The EuroBiotech Journal
RESEARCH ARTICLE
Animal Biotechnology

Evaluation of results in research made in order to obtain a phytotherapeutic product for the prophylaxis and fight against *nosema* in bees

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Abstract

In order to obtain a natural apiphytotherapeutic formula with anti-parasite effect, as an alternative in the control of *Nosema spp*. infection, an original formula based on hydro-alcoholic extracts from plants and propolis was tested. Experiments were made on naturally infected adult bees from private apiaries diagnosed with nosemosis (*Nosema apis/Nosema ceranae)*. Initially *hydro-alcoholic extracts from plants in Romania's flora* and respectively from *propolis (Apis mellifera),* with sensorial, physical-chemical and microbiologic characteristics, in conformity with requirements in the European Pharmacopeia VIth Edition and Romanian Pharmacopeia Xth edition. Parasitosis was demonstrated through laboratory methods, respectively through direct microscopic examination, on bee samples collected from aviaries according to O.I.E. (World Organisation for Animal Health) methodology (2008). „*In vitro*" lab testing of plant extracts and of work variants was done firstly on *naturally infested polyfloral honey* having an average concentration of 7 spores/ microscopic field of *Nosema sp*., with the purpose of selecting the most effective work variant which was afterwards tested „*in vivo*" in private apiaries, on bee colonies. *The results of the clinical study* showed the absence of disease in *75%* of the treated hives (*33 hives*), bees being declared *clinically healthy*. In *14 %* of the hives (*6 hives*) infestation was weak, in *6.82%* of the hives (*3 hives*) infestation was *medium*, and *4.55 % (2 hives*) were diagnosed with a *massive* degree of infestation and the therapeutic protocol continued.

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Published online: 27 January 2017
doi:10.24190/ISSN2564-615X/2017/01.06

Introduction

Nosemosis is a serious parasitic disease in adult bees (workers, queen, drones) and sometimes in brood (carriers), being correlated with important economic loss and colony depopulation (1) both in Europe (including Romania) and in the whole world (2, 3, 4). Etiologic agents of intracellular parasitosis being *ubiquitous* and *opportunistic* were found on the territory of our country in 1934 (*Nosema apis,* F. Begnescu*)* and 2008 (*Nosema ceranae,* I.D.S.A. Bucharest). Transmission of the parasitosis is done through orally ingested spores, having as main contamination sources bees' excrement, water, honey, pollen as well as the food exchange between bees, biological materials exchange between hives (5), through contaminated beekeeping equipment (6) and through migratory beekeeping (7). *Nosema ceranae* is involved in the CCD (Colony Collapse Disorder) phenomenon (8, 9) which causes considerable losses in the U.S.A. (10), Asia and Europe (8), possibly also in Romania.

As nosemosis is a frequently spread disease in Romania and given the fact that at present there are no efficient pharmaceutical products (to inhibit the biological cycle and stop the disease evolution) that should leave no residues in the hive products, it was necessary to obtain an apiphytotherapeutic product. The product is for the prophylaxis and treatment of this disease and is recommended for ecological beekeeping as well as for the traditional one.

Materials and Methods

In order to obtain an apiphytotherapeutic anti-parasitic that should be efficient in the prophylaxis and treatment of nosemosis in bees, we selected based on specific activity *a number of 9 species of plants listed by* the Romanian Pharmacopeia Xth edition and by specialized literature to have anti-parasitic and anti-inflammatory properties (11, 12) from spontaneous flora in Romania: *Artemisia absinthium, Mellissa officinalis, Origanum vulgare,* walnut leaves (*Juglans regia)*, *Thymus serpillum, Ocimum basilicum*, *Rosmarinum officinale*, *Rosa caninum*, walnut fruit (*Juglans regia)* and a prime apicultural matter (solid/ Propolis).

Initially, we made *hydro-alcoholic herbal extracts* and respectively *propolis extracts that had* sensorial, physical-chemical and microbiologic characteristics, in conformity with requirements in the European Pharmacopeia VIth Edition (13) and Romanian Pharmacopeia Xth edition (14). Several *work variants* were designed depending on the hydro-alcoholic concentration and volatile oils content (10 variants, with a prime apicultural matter included in the formula). In the bee Pathology Laboratory of I.C.D.A. Bucharest, for each product variant, sensorial, physical-chemical and microbiologic determinations were made, and *product cards* were done *(*15,16).

„*In vitro*" testing of herbal extracts and work variants were made on *naturally infected polyfloral honey* having an average concentration of *7 spores/ microscopic field* of *Nosema sp*. with the purpose of selecting the most effective work variants to be further preclinically/clinically tested „*in vivo*" on bee colonies. *The selection of the honey type* infested with *Nosema spp.* spores was made from 26 honey types with various *Nosema spp.* infestation degrees out of a total of 91 honey samples. A polyfloral honey type was selected having an average number of 7 spores/ microscopic field (centrifuged/non-centrifuged honey) (**Fig. 1, 2**). „*In vitro*" testing of herbal extracts determined a selection of 6 herbal extracts with anti-parasitic properties, as well as of the propolis extract and permitted putting together ten work variants, out of which variant number 7 was selected and used to establish the dosage and respectively the working concentration in clinical testing.

„*In vivo*" testing of the apiphytotherapeutic formula selected as result of "*in vitro*" testing was made in experiments on 225 bee colonies in private apiaries in the South-East area of Romania, divided into 5 lots of various *Nosema spp*. infestation degrees. From the general lot of hives positively diagnosed with nosemosis, for the clinical study 44 hives were selected, totaling 352 bee colonies. After an experimental protocol (5 treatments applied consecutively, as compared with a control lot), results were quantified by clinical testing.

The microscopic diagnosis of *Nosema spp* infestation was made through *direct microscopy* and *Gram coloration, according to OIE 2008*.

Results and Discussion

The hydro-alcoholic extracts obtained from the 10 types of raw material with anti-parasitic properties presented volatile oils concentrations of *0.46 – 0.55 (g/100g)* while extract number 7 had the highest volatile oils concentration *of 0.70 (g/100g)* compared to product M (currently used by beekeepers), in which volatile oils content was of 0.19-0.21 %, which constituted an additional argument to obtain efficient anti-parasitic and anti-inflammatory effects.

The determination of spore number in the naturally infested honey (centrifuged and non-centrifuged) (**Fig. 1 and 2**) and *the selection of infested honey types* as „*in vitro*" culture model, represents a novelty element (***originality degree***).

As result of the „*in vitro*" testing, an anti-parasitic activity was noticed after administering 3 work variants V_7 (T_1, T_2, T_3) in the honey naturally infested with *Nosema spp* spores. Thus, a reduction in the number of spores to max. 1 spore /field was noticed as compared to the Control Lot that remained constant to the initial value of *7 spores/ field*, which justifies using work variants for *in vivo* testing under field conditions on bee colonies. (**Fig. 3, 4**).

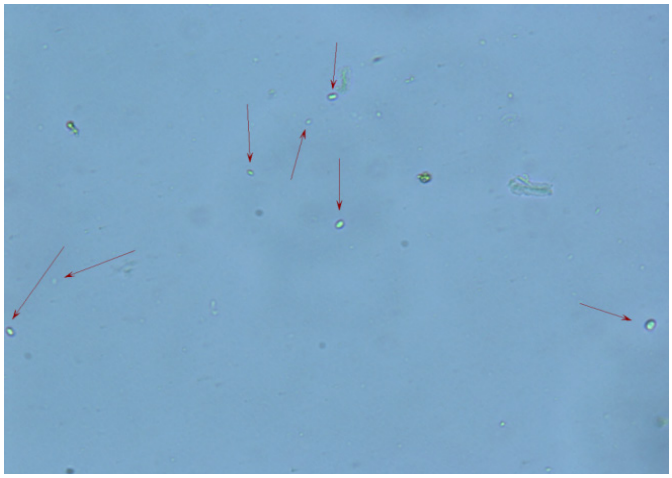

Figure 1. Non-centrifuged polyfloral honey at moment $T_{0/7}$ (spores / field) (Control Lot).

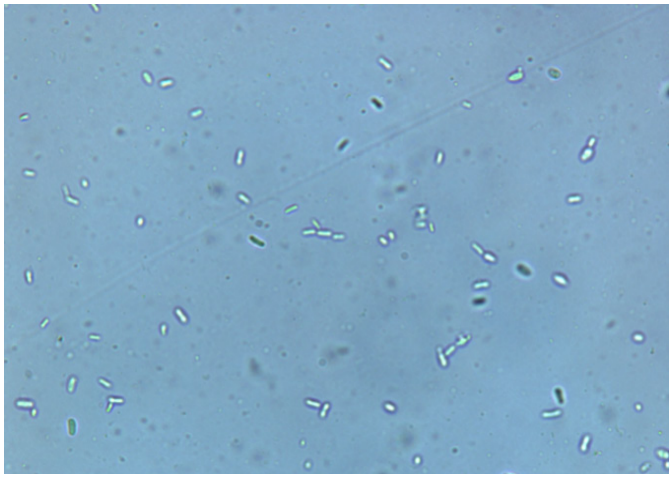

Figure 2. Centrifuged polyfloral honey at moment $T_{1/7}$ (spores/ field) (Control Lot).

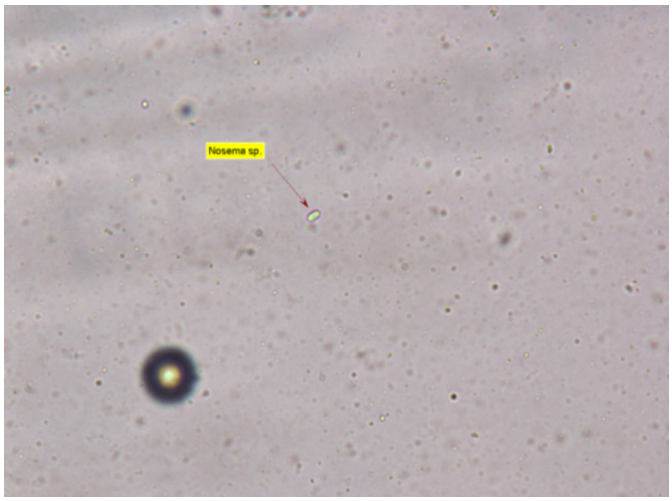

Figure 3. Sample of centrifuged polyfloral honey + V_7/ T_3 /(1 spores field) (Experimental lot).

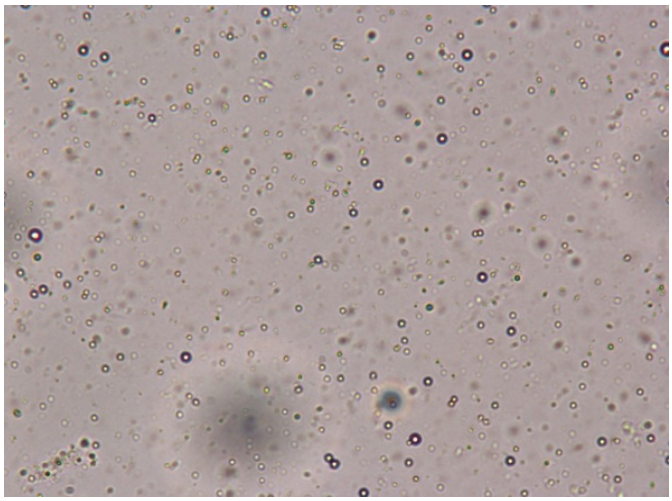

Figure 4. Sample of non-centrifuged polyfloral honey + V_7 / T_3/ (0 spores field) (Experimental lot).

Table 1. Nosema spp infestation degree in the investigated lot

No crt	Infestation degree (moment T_0)	Number of hives diagnosed with nosemosis	Percentage (%)
1	Nosema spp : N_1 – weak infestation	13	12
2	Nosema spp : N_2 – medium infestation	39	35
3	Nosema spp : N_3– massive infestation	60	53
	TOTAL	112	100

From a total of 225 examined hives for diagnose purposes, a number of 112 hives tested positive for nosemosis (**Table 1, Fig. 5**).

Out of these, 44 hives were selected for the study, in which the Experimental Lot included 220 bee colonies (5 colonies/ apiary), with various infestation degrees [Nosemosis: (N +) – weak infestation, (N++) – medium infestation, and N (+++) – massive infestation and the Control Lot included 132 bee colonies (3 bee colonies/apiary), totaling 352 bee colonies (**Fig. 6, 7, 8**).

Health evaluation of bee colonies subject to clinical testing (352 bee colonies) highlighted the presence of 168 bee colonies with manifested disease (47,73 %), 95 bee colonies clinically

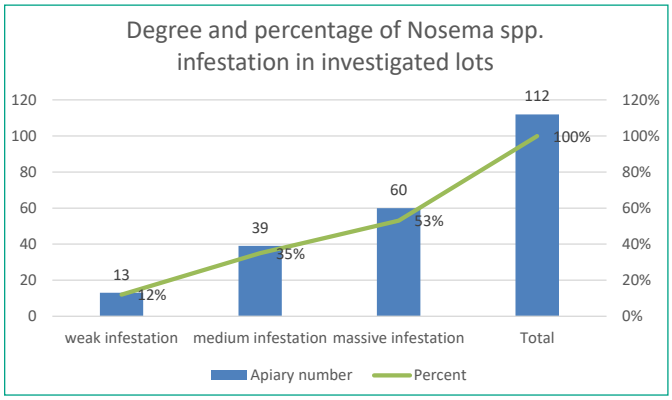

Figure 5. *Nosema spp* infestation degree in investigated lots.

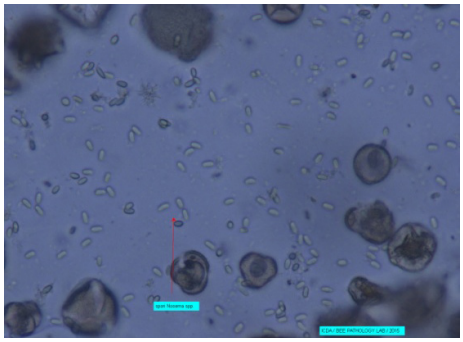

Figure 6. Microscopic image of *Nosema spp* infestation (weak infestation N+) (fresh composition) Ob x 40.

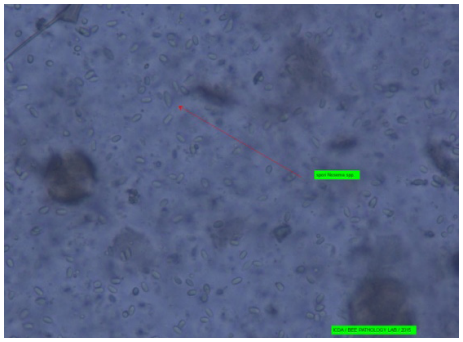

Figure 7. Microscopic image of *Nosema spp* infestation (medium infestation N++) (fresh composition) Ob x 40.

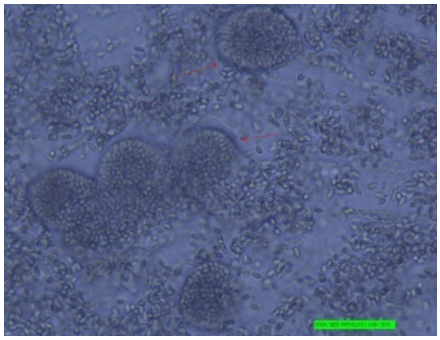

Figure 8. Microscopic image of *Nosema spp* infestation (massive infestation N+++) (fresh composition) Ob x 40.

Table 2. Results of *„in vivo"* clinical testing on bee colonies in the field of the obtained product after a period of 5 days of treatment

No. of apiaries included in the testing	Number of apiaries/FA cured	Number of apiaries with weak infestation after moment T_1 (N+)	Number of apiaries / FA with medium infestation after moment T_1 (N++)	Number of apiaries / FA with massive infestation after moment T_1 (N+++)
44	33 / 165 (75%)	6 / 30 (13,63%)	3 /15 (6.82 %)	2 / 10 (4.55 %)

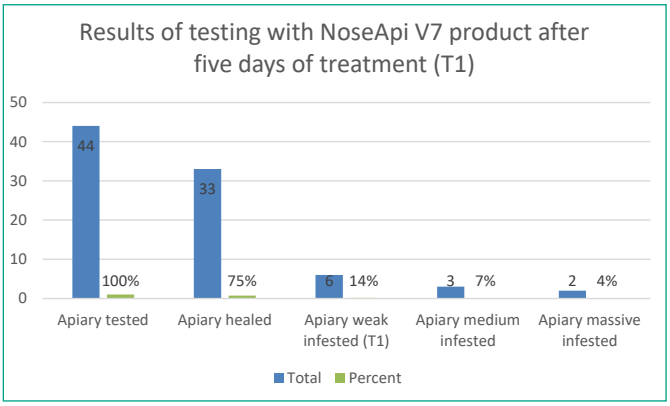

Figure 9. Graphic representation of the result of clinical testing in investigated apiaries.

healthy but carriers of *Nosema spp* (26,99 %, without clinical signs of disease and 89 bee colonies with *morbidity*, *depopulation and positive testing for Nosema spp* (25 %).

To monitor *Nosema spp* infestation and to estimate the efficiency of the *obtained* therapeutic product we selected in the Control Lot 3 bee colonies from each apiary, untreated, maintained in the same conditions and checked observing the same protocol in the same period (132 FA) as in the lots subject to clinical testing .

The treatment included administering the *obtained* product in sugar syrup *for a period of 5-7 days* depending on the infestation degree to a dosage of *10 ml*/syrup liter, administering 200-250 ml medicated syrup/bee colony.

The treatment was applied in 7 hives (35 FA) of weak infestation (16%), 11 hives (55 FA) of medium infestation (25%) and 26 hives (130 FA) of increased infestation (59%).

The results of the clinical study showed the absence of disease in *75%* of the treated hives (*33 hives*), bees being declared *clinically healthy*. In *14 %* of the hives (*6 hives*) infestation was weak, in *6.82%* of the hives (*3 hives*) infestation was *medium*, and *4.55 % (2 hives*) were diagnosed with a *massive* degree of infestation. (**Table 2, Fig. 9**) and the therapeutic protocol continued.

The general therapeutic effect of the obtained apiphytotherapeutic product is manifested in the disappearance of clinical signs of disease, correlated with a clearly diminished infestation degree and other manifestations in the bee colony (developing of brood nests, compact brood, stimulation of egg laying etc.).

As regards *product tolerability*, at the end of the treatment period with the obtained apiphytotherapeutic product, laboratory tests demonstrated the therapeutic efficiency of the product and no cases of food refusal, intoxication, behavior deviations of worker bees, negative modifications in egg laying or modifications at brood cells were noticed. The treatment did not influence in a negative way the forage capacities and bees' behavior during forage.

After the treatment in the apiaries of the experiment, the efficiency of the obtained product at moment T_1 compared to moment T_0 is presented in **Table 3**.

According to the data summed up in table no. 3, we notice that after a minimum of 5 days of treatment with the obtained product, the therapeutic efficiency is 75% (33 cured apiaries /165 FA), as compared to the initial situation in which apiaries were 100% infested.

Also, we notice a decrease in the number of hives with weak infestation, from 16 to 14%, of those with medium infestation from 25 to 6.82% and of those with massive infestation from 59 to 4.55%. This demonstrates the anti-parasitic *„in vivo"* effect by reducing especially the number of apiaries with medium and massive infestation. Respecting the therapeutic protocol, the minimal duration of treatment and monitoring the infections associated with nosemosis may lead to a therapeutic effect that may exceed the 82% showcased by *„in vitro"* testing presented in the previous phases.

Non-observance of the treatment protocol regarding minimal treatment, the existence of infested honey sources as well as of associated infections explain the 25% of apiaries with persistent infestation that did not respond adequately to the treatment. The beneficial effect of therapy with the obtained apiphytotherapeutic product is also manifested in the growth of bee colonies and brood proliferation (**Fig 10**).

Analysis of clinical test results demonstrated the efficiency of the treatment on infested apiaries, as follows: negative

Table 3. Comparative aspects of the efficiency in the treatment with the obtained apiphytotherapeutic product

Moment product is administered	Number of tested apiaries	Number of cured hives	Number of apiaries with weak infestation (N+)	Number of apiaries with medium infestation (N++)	Number of apiaries with massive infestation (N+++)
T_0	44	0	7 (16%)	11 (25 %)	26 (59 %)
$T_{1\ (after\ 5\ days)}$	44	33 (75%)	6 (14%)	3 (7 %)	2 (4 %)

Figure 10. Aspect of owner IM (IL)'s apiary at the end of the treatment (after treatment).

samples (75%), with weak infestation (14%), with medium infestation (7%) and with massive infestation (4%). "*In vivo*" testing recommends the apiphytotherapeutic formula for the control of *Nosema spp*. infections as an alternative method to the veterinary therapy presently used. Presently, in Romania, products used as antiparasitic drugs in bees have no action the infestations with *Nosema ceranae* being used as stimulator, antidiarrheal, detoxifying and there were no scientific data on the outcomes of prophylactic and curative in infestations. Preclinical and clinical testing of the apifitoterapeutic product obtained there were made after diagnosis of *Nosema ceranae* parasite by qPCR and showed its effectiveness in preventing parasites. In Europe there are synthetic products or herbal products that have a totally different composition of the product (*Nosestat - Bulgaria, Nozervit-Croatia, ApiHerb -Italy, Vita Feed Gold-United Kingdom Tymol and Garlic syrup- Greece*) without marketing approval in our country (www.icbmv.ro).

Conclusions

Determining the number of spores in the naturally infested honey infested honey types as „*in vitro*" culture model represent a novelty element (*originality degree*). The infestation degree established through parasitological testing of bees in the experimental lot showed a weak infestation (16 %), a medium infestation (25 %) and a massive infestation (59 %). Result analysis at the end of the experiment demonstrated the efficiency of the treatment in infested apiaries, as follows: negative samples (75%), the remaining 25% positive, with various infestation degrees. *In vivo* testing recommends the apiphytotherapeutic formula to control *Nosema spp*. infections as an alternative method to the veterinary therapy currently in use in beekeeping mainly for prophylaxis.

The results of the research and of the investigations *do not constitute official documents and are not part of the national programs for sanitary-veterinary supervision*.

Acknowledgements

This work was supported by a grant of the Romanian National Authority for Scientific Research, CNDI–UEFISCDI, project number PN 108/2012.

Conflict of interest statement

The authors declare no conflict of interest.

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