Dietary *Rosmarinus officinalis* as a natural alternative to reduce varroosis in *Apis mellifera*

Uso dietético de Rosmarinus officinalis *como alternativa natural para reduzir a varroose em* Apis mellifera

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Abstract

The objective was to evaluate the effect of using *Rosmarinus officinalis* essential oil for the control of *Varroa destructor*. Previously, the subspecies of *Apis mellifera* to which the studied colonies belonged was identified by phylogenetic inference. Forty experimental units were prepared, each consisting of a nucleus hive with the following characteristics: a) three brood frames, b) one food frame, c) one kilogram of bees, and d) a fertilized queen (two months old). The experimental units were divided into four groups, each receiving different concentrations of rosemary essential oil (0, 5, 10, and 20%) added to 200 g of protein food. The percentage of *Varroa* mite infestation, quantification of fallen mites, protein food consumption, and hygienic behavior were measured. Significant differences were found in varroosis incidence ($p = 0.033$), associated with mite count ($p = 0.012$), demonstrating the efficacy of treatments with rosemary oil, without affecting protein food consumption ($p = 0.067$). This indicates that the use of rosemary essential oil could be a control method with potential as a safe acaricide.

Keywords: Essential oils. Acaricide. Safety. Rosemary.

Resumo

O objetivo deste estudo foi avaliar o efeito do uso do óleo essencial de Rosmarinus officinalis *no controle de* Varroa destructor. *Previamente, a subespécie de* Apis mellifera *à qual pertenciam as colônias estudadas foi identificada por inferência filogenética. Foram preparadas 40 unidades experimentais, cada uma composta por uma colmeia núcleo com as seguintes características: a) três quadros de cria, b) um quadro de alimento, c) um quilograma de abelhas e d) uma rainha fecundada (com dois meses de idade). As unidades foram divididas em quatro grupos, que receberam diferentes concentrações de óleo essencial de alecrim (0, 5, 10 e 20%) adicionadas a 200 g de suplemento proteico. Avaliaram-se a porcentagem de infestação por ácaros* Varroa*, a quantidade de ácaros caídos, o consumo de*

alimento proteico e o comportamento higiênico. Foram encontradas diferenças significativas na incidência de varroose (p = 0,033), associadas à contagem de ácaros (p = 0,012), demonstrando a eficácia dos tratamentos com óleo de alecrim, sem afetar o consumo de alimento proteico (p = 0,067). Isso indica que o uso de óleo essencial de alecrim pode ser um método de controle promissor, com potencial como acaricida seguro.

Palavras-chave: *Óleos essenciais. Acaricida. Segurança. Alecrim.*

Introduction

Varroa destructor, the causative agent of varroosis, is one of the most serious sanitary threats to apiculture worldwide. Originally identified in *Apis cerana*, a species that has evolved defensive behaviors such as grooming — which allows it to effectively remove mites without compromising colony health — *V. destructor* has successfully adapted to *Apis mellifera*, a species lacking similar natural defenses. In *A. mellifera*, the mite infests both brood (larvae and pupae) and adult bees, causing physiological damage, shortened lifespan, virus transmission, and an overall decline in colony performance (Noël et al., 2020; Traynor et al., 2020).

Currently, varroosis is considered the primary factor responsible for colony losses in various regions around the globe (Guichard et al., 2020; Hristov et al., 2021; Reyna-Fuentes et al., 2022). Infestation levels exceeding 10% significantly weaken colonies and increase susceptibility to secondary diseases, while infestation rates above 30% may lead to complete colony collapse (Medina-Flores et al., 2011).

In response, many beekeepers rely on synthetic acaricides, including three main molecules: tau-fluvalinate (Apistan, Vita, UK), coumaphos (CheckMite, Bayer AG, Germany), and amitraz (Apivar, Geraldine, New Zealand). However, their continued use presents serious challenges, such as the development of resistance in mites and the presence of toxic residues in honey, which undermines product safety and marketability (Bajuk et al., 2017; Shahin Nekoei, 2023).

Due to the adverse effects associated with conventional acaricides, there is increasing interest in organic alternatives derived from natural compounds. These control methods exhibit different mechanisms of action, pose a lower risk of resistance, and align more closely with sustainable beekeeping practices. Among these, organic acids (formic, oxalic, and lactic) and essential oils with antimicrobial and acaricidal properties — such as thymol, eucalyptus, and camphor — have shown promising results (Pietropaoli and Formato, 2022). Recent studies have evaluated the efficacy of lavender and thyme essential oils on mite mortality in *A. mellifera* colonies (Hýbl et al., 2021).

In this context, rosemary essential oil (*Rosmarinus officinalis*) has demonstrated antifungal, insecticidal, and acaricidal properties. These effects are attributed to active metabolites such as cineole (21.5%) and camphor (18%), along with smaller amounts of alphapinene, limonene, camphene, and myrcene (Romeu et al., 2007; Flores-Villa et al., 2020; Lazăr and Pătriuță, 2020). These characteristics make rosemary a promising candidate in the development of natural control strategies against *V. destructor*.

This research aims to provide scientific evidence on the use of essential oils as safe, sustainable, and economically feasible alternatives for varroosis management. It offers an integrative perspective that evaluates the efficacy of non-conventional treatments, thereby offering practical tools to beekeepers seeking viable solutions beyond synthetic chemicals. Ultimately, this contributes to strengthening apiculture through management practices that support colony health without compromising honey quality or ecological balance.

Therefore, the objective of this study was to evaluate the effect of *R. officinalis* essential oil (REO), incorporated into the supplementary diet of *A. mellifera*, as a natural alternative for controlling the parasitic mite *V. destructor*.

Material and methods

The experiment was conducted in the municipality of Teocelo, Veracruz, Mexico, at an altitude of 2,457 meters above sea level (19°20´–19°24´ N latitude, 96°50´–97°02´ W longitude). The climate is classified as humid subtropical (Aw), with year-round rainfall, annual precipitation ranging from 1,400 to 2,100 mm, and an average temperature between 18 and 24 °C (INEGI, 2017). The predominant vegetation corresponds to lowland tropical evergreen forest, with dominant species such as *Cecropia obtusifolia* and *Heliocarpus appendiculatus* (Mexico, 2022).

The study was conducted during the period of minimal floral resource availability in the region (September – November). During this seasonal window, honey bee colonies typically exhibit increased dependence on supplemental feeding due to a pronounced decline in natural nectar and pollen flows. These environmental conditions were strategically leveraged to standardize the physiological status of the colonies and to ensure the adequate preparation of the experimental units, whereby nutritional management protocols facilitated the consistent and controlled allocation of treatments.

Molecular identification of biological material

Five worker bees from the maternal colony (source of queens used in the study) were collected and sent to the Seed Biotechnology Laboratory at the Colegio de Postgraduados, Montecillo campus, State of Mexico. Total DNA was extracted from the whole body of each bee (five individuals per colony) using the CTAB method (Doyle and Doyle, 1987).

Polymerase chain reaction (PCR) amplification targeted the mitochondrial COI-COII intergenic region using primers E2 (5´-GGCAAGAATAAGTGCATTG-3´) and H2 (5´-CAATATCATTGATGACC-3´).

The PCR program was as follows: initial denaturation at 94 °C for 5 minutes; 35 cycles of 94 °C for 45 seconds, 46 °C for 1 minute, and 72 °C for 1 minute; followed by a final extension at 72 °C for 6 minutes, using a Bio-Rad DNA Engine® thermal cycler. PCR products were purified with ExoSAP-IT (Affymetrix, USA), and sequencing was performed using the BigDye Terminator v3.1 kit (Applied Biosystems, USA) on a Genetic Analyzer 3130. Phylogenetic analysis of the mitochondrial COI-COII region was used to determine the bee lineage.

Experimental units

Following the Mexican standard NOM-057-ZOO-1997, 40 hives (experimental units) were selected, each consisting of one brood chamber and one super with four frames. Each unit contained three brood frames, one food frame, approximately 1 kg of bees, and a two-month-old mated queen from the same maternal colony. No prior treatment against *V. destructor* had been administered. After a two-month acclimatization period, colonies were transferred to jumbo-type hives, each containing at least six frames, allowing for a homogeneous experimental design.

Preparation of experimental units

Hive weight and mite infestation levels were measured at the beginning and end of the experiment. Mite infestation was assessed using the adult bee washing method (De Jong et al., 1982), recognized for its precision (Dietemann et al., 2013; Roth et al., 2020). The 40 hives were randomly assigned to four groups (n = 10 colonies per group). All groups received 1 L of 50% (w/v) sucrose solution and 200 g of a protein supplement composed of: 50.8% honey, 30.5% granulated sugar, 12.7% yeast, 2.5% egg flour, 1.2% powdered milk, 1% vitamin supplement, 0.7% avocado oil (with or without rosemary oil), 0.5% citric acid, and 0.1% ground cinnamon.

Treatment description

REO, used as the acaricidal agent, had a specific gravity of 0.894 – 0.912 and a refractive index of 1.464 – 1.476 (NOW® Foods, USA), extracted without solvents or diluents. Avocado oil (San Lucas, Mexico) served as the carrier. REO was diluted in avocado oil final concentrations of 0%, 5%, 10%, and 20% (v/v). A total of 14 mL of each mixture was incorporated into 2 kg of the protein supplement. Treatments were designated as: T0: 0% REO (negative control); T1: 5% REO; T2: 10% REO and T3: 20% REO. The protein supplement was administered daily for 7 consecutive days, during which acaricidal efficacy was evaluated.

Mite drop quantification and protein supplement consumption

A white plastic sheet (27 × 52 cm) coated with a thin layer of vegetable oil was placed at the bottom of each hive to immobilize fallen mites and prevent their reintegration into the colony (Ismaili et al., 2019). For seven consecutive days, mite drop was recorded manually at 2 pm by counting the mites trapped on the sheet. At the end of the seven-day period, the remaining protein supplement in each hive was collected and weighed. The difference between the initial and final weight of the supplement was used to estimate the amount of protein supplement consumed by the colony during the treatment phase.

Mite infestation rate

To confirm infestation levels, an auxiliary method was used involving an acaricide with > 95% efficacy. A mesh-protected bottom board (3 – 4 mm mesh) prevented bees from accessing fallen mites. Mite drop was recorded daily over three weeks, covering the Varroa reproductive cycle and including both phoretic and emerging mites from capped brood.

Hygienic behavior assessment

A metal cylinder (8 cm diameter × 10 cm height) was placed on a frame containing capped brood. Capped cells inside the cylinder were counted, and pupae were sacrificed by two applications of 150 mL liquid nitrogen, spaced one minute apart (Medina-Flores et al., 2014; Vargas et al., 2019). After 24 h, cleaned and removed cells were counted. The hygienic behavior index (HBI) was calculated as:

HBI = (Number of cleaned cells at evaluation − Number of empty cells)/Total number of capped brood cells at the beginning.

Data analysis

Lineage determination: Consensus sequences were assembled using Bio Edit Sequence Alignment v7. 0.9.0 and compared via BLASTN (NCBI). Sequences were aligned using ClustalW, and a phylogenetic tree was constructed using maximum parsimony with 5,000 bootstrap re-plicates in MEGA v6.0.

Colony performance variables: The variables evaluated included protein feed consumption, hygienic behavior, mite drop count, and varroosis incidence. Data were analyzed using PASW Statistics 18.0.0. Logarithmic transformations were applied when necessary to meet assumptions of normality. Both linear and quadratic responses in mite drop were assessed through complementary analyses using the same software.

Results

Mitochondrial lineage identification

A nucleotide sequence obtained from the isolated mitochondrial DNA (mtDNA) was deposited in the NCBI database under accession No. MK703807. Since worker bees are the progeny of a single queen, the mitochondrial lineage of the colony can be considered equivalent to that of one individual. Phylogenetic analysis using the maximum parsimony method allowed the construction of a phylogenetic tree (Figure 1), indicating that the colonies belong to mitotype C, exhibiting 87% homology with *Apis mellifera carnica*.

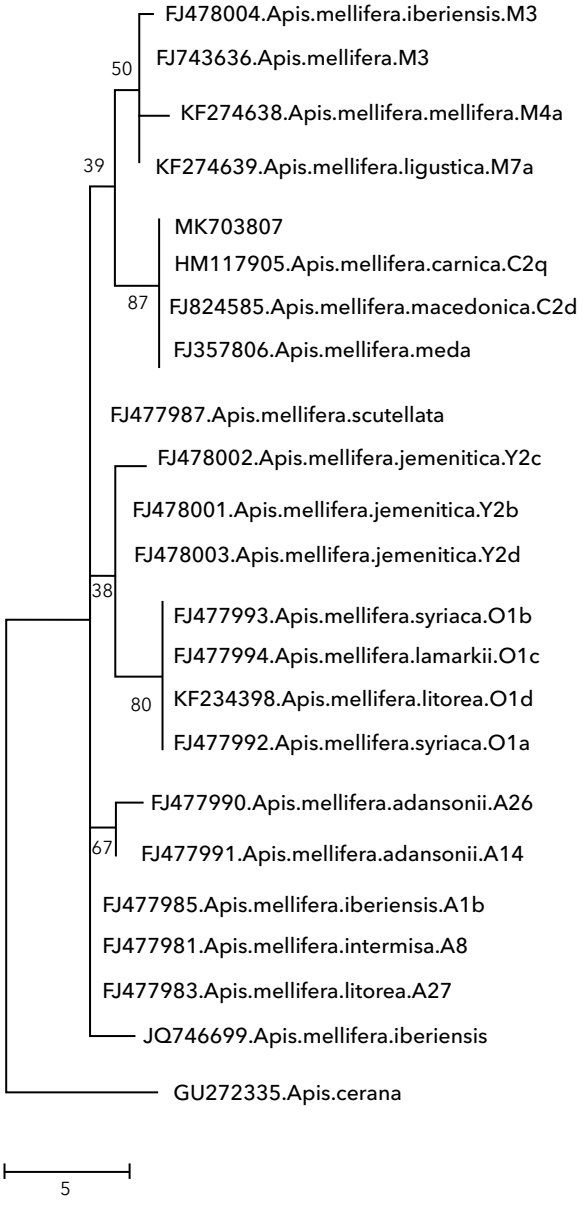

Figure 1 - Phylogenetic tree of *Apis mellifera* haplotypes based on the mitochondrial COI-COII intergenic region. Bootstrap values are shown at each node.

Initial colony conditions

No statistically significant differences were observed among treatments at the beginning of the experiment for hygienic behavior (p = 0.578), hive weight (p = 0.068), or Varroa infestation rate (p = 0.946), confirming homogeneous initial conditions across experimental units.

Final *Varroa* infestation and mite drop

At the end of the trial, *Varroa* infestation rates differed significantly among treatments (p = 0.033). Mite drop was also significantly influenced by dietary supplementation with REO (p = 0.012), with the highest acaricidal efficacy observed in treatment T2 (10% REO) (Table1).

Table 1 - Effect of dietary supplementation with *Rosmarinus officinalis* essential oil (REO) on daily mite drop, weekly protein supplement intake, and hygienic behavior in *Apis mellifera* colonies

Treatment	Daily mite drop (mean ± SEM)	Weekly supplement intake (g ± SEM)	Hygienic behavior (ratio ± SEM)
T0 (0%REO)	13.18 ± 1.79[a]	148.2 ± 47.5[a]	0.898 ± 0.0519[a]
T1 (5%REO)	12.68 ± 4.37[a]	190.3 ± 14.9[a]	0.935 ± 0.0349[a]
T2 (10%REO)	20.61 ± 5.97[b]	146.5 ± 33.1[a]	0.936 ± 0.0311[a]
T3 (20%REO)	17.02 ± 6.61[ab]	168.4 ± 53.6[a]	0.956 ± 0.0231[a]

Note: SEM = standard error of the mean. Each colony received 200 g of a protein supplement, formulated on a weight/weight basis, and composed of 101.6 g honey, 61.0 g powdered sugar, 25.4 g yeast, 5.0 g egg flour, 2.4 g milk powder, 2.0 g vitamin complex, 1.4 g avocado oil (with or without rosemary oil), 1.0 g citric acid, and 0.2 g ground cinnamon.[a,b]Different letters within the same column indicate significant differences ($p < 0.05$).

A quadratic response pattern was detected (p = 0.045), suggesting that increasing REO concentration beyond 10% (e.g., 20%) may not enhance — and could potentially reduce — acaricidal effectiveness.

Supplement intake and hygienic behavior

No significant differences in protein supplement intake were detected among treatments (p = 0.067). Likewise, hygienic behavior indices did not vary significantly across treatments (p > 0.05), indicating that REO supplementation did not adversely affect this essential colony defense behavior.

Seasonal context during the experiment

The experiment was conducted during a seasonal phase in which colonies exhibit a high dependence on supplemental feeding due to reduced nectar and pollen flows. During this interval, protein patties were consistently consumed across all treatments, and no atypical fluctuations were detected in colony strength or foraging activity. The homogeneity of these ecological conditions ensured that all colonies were subjected to comparable seasonal stressors, thereby providing a stable environmental baseline against which the effects of REO supplementation and the associated behavioral responses could be reliably interpreted.

Discussion

Sequencing of the mitochondrial COI-COII intergenic region allowed detection of nucleotide variations that enhanced the identification of genetic lineages. The colonies analyzed in this study belong to mitotype C, with 87% homology to *A. mellifera carnica* (Luna-Rodríguez et al., 2016). This result aligns with previous findings from central Mexico, where at least two mitotypes — lineages C (including *A. mellifera carnica*) and M (*A. mellifera mellifera*) — have been reported in beekeeping systems of the Chalco-Amecameca sub-basin, based on COI-COII sequence data (Luna-Rodríguez et al., 2016).

The results demonstrate that dietary supplementation with REO effectively reduces *V. destructor* infestation in *A. mellifera* colonies. The highest acaricidal

effect was achieved at the 10% REO concentration (T2), consistent with prior studies that documented REO's miticidal potential (Islam et al., 2016; Lazãr and Pãtriucã, 2020). The observed quadratic response suggests an optimal concentration threshold, above which acaricidal efficacy diminishes, potentially due to repellency effects or sublethal toxicity impacting bee behavior or physiology (Gashout and Guzmán-Novoa, 2009; Bendifallah et al., 2018). These findings imply that moderate REO doses may be preferable for field applications, optimizing safety and effectiveness.

The absence of significant differences in protein supplement consumption across treatments indicates that REO inclusion does not reduce palatability, thereby maintaining adequate colony nutrition during treatment. Furthermore, the consistent hygienic behavior across all groups suggests that REO does not interfere with this crucial social immunity mechanism.

While previous research has shown high acaricidal efficacy of REO through topical application or fumigation (Koumad and Berkani, 2019), this study proposes a novel, less invasive oral administration route via dietary supplementation. This method could facilitate wider application, reduce colony stress, and minimize beekeeper exposure to chemicals.

The incorporation of natural bioactive compounds such as REO is particularly relevant in organic apiculture, where residue-free products and sustainable pest management are increasingly demanded. Moreover, natural acaricides offer potential solutions to the growing issue of resistance to synthetic miticides, supporting resilient and ecologically sound beekeeping systems.

Overall, this research contributes empirical evidence toward transitioning apiculture to environmentally compatible production models that comply with organic certification standards and promote biodiversity conservation. Considering the critical ecological role of honey bees as pollinators, such sustainable management practices also support broader ecosystem services and agricultural productivity.

In the Mexican beekeeping context, the interpretation of *V. destructor* infestation levels gains particular importance due to the thresholds established in NOM- 001-ZOO-1994 (amended in 2005), which classify colonies exceeding 5% infestation as being at sanitary risk. This threshold is especially relevant during periods of reduced brood production, when a higher proportion of mites remain in the phoretic phase. In central Mexico — including Veracruz — infestation peaks typically occur during the low-floral period (September – November), when limited pollen availability constrains brood development and intensifies *Varroa* population pressure.

Because the present study was conducted precisely during this critical seasonal window, the infestation values obtained reflect a high ecological challenge and thus provide a rigorous context for evaluating treatment performance. Under these demanding conditions, the superior efficacy observed with the 10% REO treatment indicates its potential utility as a complementary tool within integrated varroosis management programs in Mexico, particularly during the periods explicitly identified as high-risk in national veterinary regulations (DOF, 2005).

Conclusion

The use of natural products such as REO represents a viable alternative for managing hive health in *A. mellifera* colonies. Incorporation of REO into supplementary feeding effectively reduced *V. destructor* infestation without adversely affecting food intake or hygienic behavior. These strategies are particularly relevant given increasing international demands for food safety and organic certification. Additionally, they enhance the resilience of the beekeeping sector and contribute to the well-being of honey bees — key agents in maintaining ecological balance and ensuring food security through pollination services.

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Authors´contributions

JMVR: conceptualization of the study, methodological design, analysis and interpretation of results, and critical revision of the manuscript. VAL: experi-

mental work, data collection, data processing, and initial drafting of the manuscript. JCZ: statistical analysis, interpretation of results, and technical revision of the manuscript. LECB: methodological support, validation of experimental procedures, and contribution to the discussion of results. XVSH: data collection, database organization, and support in preliminary data analysis. LLR: conceptualization of the study, overall project direction and supervision, final manuscript writing, and editorial management. IGGE: literature review, contextual interpretation of results, and critical revision of the manuscript.

Data availability statement

The research data are not publicly available due to institutional restrictions, but are available from the corresponding author upon reasonable request.

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