ESTIMATION OF FUNGICIDAL AND FUNGISTATIC PROPERTIES OF DIFFERENT CONCENTRATIONS OF BEEVITAL KALKBRUT ON *ASCOSPHAERA APIS* IN VITRO

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Chalkbrood, *ascosphaeriosis apium,* is one of the most dangerous and regularly occurring diseases of honeybee colonies throughout the world. It is caused by the fungus *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir. The development of chalkbrood disease is connected with the prevalence of the fungus in honeybee colonies, in the hive environment, in populations of solitary bees and other wild insects, as well as with factors that decrease colony immunity (Harbo 1995, Spivak and Downey 1998, Gilliam 1986, Spivak and Gilliam 1993).

The presence or absence of susceptibility to chalkbrood in honeybee (*Apis mellifera* L.) is directly connected with behavioural immunity of honeybee colonies. This feature has a genetic character and is passed on by the queen to her offspring. Behavioural immunity can be defined, with a great dose of simplification, as the bees' ability to quickly detect and uncap dead brood and remove it from the cells before the pathogen reaches an invasive potential. (Gilliam et al. 1983, Oldroyd 1996, Spivak and Downey 1998).

The recurring outbreaks of chalkbrood in apiaries are the reason why beekeepers readily reach for chemical compositions to control it. A common beekeeping practice is to raise the acidity of hive environment by means of organic acids, e.g. formic, acetic, sorbic

or citric acid (Kaftanoglu et al. 1992). Administration of fungicides, which are based mainly on nystatin or clotrimazolum (Gliński i Chmielewski 1979, 1996, Gliński et al. 1988), to bees is virtually impossible because of the danger of these substances being transferred to honey.

In the EU countries honey is numbered among alimentary products that have medicinal properties. The level of medication residues has been set at 0 MRL. This decision led to a ban on anti-fungal medicine like antibiotics and sulphonamides. In compliance with the law, no anti-fungal medicine that might contaminate honey can be used in bee colonies.

The assessment of anti-fungal activity of a given preparation is possible, among others, by determining its MIC (Minimal inhibitory concentration) and MFC (Minimal fungicidal concentrations) in relation to the analysed species of fungus. According to available literature, MIC and MFC can be determined by disc methods (Kowalska 1984) or using a series of concentrations of the fungicidal preparation on mediums (Gliński et al. 1988).

The aim of this study was to determine susceptibility of different strains of *Ascosphaera apis* to Beevital Kalkbrut (F&B GmbH Austria).

Material and methods

Twelve strains of *Ascosphaera apis* were analysed. The material was acquired in apiaries affected by chalkbrood, located in different areas of Poland. The strains of fungus were isolated from dead bee larvae showing the posthumous symptoms of chalkbrood (mummies). Cultures from fragmented larvae were inoculated onto Sabouard's medium SDA-YE (with 0.2% yeast extract and 0.1% chloramphenicol, ph 7.0) and incubated at

25°C in CO_2 enriched atmosphere. Obtained pure cultures of the strains were examined macroscopically and microscopically in order to identify the strains on the basis of their morphological traits, mycelium growth and ascospores production.

The preparation used in this study was Beevital Kalkbrut (F&B GmbH Austria). The composition of this preparation is a trade secret.

MIC was determined by means of cylinder dilution method, according to Butty (Butty et al. 1995). 30ml of Sabouraud's medium containing specific concentrations of the preparation was poured onto Petri dishes (diameter 10cm). In the preliminary research the following concentrations (by volume) of Beevital Kalkbrut were analysed:

sample	Volume of Beevital Kalkbrut	Volume of Sabouraud's medium	Tested concentration of Beevital Kalkbrut
1.	25 ml	75 ml	25%
2.	12,5 ml	87,5 ml	12,5%
3.	6,25 ml	93,75 ml	6,25%
4.	3,125 ml	96,875 ml	3,125%
5.	1,562 ml	98,438 ml	1,562%
6.	0,781 ml	99,219 ml	0,781%

The strains of *Ascosphaera apis* were inoculated onto the central part of 8cm dishes containing Sabouraud's medium. After on average 6-9 days of incubation of the strains, samples were taken for identification. The identified samples were mycelium inocula from colonies of a few centimetres diameter, obtained before the moment of ascospores production. Inocula were obtained with a Pasteur pipette (5mm in diameter) from places equally distant from the centre of the colony. The aim of these procedures was to obtain agar cylinders covered with fungus microculture containing elements of mycelium comparable with regard to their quality and quantity.

In each of the mediums containing specific concentrations of Beevital Kalkbrut three hollows of 5mm diameter were cut out, where the obtained inocula of fungus were

transferred in way that ensured sterility. The control samples were Sabouraud's mediums not containing the tested preparation, onto which inocula of the tested strains were transferred in an analogical way.

The results were read after 1, 2, 3, 4 and 9 days of growth at 25^{o}C. The diameters of cultivated colonies were measured, and the MIC value was determined as the concentration of the preparation that limited the growth of fungus colony to 7mm, which is 2 mm outside the diameter of the incorporated inoculum. This value was established after the 9th day of incubation, according to methodology described by Wawrzkiewicz et al. (2000), and each assessment was performed in duplicate.

Results and discussion

The preliminary in vitro analysis demonstrated a clear effect of different concentrations of Beevital Kalkbrut on the growth of strains of *Ascosphaera apis* used in the experiment.

Data obtained during the experiment are presented in Table 1. (Fig. 1 and 2)

Table 1.
Assessment of susceptibility of Ascosphaera *apis* to different concentrations of Beevital Kalkbrut (diameter of colony (mm) on day 1 and 6 of the experiment)

Strain number	Concentration of Beevital Kalkbrut in medium											
	25%		12.5%		6.25%		3.125%		1.562%		0.781%	
	day 1	day 6	day 1	day 6	day 1	day 6	day 1	day 6	day 1	day 6	day 1	day 6
A7	5	5	5	5	5	5	10	30	14	43	15	45
A34	5	5	5	5	5	5	7	12	11	31	15	32
A34	5	5	5	5	5	5	8	11	10	32	11	35
A42	5	5	5	5	5	5	5	14	10	27	13	28
A44	5	5	5	5	5	5	7	16	11	30	12	30
A50	5	5	5	5	5	5	7	9	10	23	13	36
A85	5	5	5	5	5	5	5	12	11	31	15	36
A90	5	5	5	5	5	5	8	16	11	38	13	36
A101	5	5	5	5	5	5	8	21	13	42	14	41

On the basis of preliminary research, it was determined that concentrations over 6.25% inhibit the growth of all of the 9 analysed strains of *Ascosphaera apis.* At these

concentrations none of the strains exceeded 7 mm in diameter, which is a clear proof that the preparation has an inhibitory effect on the fungus. In lower concentrations Beevital Kalkbrut did not have such a distinct effect. That is why in the next stage of the study 6 concentrations of the preparation in medium were tested: 3.5%, 4%, 4.5%, 5%, 5.5% and 6%. At this stage 12 strains were used, and the growth of colony was assessed on days 1, 2, 3, 4 and 9. All tests were performed in triplicate and mean values of the results the results were calculated. Variance in growth of analysed strains in consecutive replications did not exceed 5%. The results are presented in Table 2.

The control samples in this experiment were the same strains of *A. apis* incorporated on clean Sabouraud's mediums. Their diameters on the third day of observation amounted to 35-45mm, while on day 9 the mycelium covered the whole surface of the dishes.

Table 2.
Size of *Ascosphaera apis* colonies (mm) on days 1, 3 and 9 of the experiment on mediums containing Beevital Kalkbrut

Strain number	Concentration of Beevital Kalkbrut in medium																	
	3.5%			4%			4.5%			5%			5.5%			6%		
	day 1	day 3	day 9	day 1	day 3	day 9	day 1	day 3	day 9	day 1	day 3	day 9	day 1	day 3	day 9	day 1	day 3	day 9
A7	7	13	39	5	14	34	5	9	28	5	6	24	5	5	5	5	5	5
A34	5	7	31	5	5	24	5	5	23	5	5	12	5	5	5	5	5	5
A39	10	13	26	7	12	26	5	6	15	5	5	16	5	5	5	5	5	5
A42	5	6	26	5	6	26	5	5	17	5	5	14	5	5	5	5	5	5
A44	5	7	26	5	6	20	5	5	16	5	5	10	5	5	5	5	5	5
A49	7	9	27	5	6	26	5	5	19	5	5	12	5	5	5	5	5	5
A50	5	7	24	5	5	13	5	5	5	5	5	5	5	5	5	5	5	5
A55	5	7	29	5	7	20	5	5	19	5	5	23	5	5	5	5	5	5
A71	5	7	27	5	5	20	5	5	16	5	5	14	5	5	5	5	5	5
A85	5	15	38	5	6	26	5	5	5	5	5	5	5	5	5	5	5	5
A90	7	13	31	5	7	26	5	5	21	5	5	17	5	5	5	5	5	5
A101	8	13	32	5	6	26	5	5	23	5	5	15	5	5	5	5	5	5

On mediums containing 5.5% and 6% Beevital Kalkbrut the growth of *Ascosphaera apis* strains did not exceed the diameter of 7mm, which shows a clear inhibitory effect of these concentrations. Whereas on mediums containing 5% of the preparation on day 3 only the A7 strain had grown, but reaching just 6mm, which can still be seen as an inhibiting value.

On day 9 of the experiment some growth of the fungus colony was observed, and the diameters of colonies ranged from 10 to 24 mm. Two strains, A50 i A85 did not grow at this concentration of Beevital Kalkbrut. Likewise at the concentration of 4.5% these two strains did not grow on days 3 and 9, while the A7 strain reached 9mm diameter on day 9. For the 4.5% concentration the growth of the remaining strains ranged from15 to 28 mm on day 9 of the experiment. (Table 2).

In lower concentrations (3.5% i 4%) Beevital Kalkbrut did not demonstrate a distinct inhibitory effect on the growth of Ascosphaera *apis* strains and the diameters of colonies ranged from 13 to 38mm on day 9 of the experiment.

Table 3. Percentage of *Ascosphaera apis* strains for particular MIC values

MIC in ml/ml	Percentage of strains on day 3	Percentage of strains on day 9
0.035-0.060	8.33	0
0.040-0.060	66.67	0
0.045-0.060	25	16.67
0.050-0.060	0	0.00
0055-0.060	0	83.33
0.060	0	0
Total	**100**	**100**

For all strains used in the experiment MIC (Minimal inhibitory concentration) amounted to 0.04083ml/ml (+/-0.002886) on day 3 and 0.0533ml/ml (+/-0.003892) on day 9 of the experiment. The calculated MIC corresponds to 5.34ml of Beevital Kalkbrut in 100ml of medium.

The next stage of the study was aimed at checking whether the inhibitory effect of Beevital Kalkbrut on *Ascosphaera apis* is lasting. To achieve this aim, all inocula that did not present growth in the examined concentrations (4.5-6%) of the preparation were again

transferred, in a sterile way into hollows cut out in Sabouraud's medium SDA-YE. The results were read on days 1, 2, 3, 4, and 5, to assess whether any growth of mycelium has taken place. The A50 strain was found to have grown in the cylinder obtained from concentration 4.5%, while in cylinders from higher concentrations no growth was observed. In the case of A85 strain, the relevant concentration was 5.0%, while for A90 it was 5.5% (fig.4)

Table 4. Percentage of Ascosphaera *apis* strains for particular MFC values

MFC in ml/ml	Percentage of strains
0,035-0,060	0,00
0,040-0,060	0,00
0,045-0,060	0,00
0,050-0,060	8,33
0,055-0,060	83,33
0,060	8,33
Total	**100**

For all strains used in the experiment, MFC amounted to 0.055ml/ml (+/-0.002132) on day 5 after transferring inocula onto standard Sabouraud's medium.

Results obtained in the present study indicate a significant fungistatic activity of Beevital Kalkbrut solution in concentration 5.5% against strains of *Ascosphaera apis.* The analysed preparation also features very low fluctuation of activity against *Ascosphaera apis* strains used in the experiment. Changes in activity do not exceed 10%, which indicates that the activity of the preparation is stable. In comparison, in research on nystatin activity against the same fungus, the range of results comprised 5 orders of magnitude (from 100 to 1600). (Chorbiński 2003).

Positive results of *in vitro* research into antifungal activity of Beevital Kalkbrut constitute a basis for *in vivo* studies in bee colonies infected with chalkbrood. Application

of Beevital Kalkbrut to restrict the development of chalkbrood in colonies would be of enormous usefulness to beekeepers in view of the ban on using antibiotics and sulphonamides in the EU. This preparation could also constitute an important alternative to an auxiliary therapy used in beekeeping practice, based on evaporating organic acids (e.g. formic or acetic acid) in the hive.

Conclusions

Beevital Kalkbrut in concentrations above 6% demonstrates a high fungistatic activity against *Ascosphaera apis*

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ESTIMATION OF FUNGICIDAL AND FUNGISTATIC PROPERTIES OF DIFFERENT CONCENTRATIONS OF BEEVITAL KALKBRUT ON *ASCOSPHAERA APIS* IN VITRO

Abstract

The fungus *Ascosphaera apis* is the causative agent of chalkbrood disease in honeybee larvae (*Apis mellifera* L.). The aim of the study was to estimate susceptibility of 12 strains of *Ascosphaera apis* to Beevital Kalkbrut solution. MIC was determined by means of cylinder dilution method, as described by Butty et al.

The following concentrations of Beevital Kalkbrut were analysed: 3,5%, 4%, 4,5%, 5%, 5,5% and 6% MIC was calculated after the 9th day of the experiment and it amounted to 5.333% (+/-0.3892). Beevital Kalkbrut in concentrations above 6% shows a high fungistatic activity against *Ascosphaera apis.*

Keywords: *Ascosphaera apis,* MIC, Beevital Kalkbrut

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Figures:

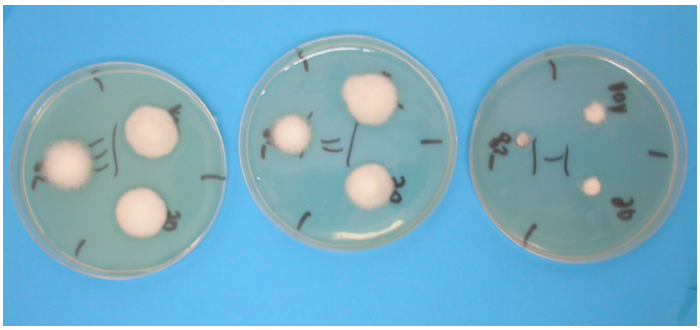


Fig.1. Cylinder dilution method, from the left Beevital Kalkbrut concentrations: 0.781%, 1.562%, 3.125%, day 3 of the experiment, inhibition of growth of Ascosphaera apis clearly visible at the concentration 3.125%.

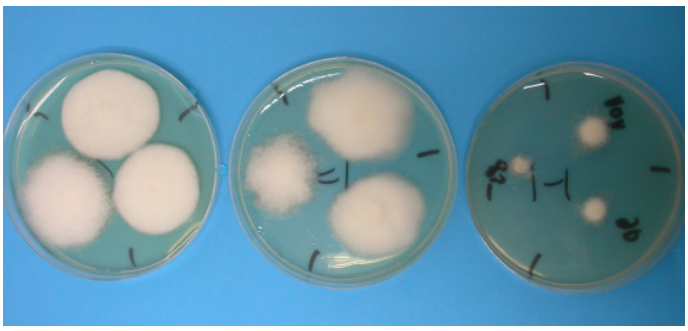
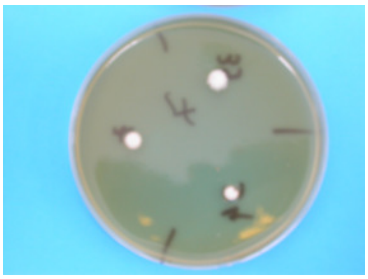
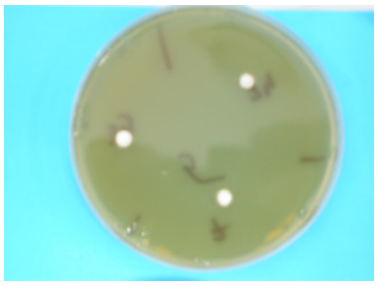


Fig. 2. Cylinder dilution method, from the left Beevital Kalkbrut concentrations: 0.781%, 1.562%, 3.125%, day 6 of the experiment, lasting inhibiting effect of concentration 3.125%.

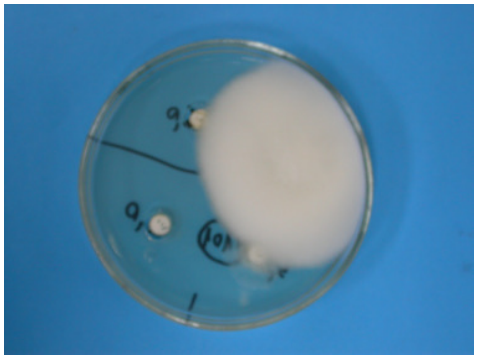


Concentration 4%

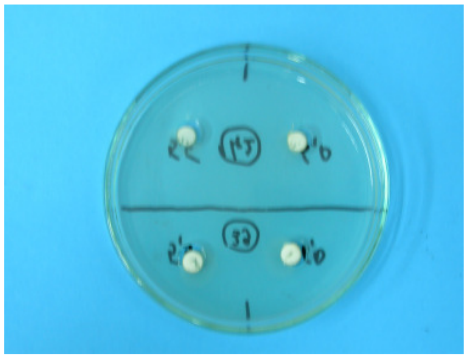


Concentration 5%

Fig.3. Cylinder dilution method, day 3 of the experiment, lack of growth of *A .apis* in the given Beevital Kalkbrut concentrations.



A



B

Fig. 4. Examination of the stability of inhibitory effect of the tested concentrations of Beevital Kalkbrut. A – analysis of two strains and two concentrations, inocula on the left of the Petri dish do not exhibit growth – a positive result. B- a positive result for two strains and two concentrations.