, counts using a Neubauer

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hemocytometer chamber under a phase contrast microscope allowed us to calculate the spore concentration in the purified suspension (Higes et al., 2007).

Experimental infections

Brood combs taken from three *A. mellifera* colonies were found to be free of *N. ceranae* infections by PCR (Higes et al., 2007) and were maintained in an incubator at 34 ± 1 ºC. Once hatched, new adult bees were carefully transferred into 9 cylindrical mesh cages (base: 71 mm^2, length 175 mm) and stored at 33 ± 1 ºC for five days. Syrup (50% sugar water with 2% Promotor L) was supplied *ad libitum* in a plastic feeder.

Five-day-old bees were starved for 2 h. After slight anesthetization with CO_2 to make handling easier, they were artificially fed 2 µL of 50% sugar water containing 154,000 *N. ceranae* spores each. The mouthparts were stimulated as needed to induce ingestion of the droplet from the micropipette. Individuals that did not consume the entire dose were discarded (Higes et al., 2007). The same procedure was used to administer 50% sugar water that did not contain spores to control, non-infected honeybees.

Effectivity test of *C. alba* essential oil against *N. ceranae*

Groups of adult bees were formed from infected and uninfected bees as described above. Three replicate cages of 20 honeybees each were used in addition to one control cage (n = 20) in which bees were fed with 10 µl of a plain sucrose solution. To avoid cross contaminations, cages containing infected bees and cages containing uninfected bees were stored separately at 33 ±_1 ºC inside two Memmert incubators of the same age (2008) and model (Mod. IPP500).

Each group of cages was provided with 2 mL of syrup (50% sucrose water with 2% Promotor L) daily for twelve days via a feeder. The cages of the fumagillin group received fumagillin syrup (240 µg/bee), while the EO groups received the *C. alba* essential oil at varying doses (1, 2, 3 and 4 µg/bee) selected for low toxicity. On days 4, 8 and 12 post-infection (p.i.), 5 bees were randomly removed from each cage. To assess their degrees of infection, each abdomen was placed in a sterile Eppendorf microtube filled with 200 µL of PCRq water. After thorough grinding, spores were counted using a hemocytometer and a phase contrast microscope. PCR was then used to confirm that the spores

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belonged to the *N. ceranae* species (Bravo, 2014). ANOVA was used to identify significant differences between treated and untreated groups using the GraphPad Prism 5 program.

Effectivity test of the major compounds present in *C. alba* essential oil

Groups of 60 adult bees each were formed from infected and uninfected bees as described above. The individuals in each group were split into three subsets of 20. To avoid cross contaminations, cages containing infected bees and cages containing uninfected bees were stored separately at 33 ± 1 ºC inside two Memmert incubators of the same age (2008) and model (Mod. IPP500).

Each group of cages was provided with 2 mL of syrup (50% sugar water with 2% Promotor L) daily for twelve days via a feeder. The cages for the fumagillin group (positive control) received a fumagillin syrup (240 µg/bee), while the experimental groups received the *C. alba* EO (4 µg/bee) or monoterpenes (Sigma ®) α -terpineol (20 µg/bee), eucalyptol (20 µg/bee) or β - phellandrene (20 µg/bee); one group was infected without treatment (negative control). On days 5, 6 and 7 post-infection (p.i.), 5 bees were randomly removed from each cage, pooled in groups of 15 individuals and dissected. To assess their degree of infection, the abdomens were placed in sterile Eppendorf microtubes filled with 200 µL of PCRq water. After thorough grinding, the spores were counted using a hemocytometer and a phase contrast microscope. Then, PCR was used to confirm that the spores belonged to the *N. ceranae* species (Bravo, 2014). ANOVA was used to identify significant differences between treated and untreated groups using the GraphPad Prism 5 program.

RESULTS

Essential oil analysis using gas chromatography-mass spectrometry (GC-MS)

C. alba essential oil, selected because it has a high yield and activity against *N. ceranae*, as well as because of its safety for bees, was qualitatively characterized by gas chromatography (GC) coupled to a mass spectrometer (MS) (Figure 4). Total composition of the essential oil of *C. alba* was determined, and the relative areas of the chromatogram for the major compounds were established. Determination of the compounds present in the sample was performed by comparing the mass spectra obtained from the sample with the spectra of the NIST 2008 database as well as by using a commercial standard from

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SIGMA ALDRICH® (99,9% purity). The Kovats index was calculated for each compound and then compared with the literature (Table 1).

Acute oral toxicity test of the *C. alba* essential oil in honeybees

The results of toxicity of the EO of *C. alba* for honeybees, as evaluated by the dietary application method of *β*- phellandrene, eucalyptol, *α*- terpineol and EO of *C. alba*, demonstrated that the samples were not toxic over 8 days of treatment (Figure 1). Bee mortality was only evident during treatment with dimethoate at 0.35 μg/bee (positive death control group).

Effectivity test of the *C. alba* essential oil against *N. ceranae*

Four colonies that had high *Nosema* spp. spore counts were used as source colonies after confirming that all were infected with *N. ceranae* only as per the species-specific PCR assay (Figure 2).

The effectiveness of the treatment at doses of 1, 2, 3 and 4 μg EO/bee was calculated as the diminishing percentage of spores of *N. ceranae* in the treated group with respect to the control group (bees treated with fumagillin at 240 μg/bee). Essential oil from *C. alba* at a dose of 4 μg/bee was the most effective treatment for diminishing the presence of spores at days 4, 8, and 12 of treatment (Figure 3).

Effectivity test of the major compounds present in the *C. alba* essential oil

The antifungal activity of the major monoterpenes present in the *C. alba* EO against *N. ceranae* (specifically monoterpenes *β*- phellandrene, eucalyptol and *α*- terpineol), as evaluated by the 2 mL of syrup application method, demonstrated that monoterpenes are effective over 7 days of treatment ($p < 0.05$, Figure 5).

DISCUSSION

This is the first study to report on the use the EO and monoterpenes of *C. alba* for the treatment of *N. ceranae* - affected beehives. The results indicate that the EO of *C. alba* contains 39 compounds, 17 of which are shown in Table 1. The three major compounds identified in the *C. Alba* essential oil were the monoterpenes *β* - phellandrene (16,28%, corresponding to 14262 ppm, with a peak in its $[M]^+$ 136),

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eucalyptol (23,27%, corresponding to 87799 ppm, with a peak in its $[M]^+$ 154) and α - terpineol (27,38%, corresponding to 13509 ppm, with a peak in its $[M]^+$ 154) (see Table 1 and Figure 4). Some of these are described by Muñoz et al., 1999.

In this research, the essential oils of *C. alba* were added to the diet of *A. mellifera* to verify their possible toxic effects. The EO of *C. alba*, eucaliptol, α-terpineol and β-phellandrene, presented similar results to the control group, causing no toxic effects or animal death (Figure 1). The EO of *C. alba* have been used in traditional medicine as an insect repellent, and some studies verified the antifungal properties of the EO against *Penicillium* sp. and *Fusarium oxysporum* (Ladio, 2009; Simosen, 2011; Avello, 2012). The *C. alba* oil appears to be nontoxic to *A. mellifera* adults at the tested concentration (the concentration inhibits the growth of *N. ceranae*), showing that this oil can be used for the treatment of nosemosis.

In relation to the effectiveness studies of the *C. alba* EO against *N. ceranae*, it was first determined that hives were infected with *N. ceranae*, which were used to later infect healthy bees in the laboratory (Figure 2). The effectiveness of the treatment at doses of 1, 2, 3 and 4 µg EO/bee was calculated as the diminishing percentage of spores of *N. ceranae* in the treated group with respect to the control group (bees treated with fumagillin at 240 µg/bee). The essential oil from *C. alba* at a dose of 4 µg/bee was the most effective at diminishing the charge of spores at days 4, 8, and 12 of treatment (Figure 3), with a significant difference ($p < 0.0001$) between the group treated with the *C. alba* EO (4 µg/bee) and negative control group appearing at day 12. The EO inhibited the spore growth of *N. ceranae* by 80% on day 7, similar to the reference drug. From the results, we also observed that the effectiveness of the *C. alba* EO as an antifungal was dose dependent (Figure 3).

Prevention and control treatments associated with this pathogen are scarce worldwide. Fumagillin, an antibiotic derived from the metabolism of the fungus *Aspergillus fumigatus*, is the alternative and specific control that has strong effectiveness and efficiency against *Nosema*. However, its use in Chile was banned in 2009 due to traces left in honey, which could influence pathogen resistance to antibiotics. It is noteworthy that in Europe, the use of fumagillin is also prohibited (Regulation 2377/90, European Commission, 2009). As an example, in Germany, products such as Nosemack Fumidil B (based on fumagillin) are prohibited. Australia has restricted the use of fumagillin for commercial queen bee breeders (Botías, 2012).

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Various studies have shown the genotoxic and mutagenic action of this substance under experimental conditions for bees (Stevanovic et al., 2011). A study by Peng (2001) revealed that exposure of human lymphocytes to low concentrations of fumagillin caused a significant reduction in their proliferation, thus indicating that this drug presents a genotoxic risk for honey consumers and beekeepers. Recent studies have suggested possible adverse short-term effects on the general state of bee colonies after treatment with fumagillin (Eischen et al., 2012). Furthermore, the possibility of a negative effect of this antibiotic on the bacterial microbiota of bees, and thus on their defenses against certain pathogens, should also be evaluated considering that their intestinal microbiota is capable of forming a protective barrier that is essential against intestinal pathogens (Koch and Schmid-Hempel, 2011).

Other alternatives for anti-nosemosis treatments have been tried abroad, specifically formulations based on natural products with fungicidal effects. These include Nozevit®, Protofil®, Vitafeed Gold® and Api Herb®. They are composed of extracts or essential oils of the native microbiota of their place of origin (Botías et al., 2011). The absence of these products in the domestic market could be due to the high costs associated with importing them (INDAP, 2007).

In the records of Servicio Agrícola y Ganadero (SAG), there are no products of natural origin authorized to be sold in the domestic market, mainly due to the lack of scientific studies that evaluate their efficacy against nosemosis and their safety for bees (Registration SAG, Organic Law of Service (art. 2), 2009).

Moreover, the presence of chemical residues is one of the main obstacles for the exportation of honey and other products of beehives to markets with high valuations of these products, particularly Europe (Manquian et al., 2007). These wastes are mainly due to a) antibiotics (fumagillin, chloramphenicol, streptomycin, tetracycline, sulfonamide), b) miticides (organophosphates, organochlorines, pyrethroids, amidines, etc.) and c) pesticides and heavy metals (lead and cadmium).

Therefore, the *C. alba* essential oil is a good candidate for alternative treatments against *N. ceranae* because it is a complex and volatile mixture that does not leave residues in honey.

Our results demonstrate the antifungal activity of the major compounds present in the *C. alba* EO, specifically monoterpenes β- phellandrene, eucalyptol and α- terpineol, against *N. ceranae* ($p < 0.05$,

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Figure 5). Notably, the EO of *C*. *alba* inhibits the growth of *N*. *ceranae* spores by nearly 80% ($p < 0.0001$), similar to the effect of the reference drug (fumagillin) (see Figure 5). The effectiveness of the *C*. *alba* EO was significantly higher than that of the major compounds evaluated, which could be explained by the contribution to the antifungal effect of its component monoterpenes (see Table 1 and Figure 5). The major compounds of the *C*. *alba* EO, α-terpineol, eucalyptol and *β*-phellandrene, showed significant effects against infection by *A*. *mellifera* with *N*. *ceranae*, and the antifungal effect of the complete essential oil on *N*. *ceranae* was larger than the individual effects of *α*-terpineol, eucalyptol and *β*- phellandrene.

In a study by Damiani et al. (2014), the compound 1,8-cineole (eucalyptol), obtained from Sigma® (99.9% purity), was evaluated against infection with spores of *N*. *ceranae*. They found no significant effect in controlling infection of *N*. *ceranae* when applying 1,8-cineole. However, our results showed significant activity in controlling infection of *N*. *ceranae* in bees ($p < 0.05$). It has been proposed that the antimicrobial activity could be due to the synergism between its different components since it was observed that no single component has shown an activity higher than the EO of *C*. *alba*. Boligon (2013) also attributes the inhibitory effect of the *Scutia buxifolia* extract again *P*. *larvae* to the synergism between flavonoids and phenols.

In the area of honeybee health, alternative natural substances to control nosemosis are unexplored and warrant future research. Elimination of *N*. *ceranae* in *A*. *mellifera* colonies requires treatments with acceptable antifungal activity and no side effects on *A*. *mellifera* as well as that minimize residues in honey and wax and act as a viable alternative to reduce antimicrobial resistance. The present study lays the foundation for the use of the *C*. *alba* essential oil against *N*. *ceranae* and will encourage new studies in the field to determine the effect that it has on naturally infected hives.

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CONFLICT OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this publication and that there has been no significant financial support for this work that could have influenced its outcome.

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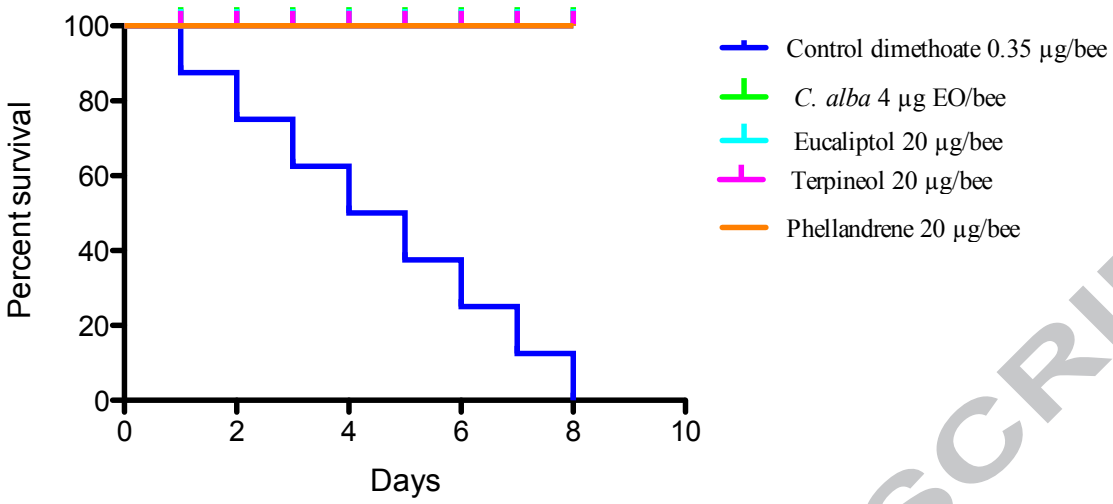

Figure 1. Effects of *C. alba* essential oil applications on bees.

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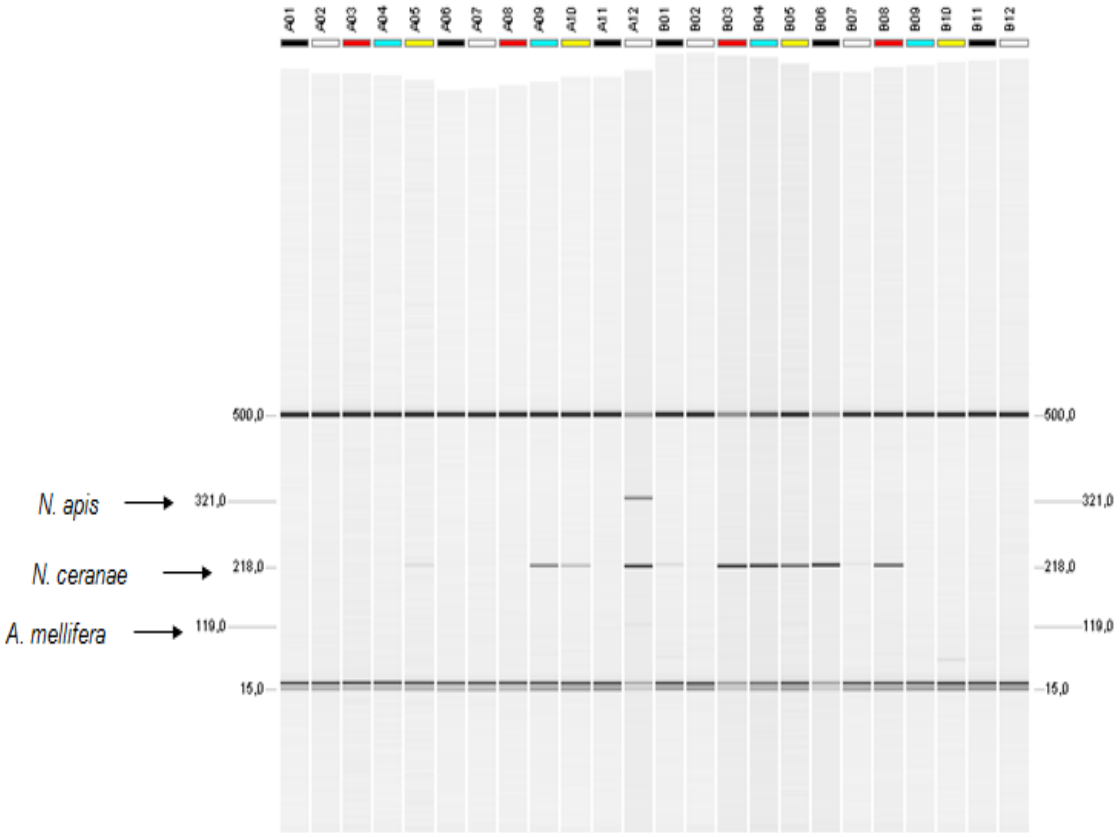

Figure 2 Electrophoresis of the PCR product in nucleic acid analyzer QIAxcel (QIAGEN). The samples A1 - A10, and B1 - B12 is from Chilean apiaries of the Valparaíso Region, with A11 as a negative control and A12 as positive control corresponding to patterns of *Nosema Apis* (321pb) and *Nosema ceranae* (218pb) and internal control COI *Apis mellifera* (119pb).

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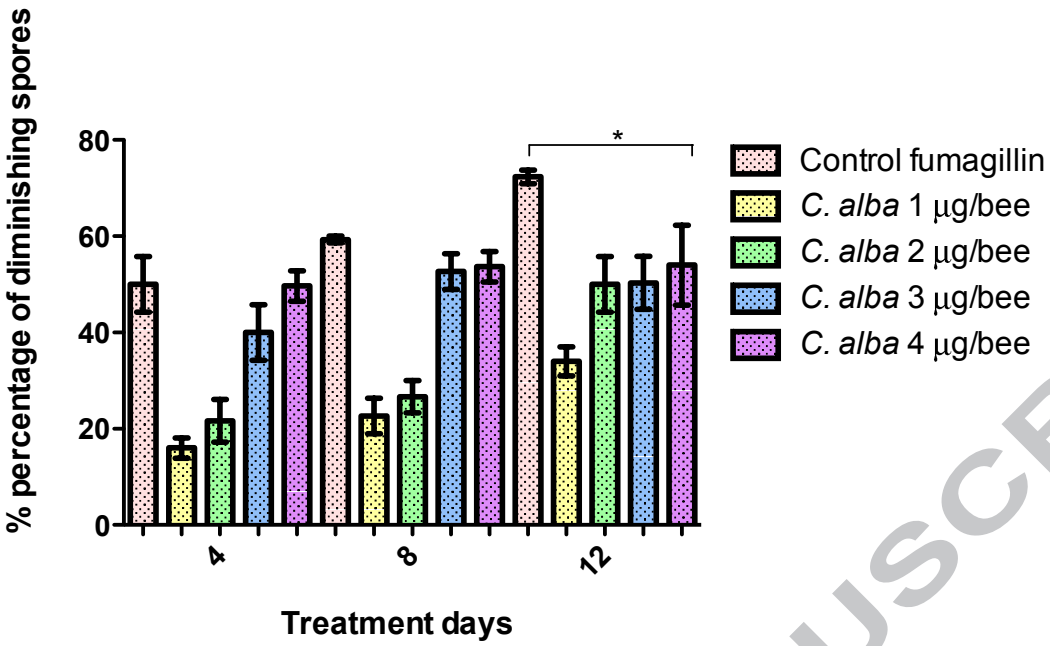

Figure 3. The effectiveness of *C*. *alba* essential oil calculated as a percentage of diminishing spores of *Nosema ceranae* on days 4, 8 and 12 of different dosage treatments (1, 2, 3 and 4 µg/bee), the reference drug (fumagillin) was applied at a dose of 240µg/bee. (Anova Test * $p < 0.0001$ vs fumagillin). Each value is the average effective rate of 3 determinations.

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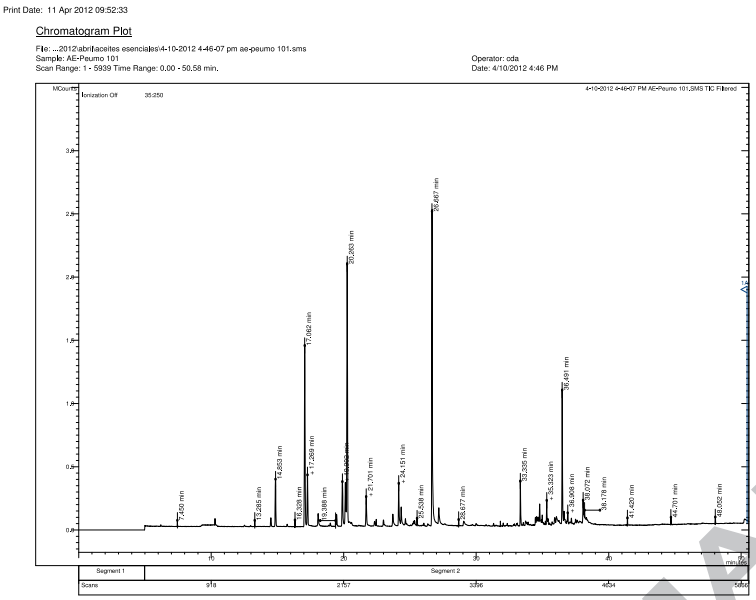

Figure 4. Chromatographic GC profile of *C. alba* essential oil.

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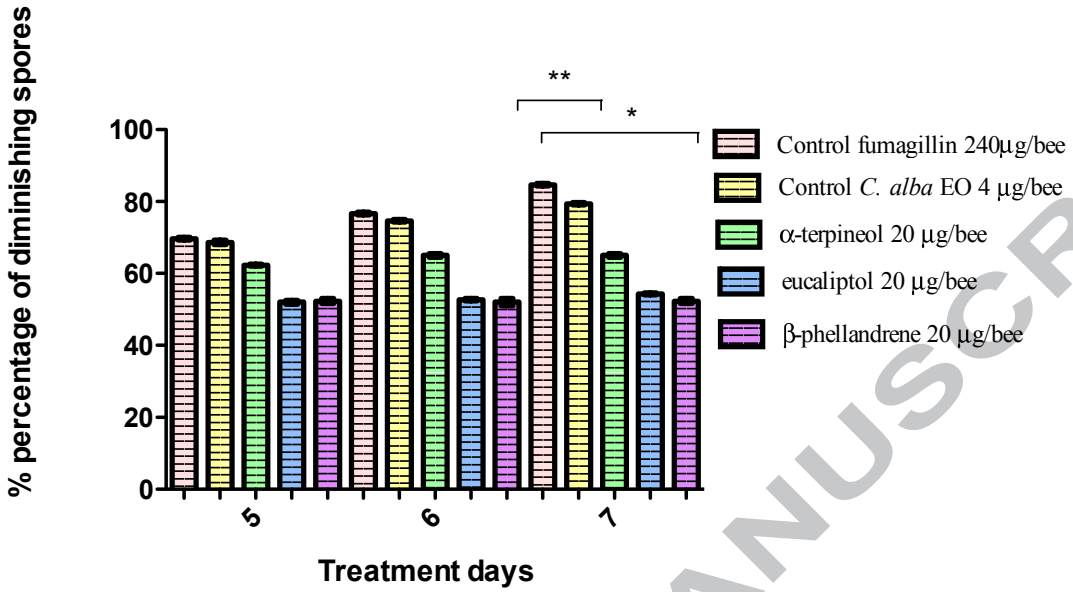

Figure 5. Effect of the major compounds present in *C. alba* essential oil in the percentage of diminishing number of spores of *Nosema ceranae* on days 5, 6 y 7 of treatment, when applying a daily dose of 20 µg/bee of the monoterpenes α-terpineol, eucalyptol or β- phellandrene. The reference drug (fumagillin) was applied at a dose of 240 µg/bee and the *C. alba* essential oil at 4 µg/bee. (Anova Test * $p < 0.05$ monoterpenes vs fumagillin, Anova Test ** $p = 0.0001$ EO vs fumagillin). Each value is the average effective rate of 3 determinations.

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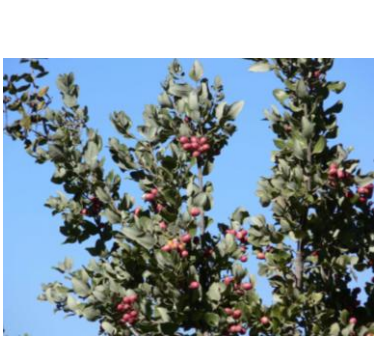
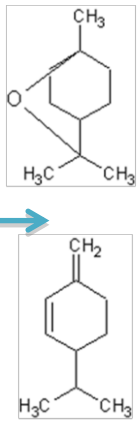
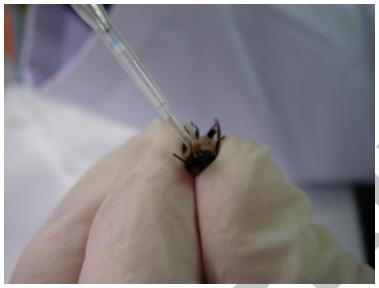
Graphical Abstract







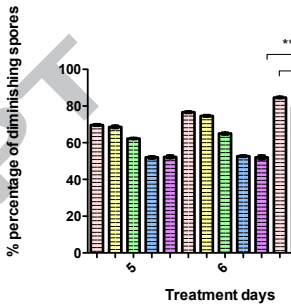


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Table 1: Summary of compounds from CG- MS analyses of essential oil of *C. alba* and the respective Kovats Indexs (KI) calculated (KI cal) and from literature (KI lit)

Fraction Number	Retention time (min)	CAS	KI cal	KI lit	[M+]	Fragment	Name
1	14.5	99-83-2	-	1007	136	91 (100), 93 (62), 77 (52)	d-phellandrene
2	14.8	7785-70-8	1025	939	136	91(100), 93 (88), 92 (50)	1R-α-pinene
3	15.7	79-92-5	1041	953	136	44 (100), 40 (77), 39 (46)	Camphene
4	17	555-10-2	1081	1035	136	93 (100), 91(88), 136 (42)	β-phellandrene
5	17.3	18172-67-3	1012	981	136	93 (100), 91 (80), 39 (51)	L-β-pinene
6	19.5	586-62-9	1085	1088	136	40 (100), 44 (85), 93 (49)	Terpinolene
7	19.9	527-84-4	1067	1014	134	119 (100), 91 (29),134 (19)	o-cimol
8	20.1	5989-54-8	1050	1031	136	67 (100), 93 (67), 91 (50)	Limonene
9	20.3	470-82-6	1013	1030	154	43 (100), 93 (80), 81 (62)	Eucalyptol
10	21.7	99-85-4	1035	1057	136	91 (100), 93 (88), 40 (60)	γ-terpinene
11	24.3	54410-94-	1053	1116	170	68 (100), 57 (43), 41 (39)	3 methyl 3 butenyl
12	26.6	562-74-3	1079	1177	154	37 (100), 38 (43), 40 (12)	4-terpineol
13	27.2	98-55-8	1009	1189	154	93 (100), 59 (90), 121 (86)	α- terpineol
14	33.3	17699-05-7	1580	1434	204	93 (100), 41(88), 119 (85)	α-bergamolene
15	34.8	339154-91-5	1575	1430	204	121 (100), 93 (69), 41 (63)	γ-elemene
16	35.3	483-77-2	1515	1523	202	159 (100), 40 (24), 160 (13)	calamenene
17	38.1	473-15-4	1733	1645	222	59 (100), 149 (46), 108 (25)	β-eudesmol

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