Inhibitory effect of selected botanical compounds on the honey bee fungal pathogen *Ascosphaera apis*

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Abstract: *Ascosphaera apis* is a heterothallic fungus causing widespread honey bee disease called chalkbrood. In many countries, beekeepers use fungicides to control this pathogen. However, this approach results in residues in bee products as well as in resistance which are serious problems. For these reasons, in European Union countries is not allowed to use nor fungicides, neither antibiotic to control bee diseases. Therefore, there is an increasing need to find environmentally friendly methods to control honey bee pathogens. One of the most promising approaches seems to be using natural botanical compounds with antifungal effects. In this paper, 2 essential oils and 2 main components were tested for *A. apis* inhibition in *in vitro* conditions. Local strain of *A. apis* was cultivated on Sabouraud dextrose agar (SDA) with different concentrations of tested botanical compounds. Cultivation was carried out at 30 °C for 21 days. The greatest inhibitory effect reached thymol (MIC 500 µg/ml). Very promising seems to be also clove bud oil (MIC 2500 µg/ml) and eugenol (MIC 2500 µg/ml). Cedarwood oil did not stop the growth of *A. apis* even in the highest concentration (MIC >5000 µg/ml). This experiment confirmed that these plant substances are efficient as an antimicrobial agent against chalkbrood disease. Despite problems with unstable botanical compounds composition in the same plant species, application of essential oils and their main components could be gentle and safe way how to control a lot of bee diseases in the future.

Key Words: Essential oils, chalkbrood, cultivation media, cultivation, antifungal effect

INTRODUCTION

More than three quarters of agricultural crops are dependent on pollination and approximately one third of food production is influenced by pollinators (Klein et al. 2007). Therefore, reduction in abundance of insects and their extinction draw the attention to many scientists because it poses a significant threat. The same, if not worse situation affects also wild plants (Biesmeier et al. 2006). One of the most important pollinators is honey bee (*Apis mellifera*) which is currently affected by many diseases more frequently than before. It is the result of exposure of bees to pesticides applied to the fields and antibiotics to bee hives (Sandrock et al. 2014), decline of biodiversity and pollen mono diet, transfer of bee colonies for long distances, inadequate beekeeping practice and also anthropogenic modifications in habitats and climate changes (Biesmeier et al. 2006). One of the very common and worldwide spread diseases is chalkbrood. Its incidence is on the rise and may cause significant economic losses, especially in cold and damp weather conditions (Aronstein and Murray 2010). There are some reports from East Asia where beekeepers deal with this disease very often (Chantawannakul et al. 2005).

Chalkbrood is caused by heterothallic fungus *Ascosphaera apis* (Maassen ex Claussen) (Spiltoir 1955) which is closely specialized to honey bees and it can infect only bee larvae. Spores of this fungus can be consumed with food by honey bee larvae. In the suitable conditions in a gut, spores are activated by higher concentration of carbon dioxide and the mycelium starts grow. At the beginning of infection, the larvae cease food consumption and get swollen. During the time, mycelium grows throughout the larvae and causes its dead. After some time, the larvae are going to shrink and solidify and also form so-called chalkbrood mummy. The color of cadavers can be white, black/grey or mottled depend on the presence of ascospores being created on the surface. These ascospores, which can be produced only sexually, are placed into resistant cyst and stay viable for many years. The mummies resemble chalk, from that the name of the disease originated (Aronstein and Murray 2010).

In many countries, beekeepers still use fungicides to treat chalkbrood disease. It can, however, leave residues in bee products and also causes problem with pathogen resistance (Chaimanee et al. 2017). Therefore, in European Union countries, are these substances banned and there is no registered therapeutic agent against chalkbrood. A lot of chemotherapeutic compounds have been investigated as potential substances to treat chalkbrood. Although many of them have antifungal effect, they are not efficient to spores and, in addition, they have negative effect to bee vitality and longevity (Aronstein and Murray 2010). Moreover, there is also a worldwide increase prevalence of other fungal pathogen species on fields and warehouses which are associated with higher fungicide consumption (Zabka et al. 2014). Farmers and even consumers are exposed to long-term, low-dose unnatural substances due to fungicides residues in food supply and groundwater as well as many non-target organisms, especially in developing countries. These negative substances are linked to immune suppression, hormone disruption and cancer. Thus, there is an increasing effort to use alternative, more environmentally friendly methods. Recently, one of the most promising way how to control chalkbrood seems to be using of plant essential oils (EOs) or their main components (MCs) with fungicide effect (Gabriel et al. 2018). Their next substantial advantage is that they are allowed in human food chain included honey production because of GRAS status (Chantawannakul et al. 2005, Li et al. 2019).

A various plants had been used in medicine long time before microbes were discovered. Our ancestors empirically observed the healing potential of some plant species which is currently known as the antimicrobial effect. This effect is mainly caused by essential oils which are volatile substances of plants containing a various organic bioactive compounds. They are known for its very sharp aroma and taste and have a protective function in plants (Kuzyšinová et al. 2016). Even now, these plant substances are abundantly used in preventing and treating various digestive and respiratory diseases in the form of tea, spices and traditional remedies. They are still efficient because there is no resistance against these compounds. In recent time, there is an increasing effort to utilize these plant compounds because they are easily degradable and environmentally friendly and have minimum or no side effects (Li et al. 2019). In this paper, we demonstrated antifungal effect of some botanical compounds against the local strain of *A. apis,* which have a potential to be used as agents against honey bee pathogens.

MATERIAL AND METHODS

Fungal isolates and essential oils

Ascosphaera apis isolates were obtained from mummified bee brood collected on apiary in South Bohemia. The opposite mating types were separated on the PDA (Potato dextrose agar, Himedia) with addition of chloramphenicol by sub culturing until pure culture were established and stored as the maternal cultures on PDA at 30 °C. For antifungal effect, the male mating type was chosen due to its faster growth. For fungicide activity assay, two EOs (clove bud oil, cedarwood oil) and two MCs (thymol, eugenol) were chosen based on their antimicrobial activity. All of these substances were obtained from Sigma Aldrich and stored in dry, dark conditions at 22 °C. The DMSO (dimethyl sulfoxide, Himedia) served as a solvent. Four different concentrations of each tested substances were prepared by diluting 25, 50, 250 and 500 µl of EOs or MCs in 500 µl of DMSO for 100 ml media.

Testing of EOs and MCs

For cultivation tests, SDA (Sabouraud dextrose agar, Himedia) medium was used due to its suitability for *A. apis* growth and also for the medium uniformity. A volumes of 100 ml SDA media were autoclaved at 121 °C for 30 min in Erlenmeyer flask for each variant. Diluted substances of EO/MC were added to the autoclaved SDA cooled at 45 °C aseptically to ensure final concentrations (250, 500, 2500, 5000 µg/ml) and dispersed by circular movements. An amount of 20 ml were poured into Petri dishes (90 x 15 mm). After media solidification, the 8 mm cork bores of mycelium were cut from the edge of 7 days old culture and placed in the middle of each Petri dish. Five repetitions were prepared for each concentration of tested substances. *A. apis* was cultivated under the dark condition at 30 °C for 21 days. The growth of mycelium was measured by two perpendicular diameters daily for the first 10 days, then the 15th day and the 21st day. The inhibitions effect was calculated. Data from the 10th day of cultivation were statistically analysed with ANOVA (STATISTICA 12, StatSoft Inc.) and the mean values were compared using the Tukey HSD test ($p < 0.05$).

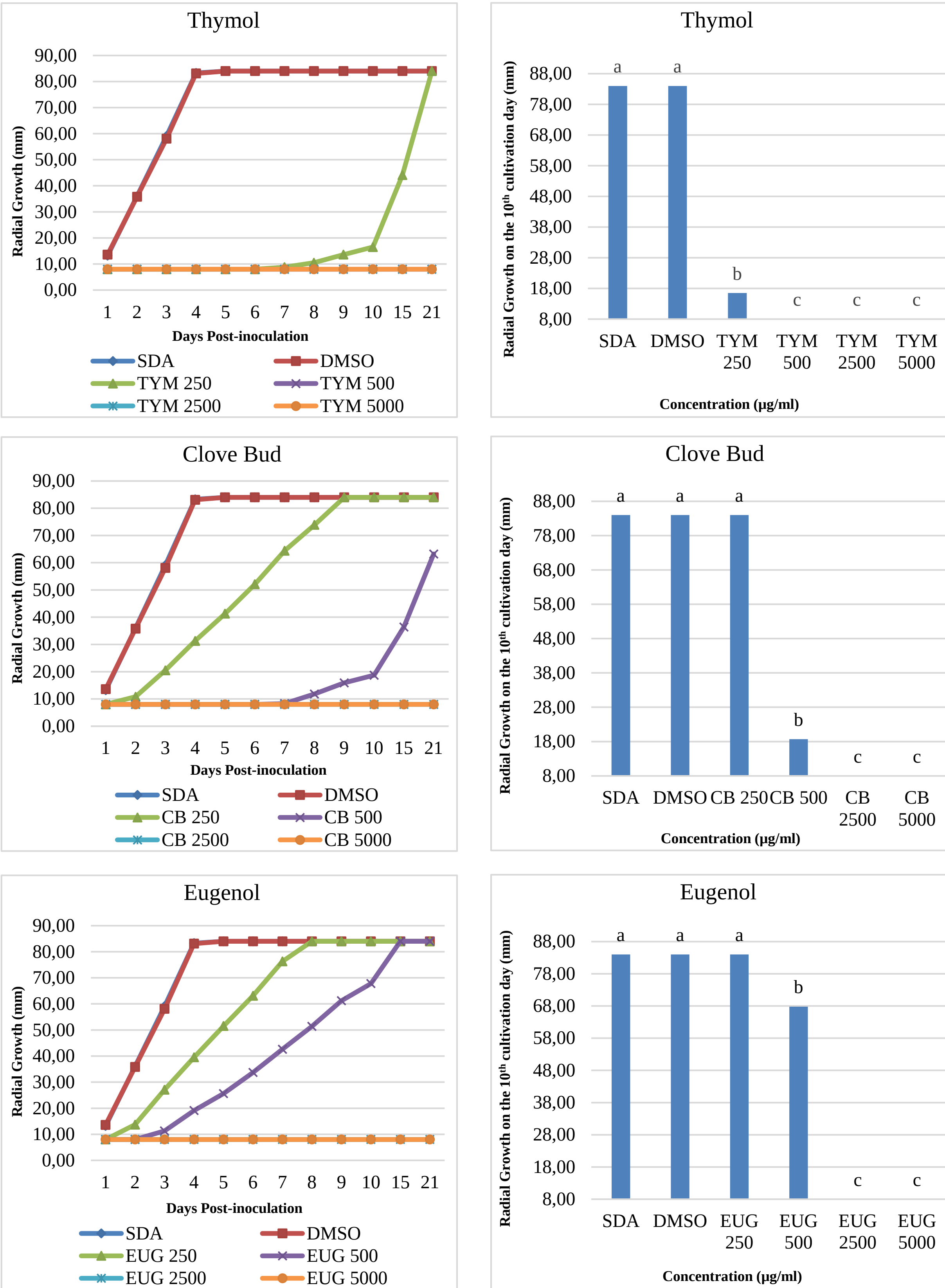
RESULTS AND DISCUSSION

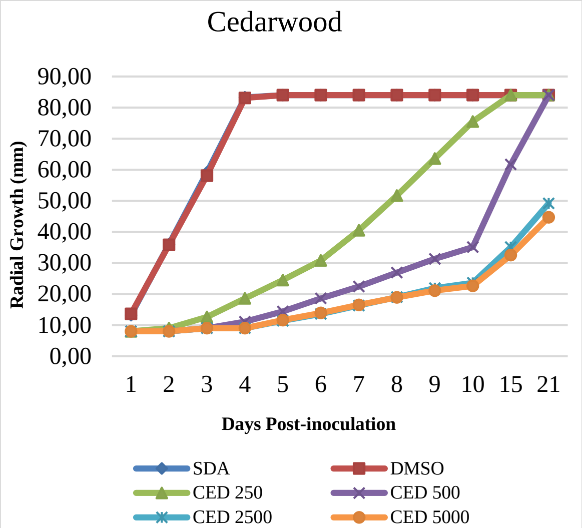
An antifungal effect of four plant substances was tested against *A. apis*. This fungus grew very well on SDA medium as well as on the SDA controls including the DMSO. All the tested substances showed an antifungal effect (Figure 1). In each case, the higher concentration of tested botanical compounds caused higher growth inhibition. Thymol ($F_{(5,54)} = 32315.7$, $p < 0.05$) has been detected as the most effective substances with the MIC < 500 µg/ml after 10 days of cultivation. However, during the first 7 days, there was no *A. apis* growth even at the lowest concentration (250 µg/ml). Calderone et al. (1994) determined its MIC as 1000 µg/ml after 7 days of cultivation. This variability may indicate different sensitivity of individual *A. apis* strains or different substances composition. Thymol is considered as a broad range inhibitor because it can suppress growth many fungal species as *Alternaria alternate*, *Stachybotrys chartarum*, *Cladosporium cladosporioides* and especially significant for beekeeping *Aspergilus niger* causing Stonebrood disease (Zabka et al. 2014) as well as *Paenibacillus. larvae* causing American foulbrood (Fuselli et al. 2006). This substance was tested against many different bacteria and yeast with very promising results (Wiese et al. 2018). Thyme, with its main component thymol, had very low MIC (300 µg/ml) also against the human pathogen as *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* (Hammer et al. 1999).

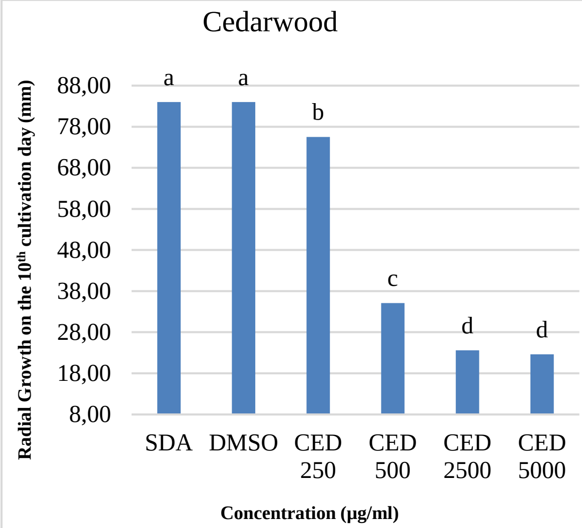
Great antifungal effect had also clove bud oil ($F_{(5,54)} = 47413.8$, $p < 0.05$) with MIC < 2500 µg/ml measured after 10 days of cultivation. According to Chaimanee et al. (2017), clove bud EO had MIC very low (32 µg/ml) after 3 days of cultivation and as an efficient oil is also considered by Ansari et al. (2016) with MIC 400 µg/ml and by Calderone et al. (1994) with MIC 1000 µg/ml, both measured after 7 days of cultivation. In our study, the lowest concentration was relatively harmless and mycelium started to grow even on the second day of cultivation. However, the clove bud concentration of 500 µg/ml inhibited *A. apis* growth for the first 7 days (Figure 1). Clove bud oil is efficient also against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* (MIC 1200 µg/ml) representing a group of common human pathogens (Hammer et al. 1999). Especially clove bud oil main component, eugenol, is known for its significant antifungal effect. In this study, eugenol inhibited 100% *A. apis* growth at 2500 µg/ml after 10 days of cultivation ($F_{(5,54)} = 11342$, $p < 0.05$). Its particular efficiency was proved also by Larrán et al. (2001) who tested eugenol as a part of basil oil against *A. apis* and also by Gende et al. (2008) who tested eugenol as a main part of *Cinnamomum zeylanicum* against *P. larvae*.

Despite cedarwood essential oil ($F_{(5,54)} = 3876.25$, $p < 0.05$) showed antimicrobial activity (Figure 1), it did not completely inhibit *A. apis* growth in any tested concentrations (MIC >5000 µg/ml). For this reason, its use can be suitable only assuming its synergic effect with other medical substances to treat chalkbrood disease. In cedarwood oil were recorded considerable diversity in its compositions, therefore the efficacy can differ. The same effect occurs in many plant oils (Paoli et al. 2011).

Figure 1 Inhibition effect of botanical compounds on the A. apis pathogen during 21 days, differences among the concentrations on the 10th day of fungus cultivation show small letters (HSD Tukey test)







CONCLUSIONS

Plants contain a diverse composition of essential oils and their main components and a lot of them have an antimicrobial effect. By these botanical compounds, plants can protect themselves against many pests and pathogens. Therefore, essential oils are used for plant protection in agriculture and it is considered as an environmentally friendly way with a minimum risk of acquisition of resistance. Moreover, there are no residues in agriculture products after their use because essential oils are easily degradable. In addition, they are convenient also for organic farming. For these reasons, botanical compounds have a large potential and represent a suitable alternative to prevent or treat different kind of bee diseases. Their antifungal effect was confirmed also by this study. There were tested four plant substances and especially thymol and clove bud essential oil had significant antimicrobial effect against *A. apis*. It is very likely, that some substances can cause a synergistic effect, which would increase antimicrobial activity and decrease costs for its practical use. However, the next studies are needed to confirm bee tolerance and also to find suitable methods for its application in bee hives.

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