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"IN VITRO" STUDIES ON USING NATURAL ESSENTIAL OILS IN TREATMENT OF NOSEMOSIS IN HONEY BEES: DETERMINATION OF THE THERAPEUTIC DOSE

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Abstract
Essentisl oils have been assigned with special properties over the time. They are used much in herbal medicine, but also in apiculture, mainly due to their emollient, calming, carminative, antispasmodic, and mainly antiseptic properties. In order to determine the therapeutic dose to be used in treatment of nosemosis in bee colonies, it has been tested the product Suppressor 1, a mixture of medicinal herbs and etheric oils obtained from melliferous plants, dissolved in ethylic alcohol 96°. The studies were carried out in laboratory conditions with microclimate parameters being permanently monitored. Five experimental modules with natural infection with Nosema spp. were organized for testing the product, together with three control ones (one positive and two negative controls, respectively); each module consisted in two groups, with at least 100 bees/group (106 - 187 bees). The bee groups were kept in wooden cages equipped with glass, mesh ventilation and nutrition systems. The tested product was used in different concentrations, from 1 to 50 ml /litre in sugar syrup, administered ad libitum. In order to determine the therapeutic dose, the mortality rate, at various intervals (24, 48, 72, 96 hours, and then at 8, 16 and 26 days, p.t.), was registered. The best efficacy was obtained for the concentration of 5 ml of product/l syrup; this was considered the therapeutic dose. The tested product was not proved to affect the viability and vitality of bees. These results will be the base for further field studies of the product, in order to provide alternative solutions for control of honey bee's diseases using natural products and to avoid drug residues in apiculture products.

Key-words*: honeybees, Nosema spp., plant extract, etheric oils, therapeutic dose.*

INTRODUCTION

Honey bee colonies grow exponentially in early spring and can use efficiently their first harvests. One of the limiting factors in the maximum development of the bee colony, particularlry for the European honey bee (*Apis mellifera*) is represented by infection with *Nosema spp*, a microsporidian parasite (Microspora: Microsporidida).

Nosema apis, the historical microsporidian parasite of European honey bees, can decrease worker longevity and cause considerable winter colony losses, whilst *Nosema ceranae*, introduced into European honey bees from its Asian congener (*Apis cerana*) within the last few decades is associated with colony depopulation and collapse in some areas of Europe (Higes et al., 2008a).

In Romanian bee colonies both species *N. apis* and *N. ceranae* exists in *Apis mellifera* colonies (Chioveanu et al., 2009).

Nosema spp. invades the epithelial cells of the intestinal midgut at its insertion with the Malpighian tubules, giving rise to large numbers of spores within a short period of time. The parasite is ubiquitous, in temperate conditions, nosemosis being considered to have a serious negative effect on the production capacity of honey bee colonies and the survival capacity of the affected colonies during the winter (Mitrea, 2011). Due to its subclinical evolution, usually, beekeepers do not always can estimate exactly the damages and losses due to *Nosema* infection (Popovich et al., 2012).

One of the goals of modern beekeeping is to obtain residue-free bee products, as well as a revival of researches related to using natural products for treatment in different pathological conditions (Bojor et al., 1984; Mitrea, 2002).
In phyto aromatherapy, etheric oils were awarded the outstanding properties over the time. They are used much in herbal medicine, but also in apiculture, mainly due to their emollient, calming, carminative, antispasmodic and antiseptic main properties (Chioveanu et al., 2004; Ion et al., 2008).
Given that the use of antibiotics to honey bees is prohibited for prevention and control of diseases, etheric oils, especially administered in their food, can be an alternative to assure, in vivo, at critical times, additional to energy or protein stimulators, a suppressive effect on infectious agents (Panizzi and Pinzauti, 1988; Roussenova, 2011).
Therefore, in this study we aimed to evaluate the efficacy, in vitro, of a natural product based on essential oils derived from herbs in treatment of nosemosis in honey bee colonies.

MATERIALS AND METHODS

In order to evaluate the efficacy of a product based on essential oils derived from melliferous medicinal herbs, usable in control of nosemosis in honey bees, it was determined the minimum dose of therapeutic efficacy (without side effects under laboratory conditions) and the lethal dose, through clinical evaluation, pathological and laboratory examinations.
The tested product (named ***Supresor 1***) is a mixture of essential oils derived from melliferous medicinal herbs (mint - *Mentha pepper*, melissa - *Melissa officinalis*, coriandrum-*Coriander sativum*, thyme - *Satureja hortensis*) extracted in 96° ethyl alcohol. The product is presented as a clear greenish-yellow liquid, with fragrant characteristic. It is composed by carvacarol, thymol, menthol, linalool, citronellol. The product contains about 200 mg volatile oil per 1ml hydroalcoholic solution.
The biological material used for testing consisted of honey bees from an apiary (GV-PH), Southern Romania. Before the experiment it was established the level of *Nosema* infection at both, colony and apiary, by determining the number of spores / bee (Table 1) (Ionita and Mitrea, 2013). In the experiment, there were used bees from a colony (ID=781) to which it was found an infection with *Nosema* spp. at 5 spores / unit which is equivalent to 1 250 000 spores / bee (Chioveanu et al., 2009).

Table 1. Honey-bee colonies examined for inclusion in the study

Explanations	Number of colonies
Examined honeybee colonies	20
Negative colonies	15
Positive colonies 1 spor/ unit (no 3; no 791)	2
Positive colonies 2 spores/ unit (no 774)	1
Positive colonies 5 spores/ unit (no 781; no 721)	2

Six experimental modules of honeybees with natural infection *Nosema spp.* were organized, of which to five (experimental modules) were administered the product in different concentration, and one module was the positive control - infected non-treated. Additionally, of the colonies where *Nosema* spores were not detected, two negative control groups (C = uninfected, non-treated; D = uninfected, treated) were selected to asses potential side effects of the product. Each module consisting in two groups (lots), with at least 100 bees/group (106 - 187 bees).
The groups were organized in wooden cages (190/150/50mm in size) equipped with glass, mesh ventilation and nutritious system; at least 100 bees (between 108-187) per each group were allocated (Table 3). Dead bees were collected from each cage daily, and counted. After the experiment, samples from dead bees were examined to determine the level of infection with *Nosema* spores.
The experiments were conducted in laboratory conditions, with the room temperature of 29 ± 0.5°C and humidity of about 28% (Fig. 1) (Williams et al., 2013).
The product ***Supresor 1*** (the etheric oil dissolved in alcoholic solution) was administered in sugar syrup 1/1 (*w/v*) in different concentrations in each module, as follows: 1 ml (module 1), 2 ml (module 2), 5 ml (module 3), 10 ml (module 4), and 50 ml (module 5) per liter of syrup.

The pH of the syrup was adjusted to 3.5-4 with vinegar 9%. Syrup was administrated daily, ad libitum (Fig. 1B). During of the experiments, daily observations on the general status of honey bees, mortality, and food consumption were performed; also, microclimate parameters (temperature, humidity) were monitorized (Figure 2).

Fig. 1. Organizing the experiment: A. monitoring temperature and humidity in experimental conditions; B. administration of the test product (syrup)

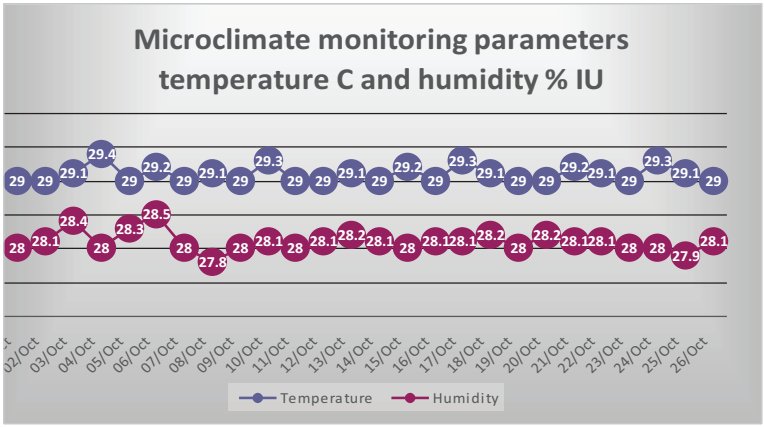

Figure 2. Microclimate monitoring parameters during the study period

RESULTS AND DISCUSSIONS

Results of the study on establishing a therapeutic dose of the tested product (*Supresor 1)* are presented in Table 2. The dose is the amount of administered tested substance. The dose is expressed as the weight of tested substance per unit. In this experiment, the dose was administered related to the quantity of honeybees' food (mg / liter). It was aimed to achieve a correlation between therapeutic dose (minimum concentration), the efficacy and possible lethal effects on honeybees, with significant clinical expression, comparative with the control groups.

The survival rate of the control groups was similar to the experimental modules 1 - 4 and higher than the 5th experimental module. The tested product mixture did not show any toxic effects on the honey bees from the experimental modules (1-4; negative control D). Referring to the toxicity scale according to HODGE and STERNER, the product can be placed in the Group 5 of toxicity, with oral administration, with limits between 5000-15000 ml / liter food for bees, practically nontoxic (OECD).

Mortality of the four experimental modules was not significant, compared with the control one; losses occurred after 4 days (96 hours) (Table 3). Considering the lots with the highest concentration (10,000 mg etheric oil / liter), after 24 hours, the losses were low (of 12.36%). Clinically, there were not registered paralyses, seizures or other signs indicating imminent or predictable death of honeybees in the experimental modules (Figure 4).

Table 2. Daily losses (number) of honeybees in experimental testing efficacy of the product *Supresor 1,* against *Nosema* infection

	Experimental modules										Positive Control		Negative Control			
	Module 1		Module 2		Module 3		Module 4		Module 5		Infected non treated		C - uninfected non treated		D - uninfected treated	
Initial number of bees	lot A	lot B	lot A	lot B	lot A	lot B	lot A	lot B	lot A	lot B	lot A	lot B	lot A	lot B	lot A	lot B
	120	**132**	**106**	**123**	**131**	**167**	**187**	**155**	**124**	**135**	**145**	**133**	**161**	**146**	**144**	**178**
Day																
1									11	21						
2									32	48						
3									48	52						
4									33	14						
5	4													2		4
6	8		7					6				8	4	2	3	5
7	8	6	10	6			12	14			10	12	7	6	7	5
8	18	10	12	14		7	5	7			7	10	10	5	8	9
9	20	17	10	16	7	6	9	12			8	11	6	7	10	8
10	10	32	12	12	6	7	7	6			9	6	8	7	6	7
11	8	15	18	18	6	11	11	8			18	13	11	9	6	11
12	5	18	9	12	9	7	9	10			14	10	12	11	10	9
13	8	22	11	17	7	9	12	12			18	12	6	8	8	11
14	9	12	17	10	7	8	11	9			22	20	12	7	9	8
15	22			18	6	12	14	11			19	16	8	11	9	10
16					10	9	11	8			16	15	9	10	10	11
17					8	9	8	11			4		11	8	9	8
18					10	11	10	7					10	10	10	9
19					9	8	12	7					10	12	7	12
20					6	12	11	8					8	9	9	10
21					8	10	12	12					6	12	11	9
22					9	9	15	7					7	10	12	9
23					8	12	18						16			8
24					4	8										15
25					5	7										
26					6	5										
LR	13s/u	12s/u	11s/u	9s/u	2s/u	3s/u	3s/u	4s/u	3s/u	5s/u	8s/u	11s/u				

LR- Laboratory results: quantitative determination of spore number of *Nosema* spp.

s/u- Spores of *Nosema spp*. Per/unit (/bee)

Table 3. Toxicity results, in terms of registered mortality rate in honeybees from the study

Supresor 1 (ml/mg ethereal oil) / l syrup)	Experimental modules Product administered/: number of ml / l syrup (mg etheral oil)					Positive Control	Negative Control	
	Module 1	Module 2	Module 3	Module 4	Module 5	Infected non treated	C-uninfected non treated	D-uninfected treated
	1 ml (200 mg)	2 ml (400mg)	5 ml (1000mg)	10 ml (2000mg)	50 ml (10000mg)	-	-	5 ml (1000mg)
Bee losses in 24/h	-	-	-	-	12.35%	-		
Bee losses in 48/h	-	-	-	-	30.88%	-		
Bee losses in 72/h	-	-	-	-	38.62%	-		
Bee losses in 96/h	-	-	-	-	18.15%	-		
Bee losses in 8 days	21.43	21.40%	2.35%	12.86%	-	14.08%	11.73%	12.73%
Bee losses in 16 days	78.57	78.60%	42.62%	46.79%	-	81.59%	46.25%	44.41%
Bee losses in 26 days	-	-	55.03%	40.35%	-	4.33%	42.02%	42.86%

Based on the recorded results on the efficacy of the administered product in different concentrations in food, the best results in terms of survival rate of the honeybees and the rate of *Nosema* infection (number of spore/unit), were obtained for the module 3 to which a dose of 5 ml/l was administered.

On this module, the mortality started 2 -3 days later than in the positive control and the rest of the experimental modules; also, the number of nosema spores was reduced (2-3s/u compared to 8-13 s/u). This dose represented by 5 ml product Supresor 1 per litre sugar syrup was considered as optimal dose for using it. The study on establishing a therapeutic dose had a prospective nature.

The product ***Supresor 1*** was not proved to be dangerous for the viability and vitality of bees, compared with the control groups, both positive and negative, respectively. It was also considered that for the dose of 10 ml (2000 mg etheric oil) / l syrup (module 4), although there were obtained good results without toxic effects for bees, however due to the strong flavour felt, it can not be used in the hives, because it could affects "the smell of the hive".

Moreover, providing of bio-stimulators of energy or protein with suppressive effect on bees, in critical times is more necessary in this respect. The present study will be extended for further observations on the apiary level.

The ultimate objective was to investigate the efficacy of medicated feed with *Supresor 1* against the development of *Nosema* spp. sporozoan in field conditions.

There are few scientific studies regarding the use of essential oils in the fight against *Nosema*. One of these experiments (Maistrello et al., 2008) shows that thymol and resveratrol undoubtedly have potential in the development of alternative strategies for the control of *Nosema* infection. In the same time a concentration of 0.12 mg/l and 0,60 mg/ l thyme esential oil obtained from Thymol (Sigma) minimum 95,5% causes decreased levels of infection with Nosema.

High concentrations of thyme essential oil – 2.5 mg/g (from *Thymol minimum* 99.5%, Sigma) can have toxic effects on honeybees (in accordance with its classification as a moderately toxic product) and may not be palatable.

Other experiments (Gherman et al., 2012) showed that propolis which contains 10% essential and aromatic oils has no effect on the spores of *Nosema* spp.

But, the majority of experiments on the use of different types of essential oils, such as: peppermint, eucalyptus, orange, lemon, etc., are performed by beekeepers, and results there were obtained in treatment of *Nosema* there are not scientifically substantiated.

CONCLUSIONS

Suppressor 1 product, a mixture of medicinal herbs and etheric oils obtained from melliferous plants, dissolved in ethylic alcohol, added to food in quantities of 1 to 10 ml per / l had good results on the survival rate of the honeybees and for control of *Nosema* infection; the product did not determined toxicological effects to honeybees. The dose of 5 ml product / l syrup was considered a therapeutic dose, whose effectiveness will be tested on the apiary level.

In conditions of an organic beekeeping, natural products based on plant extracts can be an alternative to control of honeybees' diseases; by using of these products can be also avoided risks of drug residues in apiculture products.

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****Toxicity Scale according to Hodge and Sterner https://ro.wikipedia.org/wiki/Doză_letală*