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Original Article

EFFECT OF THE PROPOLIS COMPONENTS, CINNAMIC ACID AND PINOCEMBRIN, ON *APIS MELLIFERA* AND *ASCOSPHAERA APIS*

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Abstract

The value of propolis as a disinfectant agent in the bee hive is considerable. We investigated the fungistatic effect of cinnamic acid and pinocembrin on the pathogen *Ascosphaera apis* causing chalkbrood disease in bee colonies in the laboratory with an agar diffusion test on malt extract agar. The minimal fungicide inhibitory concentration was 0.5% for cinnamic acid and 1.5% for pinocembrin (MIC-values). Both substances were tested by individual application for adult bee tolerance in a range of dosages and for brood tolerance (L1 - L5) referring to the MIC values. The dermal toxicity of adult bees for cinnamic acid and pinocembrin was low, a maximum of 3.3% even in the highest dosage of 1000 µg (concentration 20%) and 250 µg (concentration 5%) per bee, respectively. The mortality rates showed no significant differences to the controls (z-test, $p \leq 0.05$). First results on brood tolerability after application of test substances resulted in larval mortality but primarily in the group of young larvae. The mortality reduced simultaneous with the increasing larval weight. The total larval mortality 72 hours after the application over all stages was 28.7% for cinnamic acid and 13.1% for pinocembrin (positive control group 24.8%; negative control group 15.4%). The test groups showed no significant increased mortality compared to the control groups (z-test, $p \geq 0.05$). Both substances have in vitro fungicide potential and can be considered as potential active agents against *A. apis* in honey bee drugs. The results provide high tolerance of adult bees and an indication for larval tolerability most notably in elder larvae.

Keywords: *Apis mellifera, Ascosphaera apis*, chalkbrood, cinnamic acid, pinocembrin, propolis.

INTRODUCTION

Chalkbrood of honeybees (*Apis mellifera*) is a fungal disease caused by *Ascosphaera apis* (Spiltoir, 1955) affecting the honeybee larvae. The bee larvae ingest spores of *A. apis* with the food. The spores germinate in the lumen of the gut, probably activated by CO_2 from tissue (Heath and Gaze, 1987). The larvae mostly die in the L5 developmental stage. This brood disease can reduce colony productivity by lowering the number of newly emerged bees and may lead to colony losses (Jensen et al., 2013). The chalkbrood spores are very resistant to environmental conditions and can remain viable and infective for more than 15 years. There is no veterinary drug available to control chalk-brood in Germany but some substances are known to have an influence on the fungus. For example, ethereal oils containing thyme as well as citral, geraniol and citrallal inhibit growth of *A. apis in vitro* (Boudegga et al., 2010). Formic acid and oxalic acid are also known to have an influence on the fungus (Yoder et al., 2014). Natural substances from the bee colony, such as propolis could be effective in suppressing the chalkbrood pathogen. The value of propolis as a disinfectant agent in the bee hive is considerable. Propolis is used in various human medicines as an anti-fungicide (Popova et al., 2009). The use of propolis in human wound therapy, neurodermatitis and gynaecological diseases was described by Marcucci (1995).

Propolis is heterogeneous in composition. It is not known which of the more than 160 single components in propolis (Gardana et al., 2007) is responsible for the therapeutical effect. The two substances, cinnamic acid and pinocembrin, could play important roles, for example, in cosmetics (van Bergen, 2012). Pinocembrin is known to have a fungistatic effect (Mitro, 1996) and cinnamic acid

shows a germination inhibiting effect on *Candida albicans* and *Aspergillus* spec. For the use of propolis in medicinal products, it is important to test single components of the bee product to identify the active pharmaceutical ingredient of the mixture to provide standardisation for drug development.
We investigated the fungistatic effect of cinnamic acid and pinocembrin on the pathogen *A. apis*. Furthermore, the tolerability of the substances on *A. mellifera* worker bees was tested in the laboratory. Within the scope of this study, the first results on the compatibility of cinnamic acid and pinocembrin against *A. apis* larvae *in vivo* in the colony, have been reported.

MATERIAL AND METHODS

Agar diffusion test

The detection of fungistatic effects and the verification of the minimal inhibitory concentration (MIC) were conducted with an agar diffusion test on malt extract agar according to the AVID-Method (AVID X, 1998; Kettering, 2005), and also by referring to the method described by the European Community on Antimicrobial Susceptibility Testing (EUCAST, 2012). Isolation and cultivation of *A. apis* was conducted by use of a culture. Three chalkbrood-infected, white and dark spore bearing larvae (freshly removed from the colony) were crushed and suspended in 5mL of distilled water. The suspension was filtrated using a membrane filter to separate larger larval particles. The suspension (10 µL) was then transferred onto malt extract agar plates by three sector streaks and incubated for 48 hours at 30°C (Borchert, 1974). Identification of *A. apis* was conducted by light microscopy, and by comparison with Aronstein and Murray (2010). Five single colonies were removed from the agar plate and suspended in 2 mL 0.9% NaCl followed by 30 sec in a Vortex-mixing device. The confirmation of spore density was carried out with a hemocytometer in a dilution of 1:100. The start inoculum was $5x10^6$ colony forming units (CFU) per mL.
Cinnamic acid and pinocembrin were derived from the commercial chemical company Sigma-Aldrich®. The agar diffusion test was conducted with four concentrations of cinnamic acid (0.5%, 1.25, 5.0%, and 7.5%) and pinocembrin (0.5%, 1.25%, 2.0%, and 2.5%), respectively, two replicates and a repeated test (Tab. 1). The two test substances were diluted in 50% sugar syrup (w/w). The start inoculum (200 µL) was spread area-wide onto the malt extract agar. Each concentration of test substance (20 µL) was pipetted onto filter paper (ø 0.8 cm); the control papers were treated with a 50% sugar syrup. Incubation took place at 30°C in an incubator. After 48 hours, the zones of inhibition were determined with a magnifying lens. The minimal fungicide inhibition zone (MIC-value; Stock and Wiedemann, 1998) was then tested for brood tolerance.

Bee toxicity

The bee tolerance of cinnamic acid and pinocembrin was tested with *A. mellifera carnica* worker bees. Testing was conducted under laboratory conditions according to EMA (2008). Hive bees older than five days and collected from honey combs were treated individually and kept in cages. The test substances were dissolved in sugar syrup (50% w/w) and then trickled ventrally onto the abdomen in an amount of

Table 1.

Inhibition zone in the agar diffusion test with cinnamic acid and pinocembrin

Concentration [%]	1. Test:	IZ* [mm]	2. Test:	IZ [mm]	Mean [mm]
Cinnamic acid	**1. Replicate**	**2. Replicate**	**1. Replicate**	**2. Replicate**	
0.5	1.3	1.2	1.3	1.4	**1.3**
1.25	0.9	1.3	1.2	1.2	**1.2**
5.0	1.5	1.1	1.2	1.0	**1.2**
7.5	0.5	0.5	0.4	0.6	**0.5**
Control	0.0	0.0	0.0	0.0	**0.0**
Pinocembrin					
0.5	0	0.1	0	0	**0.02**
1.5	0.4	0.6	0.4	0.6	**0.5**
2.0	2.0	1.8	1.5	1.6	**1.7**
2.5	0.5	0.5	0.4	0.6	**0.5**
Control	0.0	0.0	0.0	0.0	**0.0**

*IZ: inhibition zone

5 µL per bee (Fig. 1). Different doses were tested (Tab. 2). Bees were kept in groups of 10 bees per cage, 3 cages per dose, with one replicate. This resulted in a number of 60 bees tested per dosage. All test animals were kept at a temperature of 22°C and R.H. 65% in darkness; conditions were draught free. The control groups were treated in the same way with sugar syrup (50%). The bee mortality was monitored over 3 days in intervals of 24 hours.

Larval toxicity

The brood tolerance of the minimal inhibitory concentration produced during these tests was evaluated on the larval stages (L1 to L5), under semi-field conditions, according to OECD (2007). Larval stages of four colonies were age determined (Winston, 1987) and projected onto plastic sheets. The brood combs remained in the colony during the test. Different larval stages were subsequently investigated, to avoid harm by opening the hive. Test substances dissolved in sugar syrup were pipetted onto the edge of the brood cells considering the intactness of the spiracles (cinnamic acid n = 178, pinocembrin n = 107; Fig. 2). The positive controls were treated with sugar syrup (n = 323), the negative controls were not treated (n=39). Seasonal temperature changes led to a different brood production in the four colonies and therefore, a limited sample size in the group of negative controls and older larval stages. Larval mortality was monitored over 3 days, at 24 hour intervals.

Statistical analysis

The statistical analysis was conducted with SPSS SigmaStat® 3.0 software using the Z-test. Differences were considered as significant at $p \leq 0.05$.

RESULTS

Agar diffusion test

Both test substances proved to have a fungistatic effect (Tab. 1). The minimal inhibitory concentration of cinnamic acid was 0.5% and pinocembrin 1.5%, respectively. These were the lowest concentrations showing a repression of fungi growth.

Fig. 1. Worker bee: dermal application.

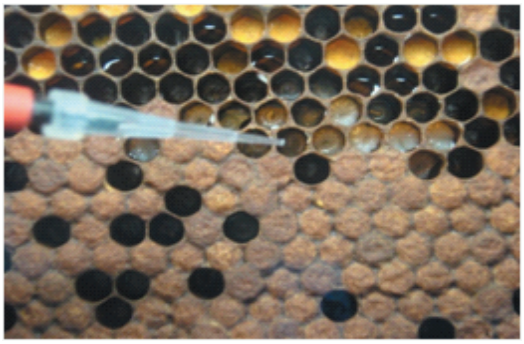


Fig. 2. Bee larvae: topical application.

Table 2.

Total bee mortality after dermal application of cinnamic acid and pinocembrin

Concentration [%]	Dosage per bee [µg]	Mortality after 72 hours [%]
Cinnamic acid		
1.25	62.50	**0.00**
2.50	125.00	**0.00**
5.00	250.00	**3.33**
7.50	375.00	**1.67**
10.00	500.00	**0.00**
12.50	625.00	**1.67**
20.00	1.000.00	**3.33**
Pinocembrin		
1.25	60.25	**0.00**
2.50	125.00	**0.00**
5.00	250.00	**0.00**
Control	0.00	**2.00**

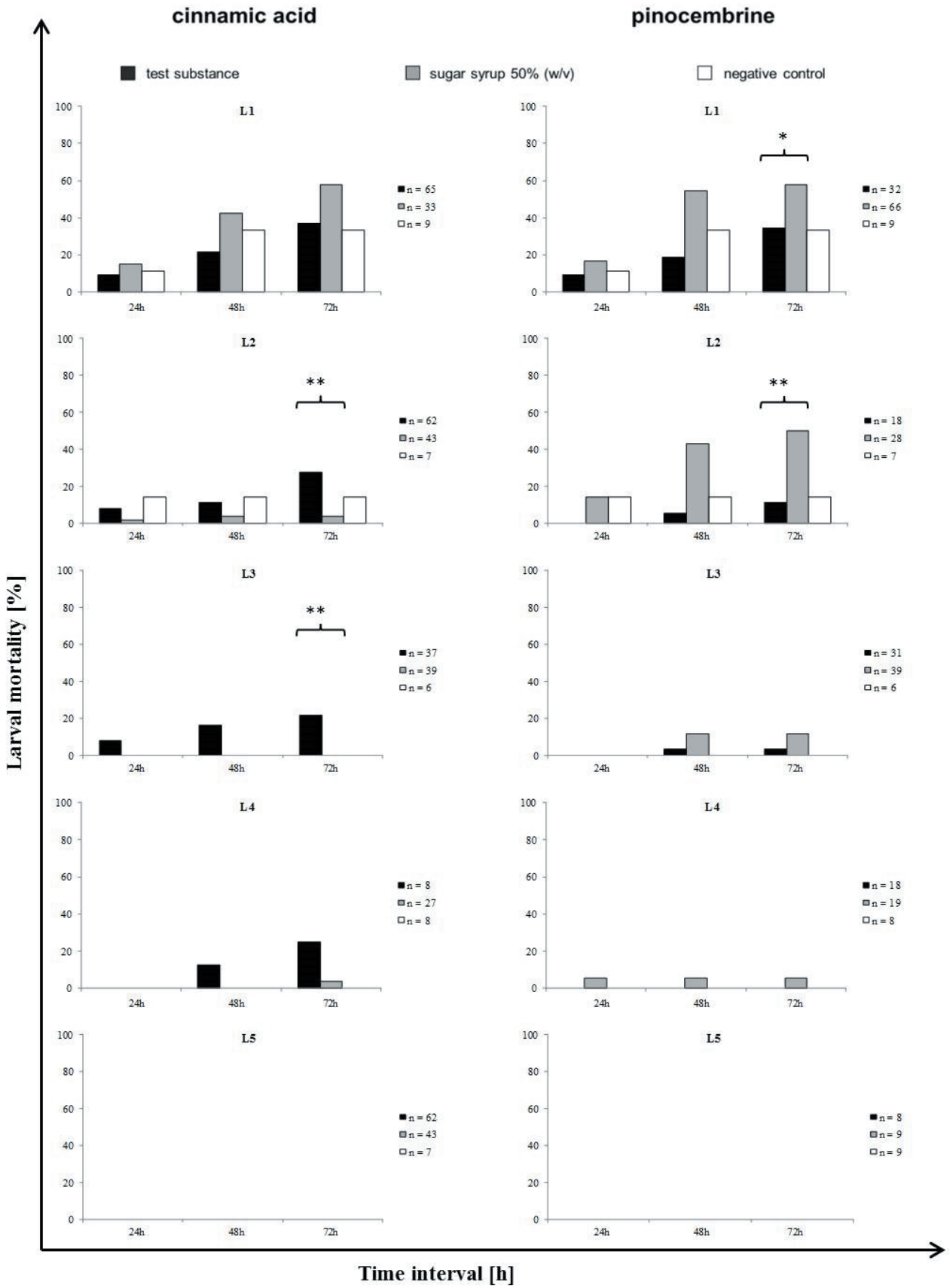
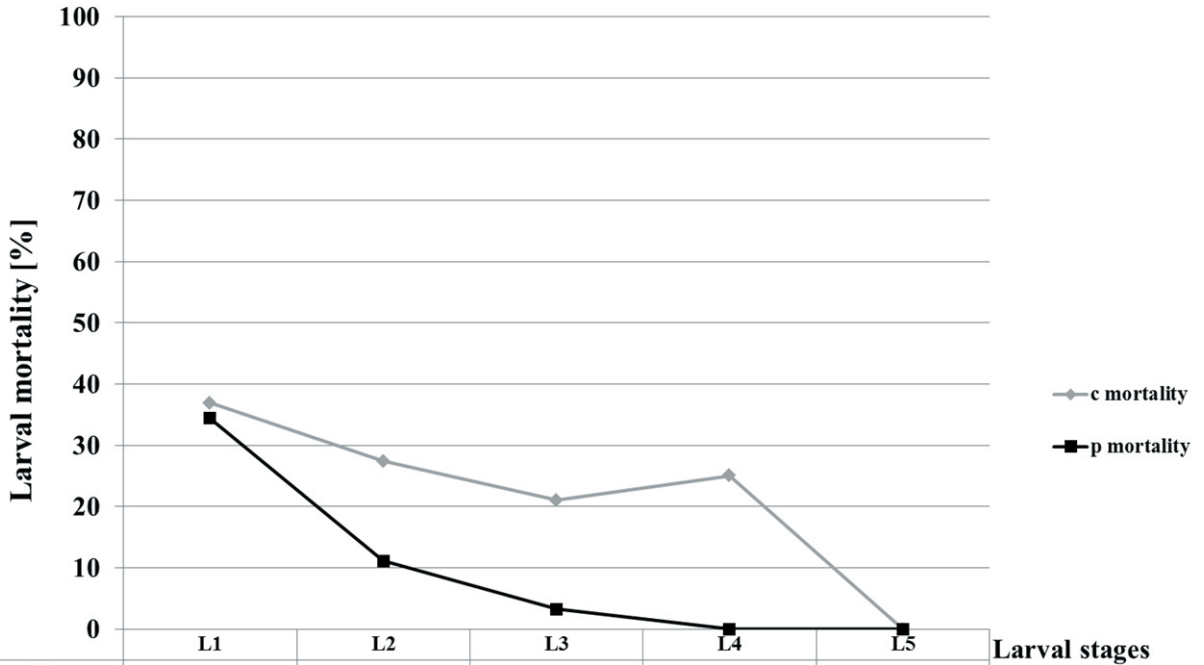


Fig. 3. The total larval mortality for the stages 1-5, 72 hours after application of 0.5% cinnamic acid solution (c1–c5, Z-test, **p≤0.02) and 1.5% pinocembrin solution (p1-p5, Z-test, **p≤0.02).



	L1	L2	L3	L4	L5
c mortality	36.92	27.42	21.05	25.00	0.00
p mortality	34.38	11.11	3.23	0.00	0.00
c dosage [µg/mg]	62.50	1.47	0.15	0.05	0.04
p dosage [µg/mg]	187.50	4.41	0.45	0.15	0.11

Fig. 4. Comparison of the larval mortality dosage relating to the specific body weight at larval stages 1-5 for the dermal application of cinnamic acid (c) and pinocembrin (p); body weight of the larval stages specified according to Snodgrass (1925).

Higher concentrations of cinnamic acid and pinocembrin dried faster and had insufficient time in the agar to inhibit the growth of *A. apis*. This effect could be the explanation for the smaller inhibitory areas at higher concentrations of both substances in the trial.

Bee toxicity

The dermal bee toxicity of cinnamic acid and pinocembrin was low. Even the highest dosages of 1000 µg and 250 µg per bee, respectively, were well tolerated by the adult bees. The mortality rates showed no significant differences to the controls (Z-test, p≥0.05, Tab. 2).

Larval toxicity

Due to the small number of tested larvae in some stages, these findings can only provide an indication on larval tolerability. The application of cinnamic acid at the MIC of 0.5% (5 µg/larva) resulted in very different mortality rates: the mortality of the youngest larvae (L1) was, in general, high (36.9%), without any clear influence of the test substance. The mortality of larval stages L2 (27.4%) and L3 (21.6%) was significantly higher 72 hours after application, than in the positive controls (less than 10% both, L2: z = 2.714, p = 0.006; L3: z = 2.766, p = 0.006; Fig. 3) but not different to the negative controls (14.3%, and 16.7%). Older larvae showed no significant differences when compared to both of the control groups. The larval mortality reduced over time as larval weight increased (Fig. 4).

The application of pinocembrin at its MIC of 1.5% (15 µg/larva) caused no significant increase in larval mortality at any of the larval stage. The mortality of the young larvae was high in the test (34.4%) and control groups (positive 57.6% and negative 33.3%, respectively); in the positive control group it was even significantly higher in L1 (z = 2.013, p = 0.044) and L2 (z = 2.394, p = 0.017) than in the test group. There was very low mortality in the test group of larval stage L3 (3.2%) and no mortality in L4 and L5 (Fig. 3). The larval mortality reduced over time with the increase in larval weight (Fig. 4).

The total larval mortality 72 hours after the application, for all of the stages, was relatively high in the test and control groups: cinnamic acid 28.7%, pinocembrin 13.1%, the positive control group 24.8%, and 15.4% in the negative control group. The test groups showed no significant increased mortality compared to the control groups (Z-test, p≥0.05).

DISCUSSION

Our work proved the fungistatic effect of cinnamic acid and pinocembrin in a 0.5 dose and a 1.5% dose (MIC), respectively, using the agar diffusion test, in the laboratory. Miyakado et al. (1976) described an inhibition of the fruit and foliar pathogens *Alternaria mali. Alternaria kikuchiana,* and *Alternaria brassicicola* by 57%, 47%, and 39%, respectively, on potato sucrose agar containing 1% of pinocembrin. Cinnamic acid dissolved in ethanol did not inactivate the growth of *Aureobasidium pullulans* on wood by 0.04%, but a mixture of ethanol, eugenol and cinnamic acid at a concentration of 0.04% controls the growth of the fungus by 83% (DPMA, 1997).
The fungistatic effect of cinnamic acid and pinocembrin was shown in dosages applicable to honey bees; adult bees tolerate cinnamic acid very well. A dosage of 20%, 40 times higher than the MIC value (0.5%), corresponding to 1000 µg/bee, does not induce bee mortality in the laboratory. Pinocembrin, the second substance tested, caused no damage on adult bees in a dosage of 250 µg/bee, this equates to an over dosage compared to the MIC (1.5%) of at least more than 3 times. Dosages of more than 250 µg could not be tested due to the insolubility of the substance in water at higher concentrations.
Brood mortality after application of the test substances in the colony, occurred in both groups but primarily in young larvae. The larval mortality reduced as the larval weight increased. This increase in weight by a factor of circa 1500 (Snodgrass, 1925) reduces the relative dosage per larva (µg/mg body weight). This could have caused the higher tolerance of the treated larvae. Ritter (1994) states that all larval stages of the honey bee can become infected with *A. apis* but older larvae are most susceptible. The total larval mortality, 72 hours after the application of cinnamic acid and pinocembrin, respectively, was less than 30%. This is slightly lower compared to Winston (1987) who described a mortality of 42% under stress conditions in the colony. Opening the hives several times and investigating the brood area is definitely stressful for the larval stages and should not be done any more than necessary during the test situation.
The results provide information about the high adult bee tolerance when treated with the test compounds. The results also indicate larval tolerability, mainly notable in the older larvae which are most vulnerable to the pathogen. Further research is needed to get a better understanding of the brood tolerance towards the tested substances during different seasons. This is a pilot study. In the future it will be necessary to extend the research to a much larger number of *A. apis* strains.

CONCLUSIONS

Both substances have *in vitro* fungicide effects and can be considered as potential active agents against *A. apis* in honey bee drugs.

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REFERENCES

AVID X (1998) Arbeitskreis Veterinärmedizinische Infektionsdiagnostik Resistenzbestimmung. AVID-Methodensammlung. 22 pp. Available at: http://www.dvg.net/avid/methoden/resistensbestimmung--x-xi-xii-1998-1999-2000.pdf

Aronstein K., Murray P. (2010) Chalkbrood disease in honey bees. Journal of Invertebrate Pathology 103(Supplement): 20-29. DOI: 10.1016/j.jip.2009.06.018

Borchert A. (1974) Schädigung der Bienenzucht durch Krankheit, Vergiftung und Schädlinge. S. Hirzel Verlag. Leipzig, Deutschland. 366 pp.

Boudegga H., Boughalleb N., Barbouche N.,Hamouda M. H. B., El Mahjoub M. (2010) *In vitro* inhibitory actions of some essential oils on *Ascosphaera apis*, a fungus responsible for honey bee chalkbrood. Journal of Apiculture Research 49(3): 236-242. DOI: 103896/ibra.1.49.3.02.

DPMA (1997) DE 196 11 825 A1 "Holzschutzmittel". Deutsches Patent-und Markenamt. Ifi Claims Patent Services. Düsseldorf, Deutschland. 4 pp. Available at: https://register.dpma.de/DPMAregister/pat/PatSchriftteneinsicht?docId=DE19611825A1

EMA (2008) Guideline on veterinary medicinal products controlling *Varroa destructor* parasitosis in bees. EMA/CVMP/EWP/459883/2008. European Medicines Agency. London, United Kingdom. 9 pp. Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/11/WC500099137.pdf

EUCAST (2012) Method for the determination of broth dilution minimum inhibitory concentration of antifungal

agents for yeast. European Comittee on Antimicrobial Susceptibility Testing. 21 pp. Available at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/EUCAST_EDef_7_2_revision.pdf

Gardana C., Scaglianti M., Pietta P., Simonetti P. (2007) Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 45(3): 390-399. DOI: 10.1016/j.jpba.2007.06.022

Heath L. A. F., Gaze B. M. (1987) Carbon dioxide activation of spores of the chalkbrood fungus *Ascospheara apis*. Journal of Apicultural Research 26(4): 243-246.

Jensen A. B., Aronstein K., Flores M. F., Vojvodic S., Palacio M. A., Spivak M. (2013) Standard methods for fungal brood disease research. Journal of Apicultural Research 52(1): 1-20. DOI: 10.3896/ibra. 1.52.1.13

Kettering M. (2005) Pilzmetabolite als chemische Botenstoffe und Inhibitoren der Infektions-relevanten Morphogenese bei Phytopathogenen. Tenea Verlag. Berlin. 51 pp.

Marcucci M. C. (1995) Propolis: chemical composition, biological properties and therapeutic activity. Apidologie 26 (2): 83-99. DOI:10.1051/apido19950202

Mitro S. (1996) Information zu Honig und anderen Bienenprodukten aus medizinischer und mikrobiologischer Sicht. Tierärztliche Umschau 51: 232-240.

Miyakado M., Kato T., Ohne N., Mabry T. J. (1976) Pinocembrin and (+)-ß-eudesmol from *Hymenoclea monogyra* and *Baccharis glutinosa*. Phytochemistry 15(5): 846.

OECD (2007) Guidance Document on the Honey Bee (*Apis mellifera* L.) Brood test under Semi-Field Conditions, Enviorement, Health and Safety Puplication Series on Testing Assessment No.75, ENV/JM/MONO 22. Paris. 27 pp. Available at: http://search.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2007)22&doclanguage=en

Popova M. P., Chinou I. B., Marekov I. N., Bankova V. S. (2009) Terpenes with antimicrobial activity from Cretan propolis. Phytochemistry 70(10): 1262-1271. DOI: 10.1016/j.phytochem.2009.07.025

Ritter W. (1994) Bienenkrankheiten. Ulmer Verlag. Stuttgart, Deutschland. 128 pp.

Snodgrass R. E. (1925) Anatomy and Physiology of the honeybee. McGraw-Hill Book Company. Michigan, USA. 172 pp.

Spiltoir C. F. (1955) Life Cycle of *Ascosphaera apis* (*Pericystis apis*). American Journal of Botany 42(6): 501-508.

SPSS SigmaStat® Version 3.0. Systat Software Inc.

Stock I., Wiedemann B. (1998) Bestimmung der natürlichen Antibiotika- Empfindlichkeit. Chemotherapie Journal 7(4): 127-135.

van Bergen A. (2012) Mit Zimtsäure gegen Bazillen. Biocom AG Biotechnologie. Deutschland. Available at: https://www.biotechnologie.de/BIO/Navigation/DE/Foerderung/foerderbeispiele,did=148418.html?view=renderPrint

Winston L. M. (1987) The Biology of the Honey Bee. Harvard University Press Cambridge. Massachusetts, London. 281 pp.

Yoder J. A., Jajack A. J., Cornacchione W. S., Dunn A. L., Cunningham E. G., Matchett C. L., Rosselot A. E. (2014) *In vitro* evaluation of sugar syrups, antibiotics, and miticides on growth of honey bee pathogen, *Ascosphaera apis:* Emphasis for chalkbrood prevention is on keeping bees healthy. Apidologie 45(5): 568-578. DOI: 10.1007/s13592-014-0274-5