INTRODUCTION

Acaricides are currently used globally for the control of the honey bee parasite, Varroa destructor, a mite that endangers the international beekeeping community. Colonies infested with V. destructor have significantly reduced worker bee populations which may eventually die off if infestations are uncontrolled (1). There are numerous drugs and modes of applications to combat Varroosis. It has been widely documented for many years that some of these drugs may cause contamination of bee products with acaricides (2, 3).

The use of synthetic lipophilic acaricides leads to an accumulation of these substances in beeswax, but less so in honey. The accumulation in wax depends on the frequency, lipophilicity and amount of active ingredient used (2). The persistence of pyrethroid and organophosphate acaricides in beeswax has led to strains of V. destructor that are resistant against fluvalinate, flumethrine and coumaphos (4, 5). These problems have led to investigations to identify alternative control methods. Some alternatives proposed have included organic acids, thymol and essential oils or any of their components (6). Essential oils are distilled from aromatic plants and possess an intense smell, exhibit low toxicity...
in mammals and bees and have less harmful environmental
effects than synthetic substances (7).

To date, over 150 essential oils and components of these
oils have been tested. However, only some of the compounds
have been found to be effective on V. destructor mites while
also remaining nontoxic to honeybees. They are applied topi-
cally either by pulverization or passive evaporation (6).

Clove (Syzygium aromaticum) oil is one of the effective
essential oils against V. destructor mites that infest honey-
bee (Apis mellifera) colonies. Promising results have been
obtained in previous studies (8, 9, 10). Active components
of this oil have been demonstrated to have antimicrobial,
insecticidal, antioxidant, antitumor and anesthetic activity
(11). It is also recommended as an insect repellent during
pediatric nursing (12).

Beeswax is a highly lipophilic medium containing high
molecular weight acids. Synthetic acaricides can be stable
and persistent in beeswax, but essential oils show less persistence
and stability (13).

This study was undertaken to determine the stability of
clove essential oil with different excipients in beeswax under
semi-field conditions, using gas chromatography/mass spectro-
photometry (GC/MS) for detection and analysis. The evapora-
tion time of active ingredient(s) from beeswax and the possible
effect of different dispensers and excipients were investigated to
determine the time of the booster treatment of Varroosis and
the convenient time for honey harvest after treatment.

**MATERIALS AND METHODS**

**Characterization of clove essential oil**

In this study, Clove bud essential oil (EU pharmacopeia
grade) was cultivated in Germany and purchased from
Fagron GMBH & CO (Barsbüttel, Germany). It was dist-
tilled in Germany as follows: isolated from the buds using
steam distillation equipment for 4 hours, separated from wa-
ter by decantation, dried with anhydrous sodium sulphate,
and kept refrigerated to avoid deterioration (14).

The characterization of the clove oil was conducted at the
National Residue Laboratory of Kimron Veterinary Institute
from the Ministry of Agriculture, Beit-Dagan, Israel, during
July 2010.

The GC/MS analysis of the oil was conducted using a
GC (Agilent Technologies 6890N, California-U.S.A.) inter-
facial with a mass selective detector (MSD, Agilent 5973B)
and equipped with an auto sampler (ALS, Agilent7673). An
Agilent HP-5 MS (5%-phenyl methyl poly siloxane) capil-
ary column (30 m x 0.25 mm i.d. and 0.25 µm film thick-
ness) was used for separation. The carrier gas was helium with
a constant flow rate of 1 ml/min. The oven temperature was
set at 50°C for 1 min, programmed at 150°C at a rate of 5°C/
min, was once more heated to 280°C at a rate of 30°C/min
and finally held isothermally at this temperature for 25 min.
Injector and GC/MS interface temperatures were 250°C and
280°C, respectively. The injection mode was splitless, and the
volume injected was 1 µl of the final extract volume. The MS
operating parameters were as follows: ionization potential, 70
eV MS source and quad temperatures of 230°C and 150°C,
respectively. The acquisition mass range was 40–400 m/z.

**Identification and quantification of constituents**

The relative percentage of the volatile oil components was
evaluated from the total peak area (TIC) using apparatus
software. Identification of components in the volatile oil was
based on a comparison of their mass spectra and retention
time with those of the authentic compounds and by com-
puter matching with the NIST and WILEY libraries as well
as by comparison of the fragmentation pattern of the mass
spectral data with those reported in the literature.

**Preparation of hives and application of clove oil**

Location of the apiary was at Beit-Dagan, 31° 99’ N, 34° 82’
E, in Israel. The study was performed in July 2010, in which
average temperature ranges were 27.5 – 31.3°C at daytime,
17.9 – 20.6°C at nights and average humidity ranges were
60 – 87% at daytime, 30 – 63% at nights. All procedures were
applied in the early morning to avoid the evaporation due to
high temperatures.

Four Langstroth hives with ten frames each were used.
All hives had a foundation of beeswax and contained no
bees in the hive at the field site. Entrances of the hives were
open. Application of the clove oil was carried out using four
methods:

**(1) Cup method with Tween 80 solution:** 50 ml of 1% clove
oil was prepared in distilled water and emulsified using a
Tween 80 solution (2 ml clove oil + 44 ml distilled water +
4 ml tween 80) and divided into two plastic cups (2×25 ml).
The cups were placed at the bottom of the hive between the
2nd-3rd and 7th-8th frames. Two frames were removed to allow
enough space for the cups.
(2) **Spray method with Tween 80 solution:** The same mixture used for the first hive was applied with a spray bottle on the 3rd–8th frames at an amount of 25 ml per frame, 12.5 ml per each surface of combs and at a total of 50 ml in the hive.

(3) **Cup method with paraffin oil:** 50 ml of 1% clove oil was prepared in paraffin oil emulsifier (2 ml clove oil + 48 ml paraffin oil) and divided into two aliquots (2×25 ml) in plastic cups. The cups were placed at the bottom of the hive between the 2nd–3rd and 6th–7th frames. Two frames were removed for the cups.

(4) **Spray method with paraffin oil:** The same mixture as used in hive 3 was applied. This mixture was sprayed on the 3rd–8th frames. It was applied at an amount of 25 ml per frame, 12.5 ml per each surface of combs and at a total of 50 ml in the hive.

(5) **Control group:** None of essential oil or any acaricide was applied on beeswaxes.

The essential oil concentrations and amounts were chosen on average according to the results of previous published research conducted on clove oil (6, 9, 10). These specific methods were used to obtain similar conditions for commercial or home-made drugs of essential oil based acaricides.

**Wax Sampling**

Using a scalpel blade, 10 cm² (approximately 4 g) of wax was taken from each hive and placed in plastic test tubes. In hives 1 and 3, samples were taken from two different frames (2×5 cm² of wax) close to the clove oil source; in hives 2 and 4, samples were taken from the combs onto which the clove oil was sprayed. In the control hive, samples were taken from two different places. All wax samples were taken at days 1, 2, 5, 7, 14, 21 and 28 after treatment.

**Sample preparation procedure**

Wax samples for GC/MS analysis were stored in a freezer (-20°C) for one night. For clove oil residue extraction, 15 ml of acetonitrile was added to the beeswax samples in 50 ml polypropylene test tubes. The samples were placed in a 70°C water bath for melting.

After 40 minutes, the samples were shaken and left to separate in the 70°C bath for 5 min. Tubes with the acetonitrile phase with extracted clove oil residues on top and beeswax at the bottom were held at room temperature for at least one hour. The liquid on top was filtered using Millipore filter paper into another test tube. Next, 1.5 ml of this liquid was transferred to Eppendorf test tubes and centrifuged for 15 min at 45000 rpm to separate possible particulate materials and wax particles. After centrifugation, all samples were transferred to the auto-sampler vials and held in the freezer (-20°C) until GC/MS analysis was performed.

**RESULTS**

Composition of clove essential oil (*Syzygium aromaticum*) obtained by GC/MS analysis resulted under our test conditions in the identification of thirty-six different components. Eugenol and trans-Caryophyllene were the two major components in the oil, together representing 84.45% of the sample; other components were present in small amounts (Table 1). None of clove oil components was found in the beeswax of control group.

**Table 1: Major components of *Syzygium aromaticum* essential oil determined by GC/MS analysis**

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eugenol</td>
<td>75.35</td>
</tr>
<tr>
<td>trans-Caryophyllene</td>
<td>9.10</td>
</tr>
<tr>
<td>(-) Caryophyllene oxide</td>
<td>4.59</td>
</tr>
<tr>
<td>beta-Selinene</td>
<td>2.15</td>
</tr>
<tr>
<td>delta-Cadinene</td>
<td>0.98</td>
</tr>
</tbody>
</table>

* Relative percentage obtained from GC peak area

Table 1 illustrates the amounts of the major components of clove essential oil in beeswax and Figure 1 illustrates the levels of eugenol and its depletion compare with beeswax different excipients and modes of applications in hives, over time obtained using GC-MS. Levels (mg/g) were calculated based on 10 cm² of beeswax foundation and corrected to 1 g of beeswax foundation in each test hive.

As can be observed from the data, the amount of eugenol decreased until the 5th day. After this point, levels increased up to the third week in all groups.

The role of excipients can be seen in Figure 1 and Table 2. The amount of eugenol in clove oil mixed with paraffin oil (hive three) was evaluated as very low, and it had evaporated at the earliest time (two weeks) when compared to the others. The amount of eugenol in beeswax with the other usage methods was exactly at the same level in the 4th week. This indicates that 1% clove oil is trapped and accumulated in the wax for at least three weeks if it is mixed with tween 80 and is applied by evaporation from cups or spraying. This is also true if it is mixed with paraffin oil applied by spraying.
DISCUSSION

Knowledge regarding the stability of the active ingredients of acaricides is very imperative as residue risks in bee products and residue studies should always include an investigation into the stability of the analyzed active ingredient.

Eugenol, as well as thymol, eucalyptol, camphor and menthol have a ‘Food and Agriculture Organization-Generally Recognized as Safe (for human consumption) (FAO-GRAS)’ status at concentrations up to 50 mg/kg. According to EU regulation No.2377/90, eugenol is within group II of the nontoxic veterinary drugs, which do not require a maximum residue limit (MRL) (15). However, if clove oil (eugenol) is used during honey flow, there is a significant possibility that eugenol residues in honey might reach levels above the taste threshold (16). As a result honey harvesting time after essential oil treatments has gained importance.

The primary component of clove oil was found to be eugenol, which, together with analogs and allylphenols, a member of the phenylpropanoid class of chemical compounds. The acaricidal properties demonstrated by clove oil and its major component eugenol have shown promising results on Varroa spp, both during in vitro and in vivo conditions (6, 9, 10). Promising results have also been obtained from eugenol for use against scabies mites as a topical acaricide (17). Clove oil is non-toxic to fish and acts as an anesthetic for some fish species; it has a low toxicity to mammals and is toxic to ants (11, 18). Toxicity tests have shown lethal effect of S. aromaticum on the mite of honeybees V. destructor. Its toxicity on honeybees can change depending on factors such as dose, temperature and evaporation time, but it is agreed as being safe if it is used at proper doses for Varroa mites at the concentrations of approximately 1% (10), as was done in this study.

Chemical composition of the clove essential oil may vary widely due to the origin of plant and other environmental variables (11). In our study, the major component of clove oil was eugenol 75.35%, with trans-caryophyllene 9.1% and lesser amounts of other components (Table 1). Nevertheless, some researchers have found different proportions such as, eugenol 63.37% with β-caryophyllene 15.94% (19), and in another study, eugenol 86.7% with β-caryophyllene 3.2% (10).

After the application of clove oil, there were considerable eugenol residues in the wax, especially during the first few days. The increase of eugenol levels from the 7th day in almost all groups is likely due to the trapping capacity of wax and volatility of the oil. Similar results were observed in a trial with thymol, whereby an increase was reported to be present on the 50th day in brood combs placed in a hive without bees (13).

We used only eugenol to indicate the stability of clove oil in beeswax. Because eugenol is the major compound, comprising 75.35% in this research, it can be used as a representative and reference compound for clove oil and for the less volatile compounds in clove oil. Beeswax is complex to analyze because it is composed of fatty esters and long chain hydrocarbons, which are both readily extracted by organic solvents commonly used in residue analysis. This causes important extraction interferences because these lipids cannot be fully separated from pesticides (20). Similarly, there were many components in blank beeswax samples used in our control group however none of them belonged to clove oil.

<table>
<thead>
<tr>
<th>Day</th>
<th>Hive 1 (mg/g)</th>
<th>Hive 2 (mg/g)</th>
<th>Hive 3 (mg/g)</th>
<th>Hive 4 (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.094</td>
<td>3.125</td>
<td>0.593</td>
<td>2.711</td>
</tr>
<tr>
<td>2</td>
<td>1.017</td>
<td>0.846</td>
<td>0.233</td>
<td>0.696</td>
</tr>
<tr>
<td>5</td>
<td>0.034</td>
<td>0.171</td>
<td>0.015</td>
<td>0.259</td>
</tr>
<tr>
<td>7</td>
<td>0.057</td>
<td>0.224</td>
<td>0.022</td>
<td>0.213</td>
</tr>
<tr>
<td>14</td>
<td>0.048</td>
<td>0.041</td>
<td>0**</td>
<td>0.081</td>
</tr>
<tr>
<td>21</td>
<td>0.005</td>
<td>0.007</td>
<td>0</td>
<td>0.021</td>
</tr>
<tr>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Levels were calculated based on 10cm² of beeswax foundation and corrected to 1 g of beeswax. Units are mg eugenol/g in beeswax foundation
** Levels below limit of detection

Figure 1: Levels (mg/g) of eugenol in beeswax in hives analyzed and measured in beeswax within the hives
The importance of carriers to be introduced to beehives is stated for the use of clove oil in Varroosis management program (10). Therefore the excipients used in this work and quantities of oil in the wax may help in the assessment of its bioactivity under semi-field conditions. However, it is recommended that similar studies with bees should be planned in the next stage of this project.

Clove oil or any aromatic oil residues in beeswax may pose a problem for the taste of the honey (16). However, this residue can become an advantage for the treatment of Varroosis due to the trapping and releasing capacity of beeswax for the effective compounds inside. The eugenol and other oil substances participate as sustained release dosage forms when they enter into beeswax or in situ retarding release formulation, a process that can potentially prolong the effect of clove oil against Varroa infestation (6). Both situations should be considered before harvest and during treatment period and therefore we aimed to determine the duration of this period in our study.

The results obtained show that if clove oil is used to combat Varroosis of honeybee colonies, its most active ingredient, eugenol, can be stable for two to three weeks per treatment period. This means that clove oil treatment should be repeated once or twice after two weeks. Thus, the honey harvest must be begun at least three weeks after the clove oil treatment to prevent the presence of the taste of clove in the honey. Additionally, the treatment of honeybees with clove oil to prevent/combat Varroa destructor can be effective for approximately two weeks; therefore, a booster treatment should be applied after two weeks. Additional work to determine the stability of clove or any different essential oils used as acaricide with more hives and with bees, needs to be carried out in order to broaden this research in future.

ACKNOWLEDGEMENTS

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REFERENCES