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Research Article

In Vitro Evaluation of *Eucalyptus pellita* Essential Oil against Varroosis in *Apis mellifera*

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Abstract

In order to evaluate *in vitro* the essential oil of *Eucalyptus pellita* against varroosis in *Apis mellifera,* fresh leaves were randomly collected from Eucalyptus plants. The essential oil extraction was carried out by means of the hydrodistillation method (with water and steam) at the Center for the Study of Applied Chemistry, University of Granma. The parasites were collected in an apiary located in the Santa Isabel community of the Bayamo municipality from zanganero combs. For the evaluation of the acaricidal effect, concentrations of 25, 50, 75 and 100% were used respectively. Distilled water was used as a control. 20 insects were placed in a Petri dish with filter paper impregnated with the product, less in the control. Readings were made at 4, 8 and 24 hours. The percentage of mortality of the mites was determined. The analysis of variance was carried out for the comparison of proportions. Through the use of the COMPARPRO 1.0 Program [4]. The results show greater antiparasitic activity when using the 75 and 100% concentrations during 24 hours of investigation. It is concluded that the biopreparation has an antiparasitic effect against Varroa destructor of Apis mellifera *in vitro*.

Keywords: *Oil; Essential; Eucalyptus pellita; Varroa*

Introduction

Varroosis is a disease caused by the *Varroa destructor* mite, a parasite that affects bees in all their stages of development, feeding on their hemolymph. This parasite not only causes physical damage, it also encourages the development of other infections in the colony. Currently it is considered worldwide as the most important pest, with prejudice to this species, due to the direct economic losses that they produce on them and the derived products.

Varroa destructor is present in most countries where beekeeping is practiced, both in populations of rustic hives and in those that are not. It became an economic concern in Japan and China in the 1950s and 1960s, in Europe in the late 1960s and 1970s, and in Israel and North America in 1980 [1].

In Cuba, this parasite was diagnosed in 1996 and is one of the worst problems affecting beekeeping. At this time it has spread to the entire national territory, causing millions in losses in our second exportable line. Between 1996 and 1997 in only two provinces more

than 10 000 hives were lost, a mortality that was stopped and today it is estimated as an annual average in 4% of the national apicultural park, these deaths not only directly attributable to the morbid picture caused by the mite, but to the super-added diseases as a result of the plundering action of the mite [2].

Many of the products that are universally used against *Varroa* are toxic, therefore their use is prohibited due to producing residues in honey, resistance in bees and environmental contamination. The control of the mite in our country began in the year 2000, it is based on the use of chemicals such as flumethrin (Bayvarol 4%), which generates resistance and residual problems in the products of the hives, being applied only at the time of do not harvest honey, it usually costs millions of dollars.

Both in Cuba and worldwide, the search for new methods that allow the control of the mite and do not affect the bees or represent a source of contamination to the beehive ecosystem and the environment in general is currently being carried out. Among the alternatives are the introduction of lines of insects resistant to the parasite, the use of organic acids, essential oils and plant extracts [3].

Objective of the Study

The objective of the work was to evaluate *in vitro* the essential oil of *Eucalyptus pellita* against varroosis in *Apis mellifera.*

Materials and Methods

Experimental location

The investigations were carried out in the period from October 2018 to March 2019, in the Department of Veterinary Medicine of the Faculty of Agricultural Sciences, the Center for the Study of Applied Chemistry, the Laboratory of Biological Agents of the University of Granma, and the Laboratory of the Department of Animal Health of MINAGRI in Granma, Cuba.

Selection of plant material to obtain essential oil

Was carried out taking as a criterion the bibliographic review and other works carried out by researchers from the beekeeping company of Granma, Cuba. In addition, the feasibility of *Eucalyptus pellita* in the forest around the University of Granma was taken into account.

The Plant Material (MV) consisted of fresh leaves collected at random from trees approximately six years old and eight meters high in the Eucalyptus forest present around the University of Granma and the surroundings of the city of Bayamo, between seven and eight in the morning.

The ambient temperature during was kept around 25 - 30°C and the relative humidity was 85%. The harvested foliage was stored in the Natural Products Laboratory of the Center for Applied Chemistry Studies (CEQA), in a cool, ventilated place with little lighting.

Extraction of essential oil

The extraction of essential oil was carried out in the Natural Products Laboratory of the (CEQA) by the hydrodistillation method (with water and steam) from the ground plant material. The distillation was carried out in a Clavenger type equipment [4], consisting of a hydrodistillator and a simple condenser. Water was used as the cooling liquid making use of a recirculator model ML W made in Germany.

Four extractions of three hours duration each were made from 82, 120, 189 and 200 grams of plant material respectively, weighed on a balance. BS2202S SARTORIUS technique made in Germany.

The essential oil obtained in each extraction was dried with anhydrous Na_2SO_4 of analytical quality to eliminate the remaining water from the distillation process and was subsequently weighed to calculate the percentage yield (mass /mass) of the extraction.

Formulation of the biopreparation

They were prepared in concentrations of the essential oil at 25; 50; 75 and 100% respectively, with distilled water as the base excipient, until the mixture was obtained with the desired homogeneity, using distilled water as a control. The ultrasound technique was used with an ultrasoniccleanerSB-120DT type equipment, later they were packaged in amber bottles (50 mL), to avoid the acceleration of the degradation kinetics by photolysis. All samples were labeled and identified.

Experimental location of the study development

It was carried out in the period from October to March of the year 2017 until May 2019. The mites were collected from an apiary located in the Popular Council of Santa Isabel of Bayamo, from drone combs of the *Apis mellifera* species subjected to a breeding system intensive in Langstroth boxes whose infestation occurred naturally.

Experimental design

Four concentrations of the biopreparation (25; 50; 75 and 100%) plus a control were used.

Dosage formulation

- Concentration 1: 25% 0.25 ml of essential oil and 0.75 ml of distilled water were added to a test tube.
- Concentration 2: 50% 0.50 ml of essential oil and 0.50 ml of distilled water were added.
- Concentration 3: 75% 0.75 ml of essential oil was added and 0.25 ml of distilled water was added.
- Concentration 4: 100% per quarter was formulated with 1 ml of the oils, without adding anything else
- Control 5: Apif1 ---- distilled water.

Each concentration constituted a treatment and three replications were made, in a Petri dish with filter paper impregnated with the biopreparation with the essential oil, by the method of complete exposure in a Petri dish, except in the control. 20 mites were placed on each disk. The readings were made at 4, 8 and 24 hours, after applying the product. The percentage of dead insects was determined, and they were observed using the microscope-stereoscope.

During the entire experimental period, hygiene and uniform lighting conditions were maintained and the behavior of temperature and relative humidity was evaluated every 4 hours with a hydrothermograph located in the room.

For the statistical analysis of the results, the number of dead mites was compared, according to the observation time for each concentration and between treatments at each observation moment through multiple comparison analysis of proportions. Duncan's (1955) test was used for multiple comparison of means. The processing was carried out using COMPARPRO 1.0 [5].

Results and Discussion

When evaluating the effectiveness of the different concentrations of the biopreparations on the mites (Table 1), it was observed that the concentrations 75% and 100% show similar results, which differ significantly from the rest of the concentrations and the control without treatment. Mortality percentages of 100% are achieved, which shows the effectiveness of the product for the treatment against *Varroa destructor* of *Apis mellifera,* which may be due to its chemical composition, which presents eucalyptol, with a great acaricidal effect.

Concentrations (%)	Accumulated deaths (%)
25%	82,20 b
50%	91,09 ab
75%	100,00 a
100%	100,00 a
Control apif1	19,44 c
EE	5,437
Sig	***

Table 1: *Accumulated percentage of dead mites with the use of different concentrations of the biopreparation.*

Verde and Chan [6], pointed out that in the world numerous investigations are carried out in order to determine and take advantage of the sensitive points of the development of *Varroa* to interfere with them and discover an effective long-term and respectful fighting technique for bees and their products.

Likewise [7], reported the acaricidal effectiveness of *Azadirachta indica A. Juss (Nim*), which has a similar application to vaseline, considered a tropical tree, from which the oil of its seeds is obtained, constitutes an effective option in the control of the Varroa. However, this effect is less than the use of chemicals.

The results obtained are similar to those reported by [8], in Italy when using thymol, reaching 81.2% effectiveness with the use of concentrations of 25%, 50%, 75% and 100 %. Which corroborates our results.

In this order, groups of Italian, French and Swiss beekeepers have practiced control methods based on natural molecules, in particular formic acid, oxalic acid and thymol, whose qualities are based on the non-contamination of honey and having a very low cost, this set of methods has been called as a generic name alternative control. However, very few are those that have been preceded by a laboratory study, an important element when it comes to achieving greater effectiveness of these products.

Similarly [8], in Italy, when comparing two thymol-based treatments, one being a commercial product, Api lifeVar (ia thymol, menthol, eucalyptol and camphor) with an acaricidal power of 81.2% and TAV designed by the Andalusian center of organic beekeeping with 85.8%. results much lower than what is shown in our research.

Likewise [9] obtained results similar to ours when applying the essential oil of *Piper aduncum* (Platanillo de Cuba), demonstrating that it has a promising and selective acaricidal effect against *Varroa destructor.* The major components of the oil are camphene, camphor, piperitone and viridiflorol. It is a promising candidate for the development of a botanical miticide and could be used in the future in the context of an integrated management of varroosis.

In our work, an effectiveness of 82% to 100% was obtained in 24 after the *in vitro* treatment was applied in the laboratory. Higher than that obtained by other researchers [10-12]. On the other hand [13,14], observed that within certain limits, the concentration and not the quantity of the product is critical for efficacy, although a certain influence of the product cannot be ruled out. breeding area.

Considering the large number of different groups of chemical compounds present in essential oils, it is important to take into account that their antimicrobial activity is not attributed to a specific mechanism, however there are some sites of action in the cell where the following effects occur: damage to the cytoplasmic membrane, to the cell wall, to proteins, filtration of cellular material, coagulation of the cytoplasm, decreased motive force within the cell [15].

Likewise [16] they suggest that carvacrol and thymol have several sites of action within cells and depending on the concentrations carried out when preparing it for its use, they can cause inhibition or inactivation of microorganisms. Likewise [17], they corroborated that

the targets or points of attack of these antimicrobial agents within cells include the cell wall and membrane, metabolic enzymes, protein synthesis and the genetic system.

On the other hand [18] they pointed out that thymol can change the permeability of the membrane of microbial cells, allowing the chemical constituents that are essential for metabolism, such as ATP ions, nucleic acids and amino acids, to be filtered. these effects cause a decrease in the total cell load.

According to [19], carvacrol and thymol, which are naturally present in some plant species such as oregano and thyme, present antimicrobial activity against certain pathogenic microorganisms of interest in food, basically their mechanisms of the action consists in changing the permeability of the cytoplasmic membrane, causing the intracellular material to escape and therefore the death of the pathogens.

In table 2, the replicas with the different concentrations are compared where a highly significant difference was observed, finding that at 4 hours the greatest effectiveness was at 100% of the concentration while at 8 and 24 hours the greatest effectiveness was reflected at 75% of concentration. This may be due to the chemical composition of eucalyptus essential oil, which is characterized by the presence of 1,8-cineole, limonene, and α-pinene.

Concentrations	4 hours	Deads (%) 8 hours	24 hours
25%	15.55^{c}	28.88^{bc}	37.77^{a}
50%	28.88^{bc}	37.77^{ab}	24.44^{b}
75%	40.00^{b}	48.88^{a}	11.22^{c}
100%	64.44^{a}	32.22^{b}	3.34^{c}
Control apif1	0.00^{d}	15.00^{c}	4.44^{c}
EE	4.82	4.94	3.96
SIG	***	***	***

Table 2: *Comparison between the different concentrations during 24 hours.*

According to [20], eucalyptus oils have been effective against *T. urticae*, 89% mortality of mites), however [21], reported 96.7% mortality against *Varroa destructor* of *Apis mellifera*, results higher than those obtained in our research.

On the other hand, [22] that among the chemical components of *Eucalyptus,* 1,8-cineole is very important due to the diversity of pesticidal effects that are reported for this essential oil, which also increases its action against the mite.

According to [23], in research carried out in Granma with alcoholic extract of the *Trichilia hirta* plant in concentrations similar to those applied in our research, he observed similar results to ours, obtaining a highly significant difference in concentrations of 75 and 100% respectively, with an observation period of 36 hours.

On the other hand, [24] showed that oxalic acid turned out to be a feasible option in the control of the *Varroa* mite in the conditions of Baja California. Efficacy was determined on the basis of the reduction of the infestation rate in adult bees, showing an effectiveness that ranged between 82.8 and 90.58% respectively, results lower than those obtained in this research.

Similarly, [25] evaluated the effect of two natural products (formic acid and thymol) for the control of *Varroa destructor* in colonies of *Apis mellifera L.* The highest efficacy was obtained with two applications of 12.5g of thymol (92.1%), while with formic acid, it was 66.4%. Both acaricides obtained a significant parasite mortality ($P < 0.0001$), however it decreased after the first replication, with different results from those exposed in our investigation.

Likewise, [26] reported that the use of natural oils derived from plants against mites is well founded. These material oils destroy the *Varroa brood* and lead to high mortality in adults. In preliminary tests several infested colonies were exposed for six weeks (after the

honey flow) to one of three different mixtures containing: thymol (74 - 79%), eucalyptus oil (16 - 17%) and menthol and/or camphor (3.7 - 4%). Mortality of 74 to 92% was observed, whereas in untreated controls it was 10%.

Studies carried out by [27], with the essence of *Eucalyptus resinifera*, to reduce the population of mites, the number of dead parasites after treatment was more evident at 24h, results similar to those obtained in our work with concentrations of 75% and 100%.

Furthermore [1], reported values of the same magnitude as those of our investigation, when evaluating the virucidal effect of thymol administered in two ways: a single application of 25 g and another of two applications of 12.5g each, obtaining a mortality of 94 % and 91% respectively without being affected the adult bees and the brood; Similar values were obtained by [6], when using three commercial products Apilife Var in porous substrate (95.5%), Apiguard in a gel matrix (94.3%) and Thymovar (90%).

Conclusion

The concentrations of 75 and 100% of essential oil, respectively, were the highest during the 24 hours of the *in vitro* investigation.

Conflict of Interest

No conflict of interest.

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