Experimental Treatment of Nosema Disease with "Nozevit" Phyto-pharmacological Preparation

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Abstract

Nosema disease is a microsporidian *Nosema sp.* parasitic disease of adult bees. The disease is spread worldwide, and it causes significant losses to apiculture and economy in general. EU and Croatian legal regulations prohibit the use of antibiotics in the treatment of bee diseases, due to possible development of resistance to used chemotherapeutic agents, masking of disease, possible relapses, as well as harmful antibiotic residues or their secondary metabolites in bee products. Therefore, the production and use of natural phyto-pharmacological preparations in the treatment of Nosema disease is a necessity. The aim of this research was to test the performance of the herbal preparation "Nozevit" as a preventive measure against artificial infection with *N. apis* spores, and it's curative effect in the treatment of bees affected by *Nosema* disease.

INTRODUCTION

The Nosema disease or nosemosis is a parasitic disease of adult honey bees (*Apis mellifera*) caused by microsporidium *Nosema sp.* which in adverse living conditions forms long-living spores. The disease occurs throughout the world, Croatia included, and it causes significant honey production and economic losses. The losses are manifested as reduced yields of honey and other apian products (Anderson and Giacon, 1992), and as poor quality and reduced yields in agriculture. Honey bees afflicted with nosemosis start to forage earlier (Fries, 1995), while pathological changes of their mid-gut epithelial cells, as well as digestive and metabolic disorders (Hassanein, 1951), cause malnutrition (Muresan et al., 1975) leading to premature deaths (Morse and Nowogrodzki, 1990). Nosemosis is a significant disease, which often escapes the notice of beekeepers. The affected honey bees tend to die of exhaustion away from the hive, and for lack of obvious signs, the disease can be difficult to notice. It is therefore often referred to as "the silent killer" (Hornitzky, 2005).

Bee colonies may survive the winter weakened by nosemosis and may have the silent disease in spring, leading to a chronic disease which extends to the whole foraging season. Therefore, diagnosis and control of the disease are of great importance for the economy and husbandry of any country. The disease affects entire bee colonies, as well as their members, with drones and bee queens as prone to it as the worker bees (Bailey, 1972). The afflicted bee queens often die during winter when there are no conditions for the development of a new queen bee, which ultimately leads to colony collapse. The Nosema disease develops and spreads particularly rapidly in winter, when cleansing flights of bees are prevented by bad weather conditions. Excrements of the queen bee, who defecates in the hive, are a significant factor of the disease transmission within a bee colony (Peroutka and Vasely, 1976; Sulimanović et al., 1995). For that reason, an Ordinance was adopted in the Republic of Croatia several years ago, which allows the breeding of bee queens for sale only in apiaries under veterinary-health control (Anon, 2008a). Nosema disease negatively affects the development of the fat-protein body as well as the levels of proteins and fatty acids in the bee haemolymph (Bailey and Ball, 1991). The levels of protein and fatty acids in the heamolymph are reduced, leading to an undeveloped lactiferous gland and poor nutrition of the brood, which causes delay and impediments in the development of bee colonies. Brood in heavily affected bee colonies are more susceptible to other diseases, particularly the chalkbrood disease (Sulimanović et al., 1995).

The Nosema disease may be suspected where a large number of dead bees are found on the bottom board of the hive during winter, or where weakening of the bee colony, loss of the queen, and faeces

Table 1. Spore counts (per 0.04 mm) on 10th, 15th and 22ndday after initial artificial invasion. Divide counts by 4 to obtain millions of spores per bee.

Preventive treatment of Nosema disease with Nozevit phytopharmacological preparation				
Test No. 1		10th day after artificial infection	15th day after artificial infection	22nd day after artificial infection
A		0.00	0.00	0.00
B	**Mean**	**5.80**	**21.60**	**75.10**
	Min	3.00	13.00	59.00
	Max	10.00	28.00	97.00
	Lower quartile	4.00	16.00	68.00
	Upper quartile	7.00	26.00	84.00
	Std. Dev.	2.34	5.3	12.12
C	**Mean**	**11.90**	**42.80**	**105.90**
	Min	5.00	31.00	86.00
	Max	17.00	60.00	132.00
	Lower quartile	7.00	34.00	95.00
	Upper quartile	16.00	49.00	123.00
	Std. Dev.	4.48	9.6	15.96

(A = control group, B = sugar solution + spores of *Nosema apis* + „Nozevit", C = sugar solution + spores of *Nosema apis)*

Table 2. Spore counts (per 0.04 mm) on 15th, 20th and 25th day after initial treatment with "Nozevit". Divide counts by 4 to obtain millions of spores per bee.

Curative treatment of Nosema disease with "Nozevit" phyto-pharmacological preparation				
Test No. 2		15th day after treatment with "Nozevit"	20th day after treatment with "Nozevit"	25th day after treatment with "Nozevit"
A		0.00	0.00	0.00
B	**Mean**	**57.20**	**27.70**	**6.10**
	Min	41.00	16.00	3.00
	Max	81.00	41.00	9.00
	Lower quartile	44.00	20.00	5.00
	Upper quartile	70.00	34.00	8.00
	Std. Dev.	13.54	8.32	1.85
C	**Mean**	**73.00**	**36.70**	**25.50**
	Min	66.00	33.00	21.00
	Max	83.00	42.00	32.00
	Lower quartile	68.00	34.00	24.00
	Upper quartile	76.00	38.00	27.00
	Std. Dev.	6.05	2.75	3.17

(A = control group, B = sugar solution +"Nozevit", C = sugar solution +"Nozevit")

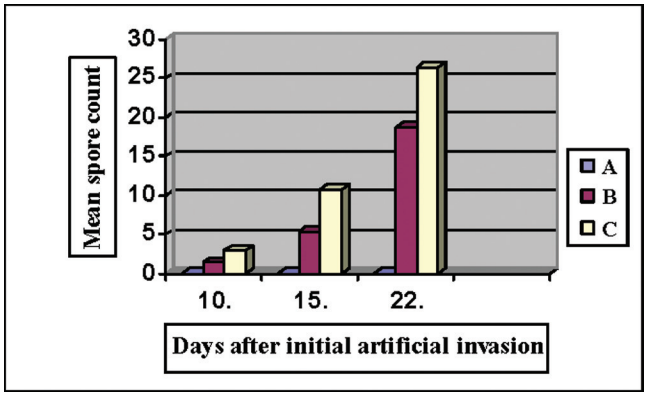


Figure 1. Mean spore counts (million spores per bee) on 10th, 15th and 22nd day after initial artificial infection.

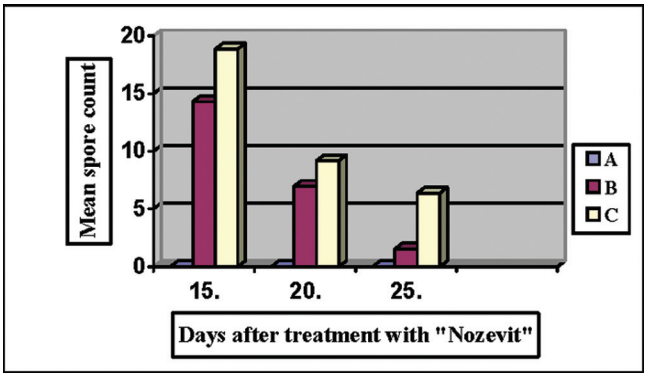


Figure 2. Mean spore count (million spores per bee) on 15th, 20th and 25th day after treatment with "Nozevit".

marks on the frames and entrance to the hive are observed. However, signs observed in individual bees and/or bee colonies are not reliable indicators of the disease presence. Nosema disease can be positively identified only on the basis of laboratory examination of winter losses of bees, and in particular by means of microscopic examination of *N. apis* spores in the digestive tract, as well as by molecular methods (RFLP – restriction fragment length polymorphism, PCR – with species specific primers, Real-time PCR – quantitative). The presence of pathogen spores can be identified with certainty in winter bees whose feaces remain in the intestines for long. The time of sample collection, both from winter losses and from live bees, depends on the climate and weather conditions, but it also differs from year to year. Based on Croatian averages, January and February have been determined as the most favorable periods for sampling (Matašin et al., 2007).

Spores enter the digestive tract of bees via infected food and drink or on the occasion of social food exchange with other bees. The most common sources of infection include unsanitary water supply, honey-comb marked with faeces of infected bees, and contaminated honey (Sulimanović et al., 1995). Factors favoring the spread of the disease include robbery in honey-bee colonies and bad beekeeping practices in the apiary, as well as sudden temperature fluctuations, poor pasture, disturbance, and frequent movements of honey-bee colonies.

According to Laere (1977), after reaching the mid-gut, *N. apis* spores germinate under the influence of diverse chemical stimuli and their vegetative form invades epithelial cells of the mid-gut where they multiply. Liu (1984) has shown that degenerative and lytic processes occur within invaded cells. In time, due to pooling of pathogens in cells, the osmotic pressure increases and causes cell membranes to burst. A part of spores is expelled from destroyed epithelial cells of the gut via excrements and a part remains in the lumen where they take vegetative form and invade previously healthy epithelial cells of the mid-gut. On average, this self-infection happens six days after the initial infection with the parasite, and the majority of spores are expelled two weeks from the onset of the disease (Bailey and Ball, 1991). Digestion disorders are the result of destroyed mid-gut, while damaged peritrophic membrane increases sensitivity to Nosema disease. Degeneration of epithelial cells inhibits the uptake of nutrients so the food just passes through the affected intestines. Besides, the lack of granules and accumulation of ribosomes in infected cells indicate that the excretion of digestive enzymes is reduced (Liu, 1984). Consequently, bees are constantly hungry and take larger quantities of food, which accumulates in their rectums as sweet faecal matter infected with spores. Signs like excited walk, wing flutter and sometimes massive bee deaths at the entrance to the hive are usually detected only when a large number of bees in the colony become infected. Abdomens of some bees may be enlarged (Somerville, 2002), and careful dissection will expose a dilated mid-gut with thin walls, milky-white in colour, and filled with pale excrements (Shimanuki et al., 1992).

The EU, as well as Croatian regulations, prohibit the use of antibiotics in the treatment of apian diseases (EU 3/01/081) because of potential development of resistance to used chemotherapeuticals, masking of the disease, possible relapses, as well as harmful residues of antibiotics and their secondary metabolites in the apian products. For that reason, the need arises for the production and utilization of natural phyto-pharmacological preparations in the treatment of the Nosema disease. The purpose of this work was to assess the effectiveness of the "Nozevit" phyto-pharmacological preparation as a preventive measure during artificial invasion with *N. apis* spores, as well as its effectiveness in the treatment of bee colonies affected with the Nosema disease. Also, the mid-gut structure was histologically analyzed in order to determine the mechanism of action of the tested preparation.

MATERIALS AND METHODS

N. apis spores were isolated from the positive collective sample of winter losses, submitted to laboratory examination in accordance with the provisions in force. To obtain spores, we separated abdomens and ground them up in a mortar with the addition of 1 ml of water per bee. The ground material was filtered through muslin and the filtrate was centrifuged for ten minutes at 3,000 rpm (Anon, 2008b). The supernatant was separated using a pipette and employed for artificial infection of bee colonies. The number of spores was determined by counting in a hemacytometer, according to Bürker – Türk (Cantwell, 1970).

Before testing, we took 30 bees per colony from the hive entrance and examined them under microscope for the presence of *N. Apis* spores.

We used three groups of bee colonies:

- The control group stimulatively fed with 1: 1 sugar solution. (A)
- The test group artificially infected with *N. apis* spore suspension and simultaneously preventively treated with Nozevit. (B)
- The test group artificially infected with *N. apis* spore suspension. (C)

Test No. 1:

Test groups of bee colonies (B, C) were infected with the suspension of *N. apis* spores (40,1 x 10spores per 1 ml) in 1:1 sugar solution prepared with water. Ten ml of the suspension was blended into half a liter of sugar solution (C) and 20 drops of Nozevit (B) was added whereupon the bee colonies were fed on the blend for five consecutive days. Instead of the *N. apis* spore suspension, the control group (A) received an equal quantity of water added to the sugar solution. Blends for individual groups were prepared immediately before placing into the feeder situated under the bee-hive roof.

Samples were taken from about 60 adult bees (Anon, 2008b) at the hive entrance on the 10th, 15th and 22nd day after artificial infection and presence of *N. apis* spores was checked under microscope. Bee samples were collected into clean plastic receptacles around noon. Bees were counted in each sample, their abdomens

were separated and 1 ml of water per bee was added. The abdomens were thoroughly crushed. Four spore samples were counted in each sample using a haemacytometer according to Bürker – Türk, and the infective dose was calculated according to Cantwell (1970). We used 400x magnification under bright field microscope OlympusBx41 and took photographs with Olympus DP12 U –TVO camera. The counting equipment was carefully washed after each sample counting in order to avoid contamination with spores from the previous sample.

Test No. 2:

The test groups (B, C) and the control group (A) from the previous test were used to assess the effectiveness of Nosemosis treatment with Nozevit phyto-pharmacological preparation. A blend of half a liter of 1:1 sugar solution and 20 drops of Nozevit was given to the test groups (B, C). The treatment was repeated four times in intervals of four days. The control group (A) received sugar solution. On the 15th, 20th and 25th, after the beginning of the treatment (or on the 36th, 40th and 47th day after artificial infection), we took samples from about 60 adult bees obtained from the hive entrance (Anon, 2008b) and examined them under microscope for the presence of *N. apis* spores. Sampling procedures and the method of laboratory examination were the same as in the Test 1.

Samples for histological preparations were taken from three groups of bees:

- Non infected bees
- Bees affected with Nosema disease and treated with Nozevit
- Bees affected with Nosema disease

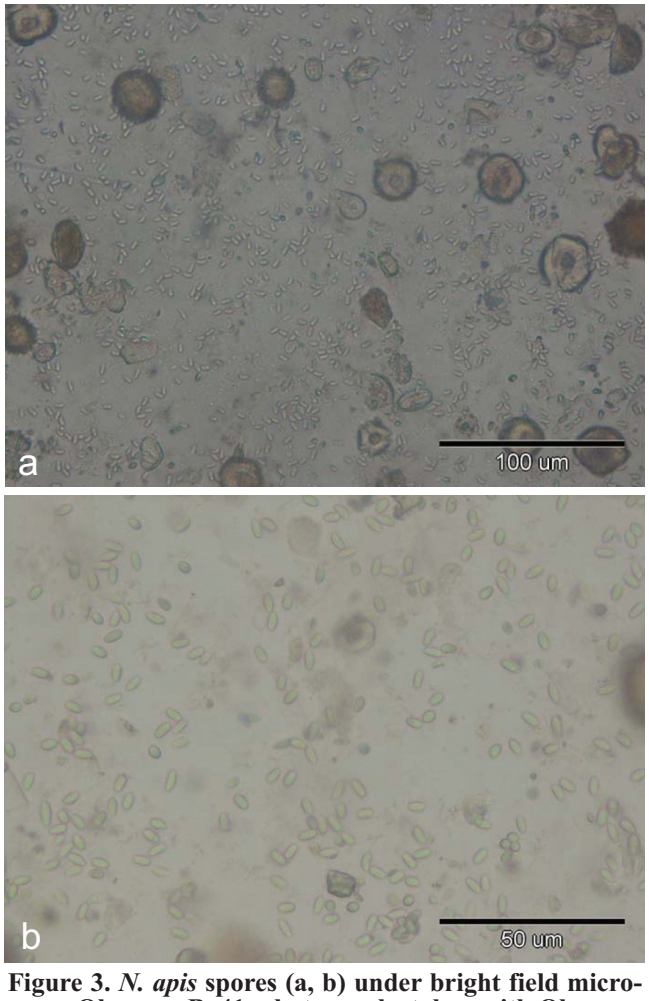


Figure 3. *N. apis* spores (a, b) under bright field microscope Olympus Bx41, photographs taken with Olympus DP12 U –TVO.

Twenty bees were taken from each group, and intestines of each bee were taken out. For the purpose, a larger pair of forceps was used to hold the head and chest of each bee, and a smaller pair of forceps to hold the top of the last abdominal segment and carefully pull out intestines. The intestines were fixed in a 4% formaldehyde solution, inserted in paraffin blocks, cut with a microtome to 6µm thick sections, and stained according to Hemalaon-Eozinic method (HE) (Roulet, 1948).

All calculations and difference significance tests were processed with Statistica Release 8 software.

RESULTS

Spores for artificial infection of bees were isolated from the pooled sample of *N. apis* positive bees (40,1 x 10spores per 1 ml). Testing of the presence of *N. apis* spores before the study gave negative results in all the three groups (A, B, C). Artificial infection with *N. apis* spores was successful and the results of microscopic examination of spore presence on 10th, 15th and 22nd day after artificial infection are provided in Table 1 and Figure 1. The results of "Nozevit" treatment on 15th, 20th and 25th day after its introduction (or on 36th, 40th and 47th day after artificial invasion) are provided in Table 2 and Figure 2. Statistically significant difference was found for the preventive treatment in the group B on the 22nd day ($p<0.05$), as compared to the 10th day after artificial infection with *N. apis* spores.

The results of histological examinations are provided in Figures 4 – 8 herein.

DISCUSSION

Nosemosis is a parasitic disease affecting adult bees. Due to its inconspicuous signs and the need for eradication by interchange of frames with brood in a disinfected hive, beekeepers devote insufficient attention or often neglect the disease. Since the EU prohibits the use of antibiotics, it appears to be necessary to introduce herbal preparations into the treatment of the Nosema disease.

The purpose of our study was to determine effectiveness of the "Nozevit" phyto-pharmacological preparation after repetitive preventive and curative treatments of bees afflicted with the disease. The study involved bee colonies kept in a mini-scale test apiary. It was divided in two parts in order to first determine preventive performance of "Nozevit", i.e. its capacity to inhibit the infection with *N. apis* spores, and then to determine effectiveness of the preparation in the treatment of affected colonies. We assumed that a preliminary small scale study would demonstrate whether "Nozevit" has potential for effective treatment of bee colonies suffering from the Nosema disease. In the first part of the study, concerned with preventive activity of "Nozevit", we used three groups of bee colonies, i.e.: the control group (A) which was free from *N. apis* spores and was not

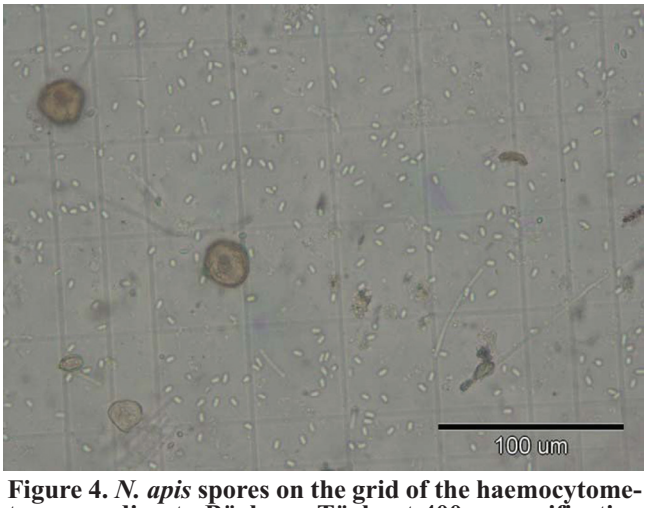
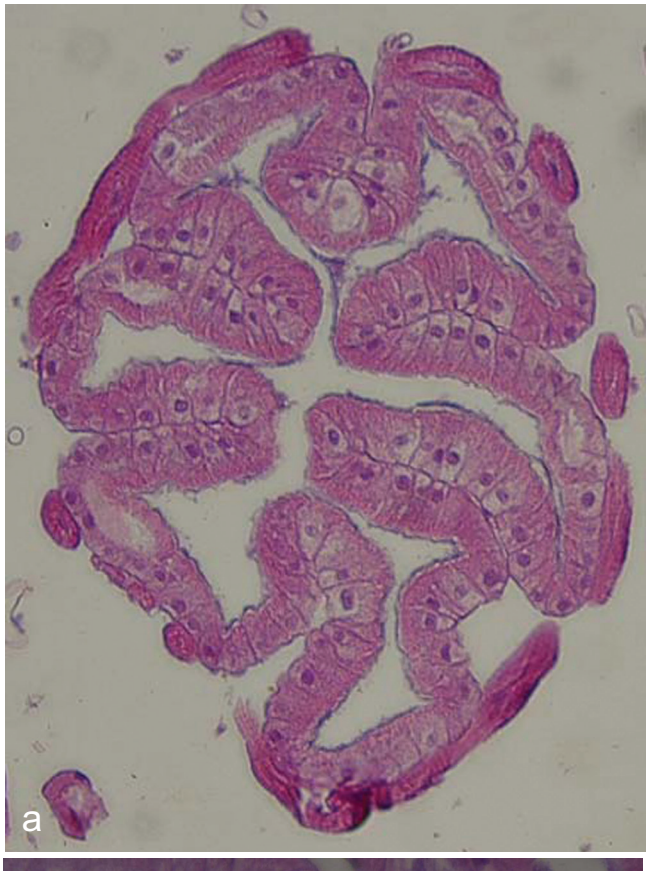


Figure 4. *N. apis* spores on the grid of the haemocytometer, according to Bürker – Türk, at 400x magnification under bright field microscope Olympus Bx41, photographs taken with Olympus DP12 U –TVO.



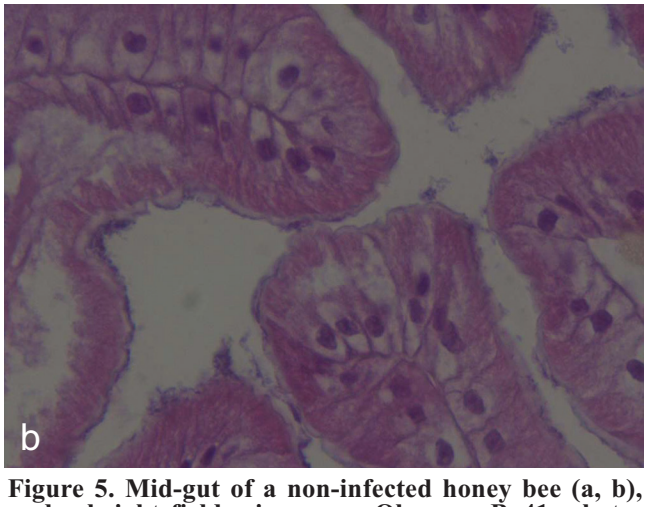


Figure 5. Mid-gut of a non-infected honey bee (a, b), under bright field microscope Olympus Bx41, photographs taken with Olympus DP12 U –TVO.

treated with "Nozevit"; the test group (B) which was artificially infected with spores and simultaneously treated with "Nozevit"; and the test group (C) which was artificially infected with the spores.

The results of that part of our study demonstrated that the disease was not prevented in the tested bee colonies. However, in comparison with the group (C), which was not treated, a considerable reduction in spores was achieved (48.73% on 10th day; 50.46% on 15th day and 70.91% on 22nd day after artificial infection with *N. apis* spores).

The results of the second part of the study demonstrated that the treatment with "Nozevit" failed to remove the *N. apis* spores, i.e. that the bee colony infected with the Nosema disease was not completely cured. Despite failure to achieve complete cure, it needs to be stressed that the group treated with "Nozevit" from the beginning of our study, first preventively and then curatively, had a reduced number of spores compared to the group which received the preparation only for curative purposes and compared to the number of spores counted at the beginning of the treatment (78.37% on 15th; 75.47% on 20th and 23.92% on 25th day after the curative treatment). It was also observed that the bees fed on sugar solution to which "Nozevit" was added consumed the offered food twice as quickly as those fed on sugar solution only (*personal observation*). Our intention was to ensure constant presence of the phyto-pharmacological preparation in mid-guts of tested bees by continuously using "Nozevit", first preventively for five days and then curatively four times in four-days intervals. However, the mode of administration may not have been appropriate, so that all bees did not evenly receive a sufficient dose. Oliver (2008) tested the activity of the same preparation using the "drench" method, by means of which each bee could receive a part of the herbal preparation bee on account of their social behavior. It involves taking of all drenched sugar syrup and its "proboscis to proboscis" sharing, so that the active substance could be spread across the entire bee colony with minimal honeycomb storage. He believed, however, that the given dose of the preparation was insufficient and as a result hungry bees could not store enough preparation to ensure continuous dose supply to their intestines between treatments.

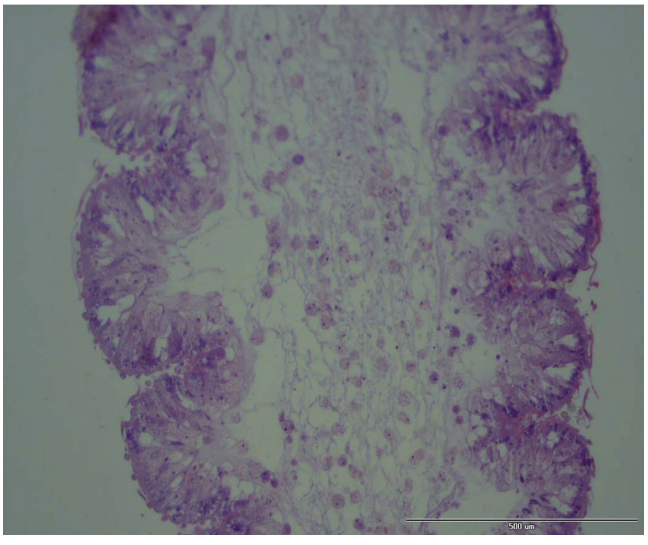


Figure 6. Mid-gut of a bee infected with *N. apis* spores, untreated, at 100x magnification under bright field microscope Olympus Bx41, photographs taken with Olympus DP12 U –TVO.

N. ceranae has not been determinated in Croatia to date, but it could be present in mixed infections because high percentage of the *Nosema* spores was detected also during summer, and because it has been diagnosed in some neighboring countries. However, this needs to be confirmed by molecular methods (Fries et al., 2006; Martin – Hernandez et al., 2007).

In view of the fact that the preliminary study of "Nozevit" effectiveness was performed on a small number of bee colonies, the results cannot be considered as conclusive. Since considerable reduction in the number of spores was achieved in treated bee colonies, a large-scale study should be carried out in a productive apiary. Also, the number of *Nosema* spores and the invasion dose should be tested over a longer period of time. It is also necessary to determine the optimum dose per bee colony, the frequency of treatments and total number of treatments required for cure. Currently, the manufacturer recommends 15 – 20 drops of "Nozevit" to be applied by spraying on bees, added to a sugar solution, or blended into a honey-sugar bread as an addition to stimulative feeding. The treatment should be carried out two or three times in 10-day intervals during summer months. So far, there have been no reports on toxicity or possible harmful residues in honey or other apian products. The possibility of concentrating the preparation was tested by dissolving the herbal extract in two-times smaller quantity of water (Manger 2008, *personal comment*), however, spectrophotometric measure-

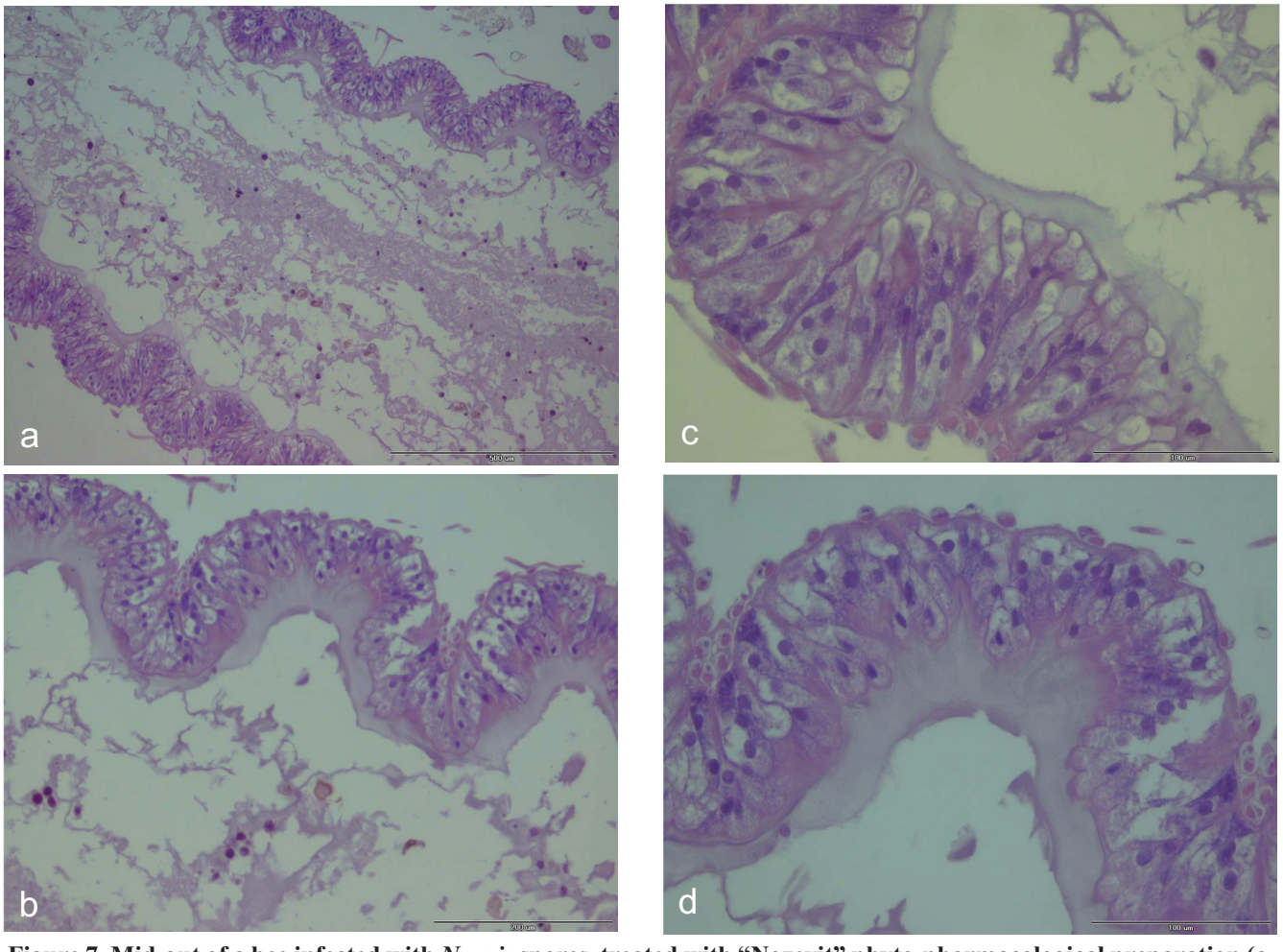


Figure 7. Mid-gut of a bee infected with *N. apis* spores, treated with "Nozevit" phyto-pharmacological preparation (a, b, c, d), under bright field microscope Olympus Bx41, photographs taken with Olympus DP12 U –TVO. The gut lumen of bees is coated with a firm layer.

ments (Beckman DU-530 UV/VIS spectrophotometer; 1100nm – 190 nm, dilution 1:20 in water) showed very similar or overlapping concentrations of the extract. The conclusion was that the herbal extract binds water to the maximum saturation point and that this was not a satisfactory method for concentrating the preparation.

"Nozevit" is a natural extract of oak bark which has been known as a rich source of tannin for many years (Wikipedia 2008). Tannins are natural, bitter plant polyphenols the main property of which is protein binding, precipitation or coagulation. They are used in human medicine to treat inflammatory diseases of the digestive tract. Tannins stick to the mucosa to form a resilient membrane (in the treatment of mouth sores) or exert anti-inflammatory activity (in alleviating inflammatory symptoms of irritable bowel syndrome). These phenols of high molecular weight contain sufficient number of hydroxyl groups to form complexes with proteins, cellulose and some minerals and thus inhibit diarrhea. If we draw a comparison with diarrhea induced by the Nosema disease, tannins from "Nozevit" should be able to stop diarrhea and thus substantially reduce spreading of the pathogen within bee colonies.

The question remains whether active substances in "Nozevit" coat the mid-gut lumen of bees or the *Nosema* spores? In case they coat the mid-gut lumen, i.e. the peritrophic membrane, then the question is whether this coat is selectively permeable? If germination of spores is prevented and they are unable to penetrate epithelial cells of the mid-gut, then there is the question normal digestion through such membrane? What happens to shed cells and digestive enzymes expelled via those cells? Also, what happens with food uptake if the food is digested at all? Do all physiological processes follow a normal course? All the above questions call for re-study under controlled laboratory conditions, with known number of bees in each group and known level of active ingredient per bee in the preparation. In this manner any extrinsic adverse effects would be eliminated, including bad weather or uneven strength of bee colonies. The results of histological examination show that the gut lumen of bees treated with "Nozevit" is coated with a firm layer, while untreated bees have a much looser and not clearly limited area of peritrophic membrane. Also, it has been observed that the intestinal content with numerous spores tends to be squeezed in the center of the lumen, due to which germination of spores is probably impeded. We assume that "Nozevit" simultaneously coats both the gut lumen and the *Nosema* spores. The mechanism of the "Nozevit" action requires more detailed biological and histological studies, and so do the differences in pathogenesis of *N. apis* and *N. ceranae* (Higes et al., 2007).

In view of the fact that the preliminary study of "Nozevit" performance was carried out on a small number of bee colonies, the results of the study cannot be considered as conclusive. However, based on the fact that the number of Nosema spores was considerably reduced upon preventive and curative use of "Nozevit", we believe that the preparation deserves further studies.

REFERENCES

Anderson, D. L. and Giacon, H. (1992): Reduced pollen collection by honey bee (Hymenoptera: Apidae) colonies infected with *Nosema apis* and sacbrood virus. *J. Econ. Entomol*. 85 (1): 47 – 51.

Anon (2008a): Naredba o izmjenama Naredbe o mjerama zaštite životinja od zaraznih i nametničkih bolesti i njihovom financiranju

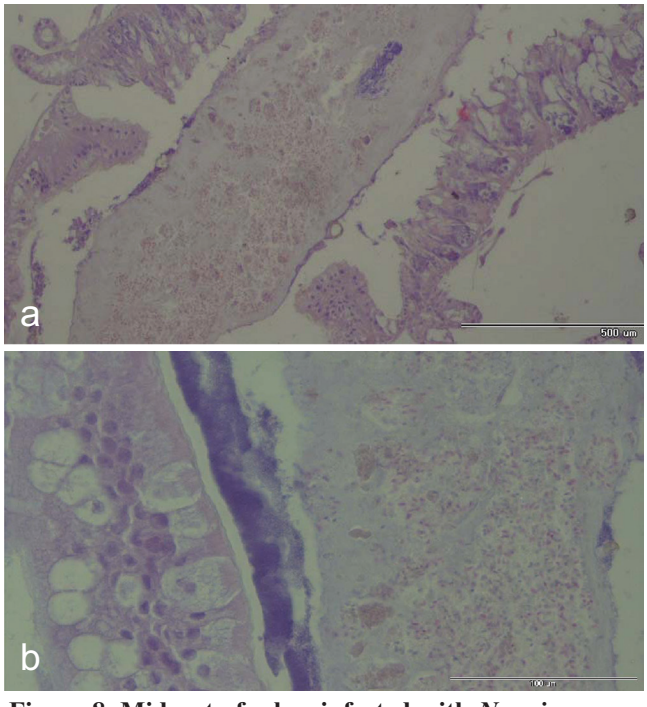


Figure 8. Mid-gut of a bee infected with *N. apis* spores, treated with "Nozevit" phyto-pharmacological preparation (a, b), under bright field microscope Olympus Bx41, photographs taken with Olympus DP12 U –TVO. Intestinal content with numerous spores tends to be squeezed in the center of the lumen.

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Anon (2008b): Nosemosis of Honey Bees. OIE Terrestrial Manual. Chapter 2.2.4., 410 – 414.

Bailey L. and Ball B. (1999): Honey Bee Pathology. Second edition. Academic Press 64 – 143.

Bailey, L. (1972): *Nosema apis* in drone honey bees. *J. apic. Res.* 11, 171 – 174.

Cantwell, G. E. (1970): Standard methods for counting *Nosema* spores. *Am.Bee J.*110, 222 – 223.

Fries I., R. Martin, A. Meane, P. Garcia-Palencia, M. Higes (2006): Natural infections of *Nosema ceranae* in European honey bees. *J. Apicul. Res.* 45, 230 – 232.)

Fries, I. (1995): *Nosema apis* – a parasite in the honey bee colony. *Bee World* 74, 5 – 19.

Hassanien, M. H. (1951): Studies on the effect on infection with *Nosema apis* on the physiology of the queen honey bee. *Q. JI. microsc. Sci.* 92, 225 -231.

Higes M., P. Garcia-Palencia, R. Martin-Hernandez, A. Meana (2007): Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). *Journal of Invertebrate Pathology* 94, 3, 211 – 217.

Hornitzky M. (2005): A report for the Rural Industries Research and Development Corporations 1 – 16.

Laere Van, O. (1977): Factors influencing the germinatione of *Nosema apis* spores. In: Biological aspects of . Apimondia Symposium, Merelbeke, Belgium.

Liu, T. P. (1984): Virus like cytoplasmic partlices associated with lysed spores of *Nosema apis*. *J. Invertebr. Pathol.* 44, 103 – 105.

Martin – Harnandez R., L. Prieto, A. Martinez Salvador, E. Garrido-Bailon, M. Higes (2007): Outcome of Colonization of *Apis mellifera* by *Nosema ceranae*. *Appl. Environ.Microbiol.* 73, 20, 6331 – 6338.

Matašin, Ž., I. Tlak, Z. Petrinec (2007): Značenje dijagnosticiranja nosemoze. Međunarodni stručno – znanstveni skup 4. Dani meda. Poljoprivredni fakultet u Osijeku. Zbornik radova 98.

Morse, R. A. and Shimanuki, H. (1990): Summary of control methods. In: Honey bee pests, predators, and diseases. Second edition. Morse R. A. and R. Nowogrodzki (Ed.). Cornell University Press. Ithica and London, 341 – 354.

Muresan, E., C. Duca, Z. Papay (1975): The study of some histochemical indicies of the mid – gut, healty and infected with *Nosema apis* Z., of the *Apis mellifica carpatica* bee. In: Proc. XXV Int. Cong. Apimondia. Public House, Grenoble, 384 385.

Oliver, R. (2008): A Test of the "Drench" Method for *Nosema* Treatment. *American Bee Journal* 148, 10, 917 – 927.

Peroutka, M., V. Vesely (1976): Apimondia symposium Biological aspects of Merelbeke. Book of abstracts 65 – 68.

Roulet, F. (1948): Methoden der pathologischen Histologie. Springer-Verlag. Wien.

Shimanuki H., Knox, D. A., Furgale, B. Caron D. M. and Williams, J. L. (1992): The hive and the honey bee. 10th edition. Graham, Dadant and Sons (ed.), Hamilton, Ilionis.

Somerville, D. (2002): in Bees. Agnote DAI, 124.

Sulimanović, Đ., Lj. Zeba, J. Marković (1995): Prepoznavanje i suzbijanje pčelinjih bolesti. PIP. Zagreb.

Wikipedia (2008): Tannins. http://en.wikipedia.org/wiki/Tannins

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