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IN VIVO TESTING OF PLANT EXTRACTS IN BEES' CHALKBROOD INFECTION CONTROL *(APIS MELLIFERA CARPATHICA)*

TESTAREA *IN VIVO* A EXTRACTELOR DE PLANTE ÎN CONTROLUL ASCOFEROZEI – BOLII PUIETULUI VĂROS LA ALBINE *(APIS MELLIFERA CARPATHICA)*

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ABSTRACT

Ascospherosis is the most important mycotic disease of brood which is widespread around the world, including in Romania. Hydroalcoholic plant extracts obtained from the wild flora and from the cultivated flora in Romania were tested *in vivo* on bee colonies diagnosed as carriers of *Ascosphera apis*, by direct microscopic examination of the intestinal contents while monitoring the bees' health in 250 apiaries, in various geographical areas of the country. Following diagnosis, 4 apiaries were selected as experimental and witness lots. The evaluation of the inhibiting effect of the selected extracts (oregano, lavender, yarrow) and of the propolis tincture was carried out on infested bee colonies without clinical signs of disease. After 12 experimental days, with 5 doses of hydroalcoholic and propolis extracts administered every 2 days, we noted a decrease in the infestation level with *Ascosphera apis*, especially for the propolis tincture and lavender and oregano extracts in all 4 apiaries, as compared to the untreated witness lot. The obtained results highlight the inhibitting effect of the three extracts and propolis tincture.Results show maximum inhibition values in the bee colonies treated with propolis followed by the ones treated with oregano.

Keywords: honey bee, *Apis mellifera carpatica,* chalkbrood, plant extracts

REZUMAT

Ascoferoza reprezintă cea mai importantă boală micotică a puietului de albine, fiind răspândită în toată lumea, inclusiv pe teritoriul României. Extractele hidroalcoolice obținute din plante aparținând florei sălbatice și florei cultivate din România au fost testate *in vivo* pe coloniile de albine diagnosticate ca purtătoare ale *Ascosphera apis*, prin examinarea microscopică directă a conținutului intestinal și prin monitorizarea sănătății albinelor din 250 de stupine, aflate în diferite zone geografice ale țării. În urma procesului de diagnosticare, 4 stupine au fost selectate pentru furnizarea de loturi experimentale și loturi martor. Procesul de evaluare a efectului inhibitor al extractelor selectate (oregano, lavanda, coada șoricelului) și a tincturii de propolis s-a desfășurat în colonii de albine infestate care nu au prezentat semne clinice de boală. După 12 zile experimentale, cu câte 5 doze de extracte hidroalcoolice și propolis administrate la fiecare 2 zile, s-a constatat o scădere a nivelului infestării cu *Ascosphera apis*, în special în cazul loturilor tratate cu tinctura de propolis și extracte de lavandă și oregano, în toate cele 4 stupine, comparativ cu lot martor netratat. Rezultatele obținute evidențiază efectul inhibitor al celor trei extracte și al tincturii de propolis. Rezultatele arată valori maxime de inhibare a infecției în coloniile de albine tratate cu propolis, urmate de cele tratate cu oregano.

Cuvinte cheie: albine, *Apis mellifera carpatica,* puietul văros, extracte din plante

Major mycoses are frequent in Romania in all geographical beekeeping areas with the *Apis mellifera carpathica* bee*,* being represented by the chalkbrood (*Ascosphera apis*), stonebrood (*Aspergillus spp*.) and melanosis (*Melanosis spp*). The etiological agents affect both bee brood and queens, causing important losses through mortality, weakened hive strength, diminished number of bee colonies, lower queen quality (quantitative and qualitative impact on the egg laying) and favours major bacterioses (12, 22, 23).

Ascospherosis, caused by *Ascosphera apis,* is the most important mycotic bee disease. *Asposphera apis* is a pathogenic fungus for the melliferous bee larvae, being an opportunistic pathogen that kills the larvae only when they are the object of the action of predisposing conditions. The *spores* and the ascosphers of *A. apis* are present in the middle intestine of adult bees,

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on the mouth, on the first pair of little legs and in general on the surface of nurse bees that got contaminated by eliminating dead larvae from the hive cells, in the intestine of apparently healthy larvae, in the faeces of healthy pupae, immediately after they turn into pupae, on the surface of hives in sick bee colonies, in their honey, in the fresh pollen as in the stored one, as well as in the brood food (3, 7, 8, 9, 10, 16, 17, 18, 19).

MATERIAL AND METHODS

The identification of the bee colonies suspected to be infested with *Ascosphera apis* was done while monitoring the bee health in the various geographical areas of the country (250 apiaries).

Table 1

Distribution of monitored apiaries of bee colonies diagnosed positive for the experiment

Location of apiary (County/area)	Number of bee colonies
Apiary VS (East area)	80
Apiary AG (South area)	120
Apiary CL (East area)	40
Apiary TR (South area)	160
Total monitored bee colonies	400

Of the total of monitored apiaries in our country in the apicultural years 2017-2018, a number of 4 apiaries were included in the research lot (Table 1) that were suspected of ascopherosis, clinically healthy but carrying etiological agents of major mycotic diseases (chalkbrood). The direct microscopic examination of living bees' intestine established the bee colonies infested with *Ascosphera apis* (with no clinical signs of ascopherosis in the brood). Based on this fact the Experimental and the Witness lots were created, out of which in turn the experimental sublots (E_1, E_2, E_3, E_4) were set in the selected apiaries in 4 counties: Vaslui (sublot E_1), Călărași (sublot E_2), Argeș (sublot E_3), Teleorman (sublot E_4) and the witness lot (Table 1, Fig. 1 and 2). The apiaries brought together 400 bee colonies.

Fig. 1. Experimental apiary VS (photo made by the beekeeper D.V.)

Fig. 2. Experimental apiary AG (photo made by the beekeeper A.I.)

Table 2

Distribution by sublots of bee colonies in the experiment (Number of bee colonies)

County	Experimental sublot E_1 (Oregano)	Experimental sublot E_2 (Lavender)	Experimental sublot E_3 (yarrow)	Experimental sublot E_4 (Propolis tincture)	Witness Lot (sublots)
Apiary VS	5	5	5	5	4
Apiary AG	5	5	5	5	4
Apiary CL	5	5	5	5	4
Apiary TR	5	5	5	5	4
Total bee colonies	20	20	20	20	
Total	80				16

The number of bee colonies selected for each apiary diagnosed positive, per each experimental or witness lot, is presented in Table 2.

The experimental lot (80 bee colonies) was made up of 4 sublots of 5 clinically healthy bee colonies each, but diagnosed positive after the direct microscopic examination, while the witness lot consisted in 4 sublots of 4 bee colonies /each sublot, a total of 16 bee colonies.

The diagnose methodology that was used was in conformity with OIE regulations (2008) and was adapted by the Bee Pathology Laboratory of ICDA in Bucharest (3, 12, 18, 19, 23). The diagnose of the etiological agent's presence (*Ascosphera apis)* was done on samples from living bee intestines through direct microscopic examination (*Nikon* ECLIPSE E400 microscope). The results of the microscopic examinations of living bees' intestines certified the presence of some evolving forms of the fungus *Ascosphera apis* (Fig. 3 and 4), at the base of the experimental model.

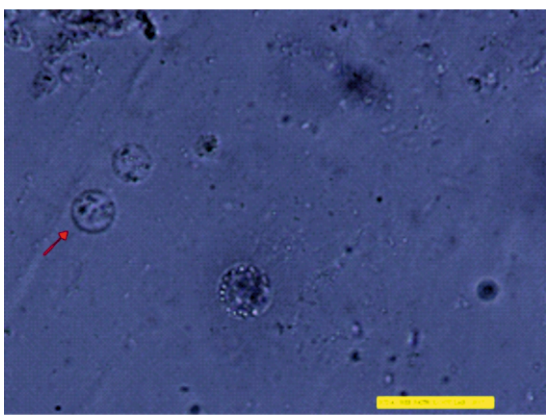

Fig. 3. Direct microscopic examination: ascospores present in the contents of living bees' intestines (Pathology Laboratory, ICDA, Nikon ob x 40) (CL Apiary)

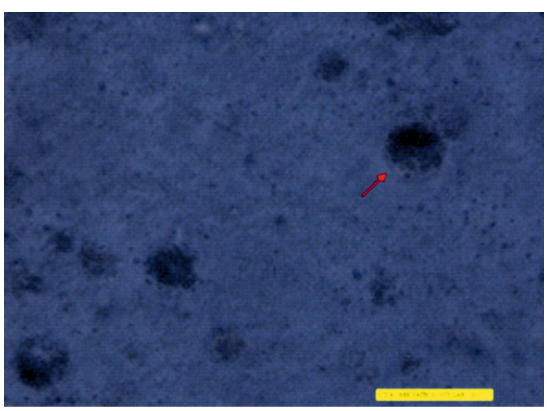

Fig. 4. Direct microscopic examination: ascopores present in the living bees' intestines content (Pathology Laboratory, ICDA, Nikon ob x 40) (AG Apiary)

The methodology of obtaining, conditioning and controlling the plant extracts used in the *in vivo* testing (4, 11, 16, 20, 21, 23) is the object of a request for national patent with OSIM.

The experimental model

The experiment was based on diagnosing and monitoring the 4 selected apiaries included in the research lot, out of which 80 bee colonies constituted the *experimental lot*, and 16 bee colonies constituted the w*itness lot*.

The experimental lot was made up of 4 sublots of 5 clinically healthy bee colonies that however were found to test positive in the direct microscopic examination (bees that had gone through the clinical evolution of „chalkbrood" with risk of disease recurrence in the apiary). These sublots were treated with extracts of selected plants with antifungic action, *in vitro* tested, in previous research (18), as follows:

- *Vaslui apiary experiment* (VS): made up of 20 clinically healthy bee colonies positively diagnosed with *Ascosphera apis* by direct microscopic examination (chalkbrood), was divided in experimental sublots consisting in 5 bee colonies each, that were treated with oregano (sublot E_1), lavender (sublot E_2), yarrow (sublot E_3) and propolis tincture (sublot E_4), compared to 4 bee colonies in the witness lot, untreated;

- *Arges apiary experiment* (AG): made up of 20 clinically healthy bee colonies positively diagnosed with Ascosphera apis through direct microscopic examination (chalkbrood), was divided into experimental sublots consisting in 5 bee colonies each, that were treated with oregano (sublot E_1), lavender (sublot E_2), yarrow (sublot E_3) and propolis tincture (sublot E_4), compared to 4 bee colonies in the witness lot, untreated;

- *Calarasi apiary experiment* (CL): made up of 20 clinically healthy bee colonies diagnosed positively with Ascosphera apis through direct microscopic examination (chalkbrood), was divided into experimental sublots consisting of 5 bee colonies each, that were treated with oregano (sublot E_1), lavender (sublot E_2), yarrow (sublot E_3), propolis tincture (sublot E_4), compared with 4 bee colonies in the witness lot, untreated;

- *Teleorman apiary experiment* (TR): made up of 20 clinically healthy bee colonies diagnosed positively with Ascosphera apis through direct microscopic examination (chalkbrood), was divided into experimental sublots consisting in 5 bee colonies each, that were treated with oregano (sublot E_1), lavender (sublot E_2), yarrow (sublot E_3) and propolis tincture (sublot E_4), compared to 4 bee colonies in the witness lot, untreated;

Table 3

Division into sublots of bee colonies from the apiaries included in the experiment at time T_0 (number of bee colonies)

Regional distribution	Experimental lot	Untreated witness lot
Vaslui apiary Experiment sublots E_1-E_4	Clinically healthy bees carriers of major mycotic disease germs (chalkbrood)	Clinically healthy bees carriers of major mycotic disease germs (chalkbrood)
Total bee colonies	20	4
Arges apiary Experiment sublots E_1-E_4	Clinically healthy bees carriers of major mycotic disease germs (chalkbrood)	Clinically healthy bees carriers of major mycotic disease germs (chalkbrood)
Total bee colonies	20	4
Calarasi apiary Experiment sublots E_1-E_4	Clinically healthy bees carriers of major mycotic disease germs (chalkbrood)	Clinically healthy bees carriers of major mycotic disease germs (chalkbrood)
Total bee colonies	20	4
Teleorman apiary Experiment Sublots E_1-E_4	Clinically healthy bees carriers of major mycotic disease germs (chalkbrood)	Clinically healthy bees carriers of major mycotic disease germs (chalkbrood)
Total bee colonies	20	4
TOTAL	80	16

Each extract was used by administration of 10 ml/liter of sugar syrup 1:1, a total of 5 doses every 2 days. The evaluation of the extracts administration efficiency was done at T_{final} time (the 12^{th} day of the experiment).

The total number of samples collected from each apiary at time T_0 was 24 samples/apiary, with a total of 80 samples for the experimental lots and 16 samples for the witness lot. (Table 3)

At the final time of the experiment (T_{final}) apiary samples were collected from each experimental sublot (E_1, E_2, E_3, E_4) treated with plant extracts and propolis tincture, were also collected samples from the witness lot (M). In total, 4 samples /apiary for the treated sublots and one sample for each sublot in the witness lot were collected.

RESULTS AND DISCUSSIONS

The prophylactic effect of plant extracts and propolis tincture was assessed based on observations on the evaluation of the infestation degree with *Ascosphera apis* spores in bee colonies without signs of disease (Table 4).

After 12 days of experiment, with 5 doses of hydroalcoholic extracts and propolis tincture, every 2 days, a decreased infestation degree with *Ascosphera apis* was found especially for the propolis tincture, lavender and oregano extracts in all 4 apiaries, as compared to the untreated witness lot in each apiary, from an initial infestation degree of "++" to a final infestation degree "0, +" (Table 4). The obtained results highlighted the prophylactic effect of the three plant extracts in the context of sublinical infections with *Ascosphera apis*.

There were obtained maximum results for the sublots treated with the propolis tincture, followed by those treated with the oregano extract. The maximum positive effects in the reduction of the infestation degree of sick bees as compared with the witness lot in each apiary, were noted for the bee colonies which received preventively doses of hidroalcoholic plant extracts and propolis tincture, after 5 treatments every 2 days.

Researchers' efforts nationally and internationally materialized in the results that were obtained regarding the *in vitro* antifungic effect of hydroalcoholic plant extracts (6, 21), essential plant oils (1, 2, 7, 10, 14, 15, 17), as well as their *in vivo* use (10, 21), on infested bee colonies. Plants species out of which plant extracts and essential oils were obtained for the *in vitro* and *in vivo* use by the researchers previously mentioned were different from the ones we studied (4, 20).

The authors of this paper also introduced the propolis tincture in the research, an important component of hive products, and comparatively followed the *in vivo* antifungic effect of plant extracts from plants selected from the Romanian flora.

The prophylactic antifungic effect of extracts in the

Table 4

Evaluation of the results of infestation degree in bee intestine samples, the average of positive samples at the time T_{final}

Apiary county	Experimental lot E_1 (Oregano)		Experimental lot E_2 (Lavender)		Experimental lot E_3 (Yarrow)		Experimental lot E_4 (Propolis tincture)		Witness lot (untreated)
	T_0	T_{final}	T_0	T_{final}	T_0	T_{final}	T_0	T_{final}	T_{final}
VS Apiary	++	+	++	+	++	++	++	0	+++
AG Apiary	++	+	++	0	++	+	++	0	+++
CL Apiary	++	+	++	+	++	+	++	0	+++
TR Apiary	++	+	++	0	++	++	++	0	+++

Legend: + (1-2 ascosphers/microscopic field); ++ (3-5 ascosphers/microscopic field); +++ (> 5 ascosphers/microscopic field)

treated bee colonies may be amplified through synergism, in future experiments of combinations with propolis tincture, lavender extract and oregano extract. The following experiments may highlight the prophylactic and potentially curative effects on bee colonies infested with clinical forms of the evolving disease, through the synergism of the respective extracts and propolis tincture.

CONCLUSIONS

The *in vivo* testing, after 5 doses every two days (12 days of experiment), demonstrated the inhibiting effect upon spores of *Ascosphera apis*, especially for the propolis tincture and plant extracts, lavender and oregano, in all 4 apiaries. The reduction of the infestation degree with *Ascosphera apis* was maximal for the bee colonies treated with propolis tincture, followed by the ones treated with oregano extract. The *in vivo* testing of plant extracts and propolis tincture showcased the prophylactic effect and the possibility to use their synergism with the propolis tincture in order to obtain an antifungic apiphytotherapeutic product with curative effect.

Conflicts of interest

There are no conflicts of interest to be declared.

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REFERENCES

1. *Ansari M.J., Al-Ghamdi A., Usmani S., Khan K.A., Alqarni A.S, Kaur M., Al-Waili N.*, (2016), In vitro evaluation of the effects of some plant essential oils on *Ascosphaera apis*, the causative agent of Chalkbrood disease. Saudi J Biol Sci, 24(5):1001-1006
2. *Ansari M.J., Al-Ghamdi A., Usmani S., Al-Waili N., Nuru A., Sharma D., Khan K.A., Kaur M., Omer M.*, (2016), *In vitro* evaluation of the effects of some plant essential oils on *Paenibacillus larvae*, the causative agent of American foulbrood. Biotechnology & Biotechnological Equipment, 30(1):49-55
3. *Aronstein K.A., Murray K.D.*, (2010), Chalkbrood disease in honeybees. Journal of Invertebrate Pathology, 103(Suppliment):S20-S29
4. *Bojor O.*, (2003), Ghidul plantelor medicinale şi aromatice de la A la Z, (Ed.) Fiat Lux, Bucharest, Romania
5. *Boudegga H., Boughalleb N., Barbouche N., Ben-Hamouda M.H., Mahjoub M.E.*, (2010), In vitro inhibitory actions of some essential oils on *Ascosphaera apis*, a fungus responsible for honey bee chalkbrood. J Apic Res, 49(3):236-242
6. *Buţu M., Dobre A., Rodino S., Butu A., Lupuleasa D.*, (2013), Testing of the antifungal effect of extracts of burdock, thyme and rough cocklebur. Seria Ştiinţele Vieţii, 23(1):65-69
7. *Colin M., Ducos de Lahitte J., Larribau E., Boué T.*, (1989), Activité des huiles essentielles de Labiées sur *Ascophaera apis* et traitement d'un rucher. Apidologie, Springer Verlag, 20(3):221-228
8. *Codreanu I., Sfetcu I., Codreanu M., Goran G.*, (2009), Correlations between the lead residues in

honey bees and the apiary location. Lucrări Stiintifice Medicină Veterinară, 42:301-305

9. *Codreanu I., Sfetcu I., Codreanu M., Goran G.,* (2009), Investigations concerning the lead residues in apiary products (honey wax). Lucrări Stiintifice Medicină Veterinară, 42:306-309
10. *Craig D., Ward W.*, (2003), Control of chalkbrood disease with natural products. Publication nr. 03/107, Project No DAQ-269A
11. *European Pharmacopoeia*, (2008), Ed. a VI-a, vol 1,2, (Ed.) Council Of Europe, Strasbourg, France
12. *Guimarães-Cestaro L., Serrão J.E., Message D., Martins M.F., Teixeira E.W.*, (2016), Simultaneous detection of *Nosema* spp., *Ascosphaera apis* and *Paenibacillus larvae* in honeybee products. Journal of Hymenoptera Research, 49:43-50
13. *Jensen A.B., Aronstein K., Flores J.M., Vojvodic S., Palacio M.A., Spivak M.*, (2013), Standard methods for fungal brood disease research. J Apic Res, 52(1):39
14. *Kloucek P., Smid J., Flesar J., Havlik J., Titera D., Rada V., Drabek O., Kokoska L.*, (2012), In vitro inhibitory activity of essential oil vapors against *Ascosphaera apis*. Nat Prod Commun, 7(2):253-256
15. *Michalczyk* A., Kazimierczak *J.,* Cieniecka-Rosłonkiewicz *A.*, (2016), Use of essential oils and their components in the treatment of American foulbrood and chalkbrood in bees. Przemysl Chemiczny, 12(12), doi:10.15199/62.2016.12.4
16. *Milea F.G., Radoi I., Şapcaliu A., Savu V., Popa O.,* (2017), Ascospherosis incidence in bees investigated for major bacteriosis in the beekeeping. Scientific Works. Series C. Veterinary Medicine, 59(4): 123-127
17. *Nardoni*, *Guido Rocchigiani, Carlo D'Ascenzi, Francesca Mancianti*, (2017), Stonebrood and chalkbrood in Apis mellifera causing fungi: in vitro sensitivity to some essential oils. Natural Product Research,32(4):1-6
18. *Ileana N.*, (2007), Researches regarding the chalkbrood disease (*Ascosphaera apis*) of the honey bees. Bulletin USAMV-CN, 64(1-2):207-210
19. *OIE (World Organisation for Animal Health)*, (2016), Manual of Diagnostic Tests and Vaccines for Terestrial Animals (mammals, birds and bees), vol.1, 2, ed. 25th Edition
20. *Pârvu C.*, (2006), Universul plantelor, (Ed.) ASAB, Bucharest, Romania
21. *Rodino S., Butu M., Petrache P., Butu A., Cornea C.P.*, (2014), Antifungal activity of four plants against *Alternaria alternata*. Scientific Bulletin. Series F. Biotechnologies, 18:60-65
22. *Sarwar M.*, (2016), Fungal diseases of honey bees (*Hymenoptera*: *Apidae*) that induce considerable losses to colonies and protocol for treatment. International Journal of Zoology Studies, 1(1):6-13
23. *Savu V., Şapcaliu A.*, (2013), Patologia albinelor. (Ed.) Fundaţiei România de Mâine. Bucharest. Romania, 31-38.