IN VITRO EVALUATION OF THE EFFECTS OF ESSENTIAL OILS ON *ASCOSPHAERA APIS*, THE CAUSATIVE AGENT OF CHALKBROOD DISEASE

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Abstract

Honeybee diseases cause considerable expenses to beekeepers for cost of maintaining apiary's inspection, colonies damaged or destroyed and drugs fed to prevent bee's infections. One of them is *Ascosphaera apis,* a fungal pathogen causing Chalkbrood disease in honey bee larvae. Chalkbrood is most frequent during damp conditions. Infected larvae turn chalky white color, become hard, and then turn black. It can be regarded as "the most widespread infectious disease" and this has led to economic loss in apiculture. We aim to find an alternative approach by using essential oils from medicinal plants in combating and controlling the disease.Therefore, current article compiled the effects of ten essential oils (citral, geraniol, citronelol, cinamaldehid, thymol, eucalyptus oil, lemongrass oil, fennel oil, clove oil and cedar oil) on *Ascosphaera apis* in the laboratory with an agar diffusion method and the method of serial dilutions in microdilution plate. *A. apis* strains were isolated from the dead honey bee larvae and the fungal strains were identified using a light microscopy. Various concentrations (0.08% - 10%) of the essential oils were experimented for determination of minimal fungistatic and also fungicide concentration. After incubation, the zones of inhibition were determined with a magnifying lens. The minimal inhibitory concentration was in range of 0.3125% - 1.25% for tested essential oils (MIC- values), and the minimal fungicide concentration was in range of 1.25% - 5%. All tested substances shows *in vitro* fungistatic and fungicide potential and can be considered as potential alternative active agents for prevention and control of chalkbrood disease without the use of antibiotics.

Keywords: fungus, pathogen, fungicide inhibitory concentration, agar diffusion test, method of serial dilutions, honeybees.

Introduction

Insect pathogenic fungi can be found throughout the fungal kingdom (Humber, 2008), all being capable of invading their hosts and overcoming their immune systems. Chalkbrood of honeybees (*Apis mellifera*) is a fungal disease caused by the spore-forming ascomycete fungus *Ascosphaera apis* (Spiltoir, 1955) affecting the honeybee larvae, producing severe damages in apiarian producers. 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). It only infects larvae that are three to four days old, which are most susceptible to the fungus, especially if these are chilled after ingesting spores of *A. apis*. The larvae mostly die in the L5 developmental stage. Worker, drone and queen larvae all are susceptible to chalkbrood disease. At first, dead larvae inside recently capped cells are covered by a fluffy white mold, and later on these dry and become black or white mummies, and at the peak of the disease, mummies are easily detected at the entrance to the hive as nurse bees remove them from their cell. This color variation is due to the presence or absence of the black fruiting bodies that are formed on the outside of the larvae. The disease may become severe in some colonies, but is not expected to be a serious problem for beekeepers. The fungus causing chalkbrood in honey bees has a narrow host range and a unique infection route, it relies solely on sexual reproduction and has many host-specific adaptations. The field diagnosis of chalkbrood is based on visual detection of diseased, mummified brood, commonly known as "chalkbrood

mummies". Chalkbrood can reduce colony productivity by lowering the number of newly emerged bees, and in some cases may lead to colony losses (Jensen et al., 2013). The disease is found infecting honey bee brood in most regions of the world, including warm and dry climates. Clinical symptoms of chalkbrood often appear for only a short time, typically under cold and damp weather conditions (Aronstein and Murray, 2010). The chalkbrood spores are very resistant to environmental conditions and can remain viable and infective for more than 15 years. Treatments for the disease involve a variety of chemicals, which have been applied in a continuous and excessive way, so the fungus may develop resistance to antibiotics, besides generating residues in honey, thus affecting quality and commercialization. Therefore, during the last years it has been appealed to natural substances, such as essential oils, to treat infected beehives (Colin et al., 1989; Floris and Carta, 1990). Essential oils are the result of a vapour hydrodistillation of plant species, which are thus separated because of being immiscible in water. They are complex mixtures in whose composition there are mainly terpenic compounds, bencenic compounds, and phenols, which are being continuously studied, e.g., as natural biocide agents. In this work *in vitro* behaviour of ten essential oils has been studied against *Ascosphaera apis*, the causative agent of chalkbrood disease.

Material and methods

Chemicals

Citral, geraniol, citronelol, cinamaldehid, thymol, eucalyptus oil, lemongrass oil, fennel oil, clove oil and cedar oil were derived from the commercial chemical company Sigma-Aldrich®.

Preparation of the inoculum

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 Sabouraud Dextrose Agar (SDA) 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.

Agar diffusion test

The antifungal oil activity was determined using agar diffusion method with paper disks. The agar diffusion test was conducted with four concentrations of essential oils (0.5%, 1.25%, 5.0%, and 7.5%) in three replicates. The essential oils were diluted in 50% sugar syrup (w/w). The start inoculum (200 µL) was spread area-wide onto the Sabouraud Dextrose Agar. Each concentration of test substance (20 µL) was pipetted onto filter paper (ø 0.8 cm); the negative control papers were treated with a 50% sugar syrup; the positive control papers were treated with a Chlorimidazole (1 mg/ml). Incubation took place at 30°C in an incubator. After 48 hours, the zones of inhibition were determined with a magnifying lens. All experiments were carried out in triplicate.

Microdilution method

Also, the antifungal activities of the essential oils were assessed using a modified version of the microdilution techniques described by Drummond and Waigh (2000). The antifungal assay was performed by using a sterile 96-well plate and the Minimal Inhibitory Concentration (MIC) and the Minimal Fungicidal Concentration (MFC) values were determined. All the assays were prepared using aseptic conditions. The growth of *A.apis* was inspected visually. Sterile Sabouraud Dextrose Broth (SDB) was used as a growth medium in the assay. A positive and a negative control were used in each plate in order to ascertain the viability of the fungal culture and the sterility of the working

conditions and solutions. Each essential oil was subjected to serial dilutions in descending concentrations starting from a concentration of 10% and finishing with a concentration of 0.08%. The microtiter plates were wrapped in sterile tinfoil in order to prevent contamination and then were incubated at 30°C for 7 days. The assays were inspected visually: a clear solution indicated absence of growth, while visual indication of mycelia indicated microbial growth or absence of inhibition. The MIC was determined to be the lowest concentration of essential oils that resulted in the inhibition of the visible growth of *A. apis* after a seven days incubation, compared with the growth control. All the essential oils that demonstrated inhibitory activities were further tested for fungicidal activity. Namely, a sample from each well that tested positive for inhibitory activity was inoculated on fresh sterile Sabouraud Dextrose Agar plates and incubated an additional 5-7 days. Absence of colonies/mycelia was regarded as positive for fungicidal activity, while growth of colonies/mycelia was regarded as negative. All the tests were performed in triplicate.

Results and discussion

Agar diffusion test

The lack of any effective control agent for chalkbrood has resulted in an increased interest in the investigation of alternative control strategies. Essential oils and oleoresins of many plants are known to exhibit significant antimicrobial activity against a wide spectrum of microorganisms. Out of the ten essential oils tested, all exhibited antifungal activity against *A. apis*, with zone of inhibition (ZI) ranging from 6.3 mm to 0.3 mm (Table 1). All essential oils proved to have a fungistatic effect (Table 1). The minimal inhibitory concentration of all tested essential oils were 1.25%. These were the lowest concentrations showing a repression of fungi growth. Higher concentrations of tested essential oils 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 all essential oils in the experiment. The zone of inhibition produced by the Chlorimidazole was 8.0 mm, which was little more than to the zone of inhibition produced by citronelol (6.3 mm). Overall, this study shows that citronelol has the best results against *A. apis* with zone of inhibition of 6.3±0.8 mm (1.25 % of citronelol).

Table 1. Antifungal activity (inhibition zone, mm) of essential oils against *A. apis* by the disc diffusion method. Data are presented as mean±SD.

concentration [%]	essential oils									
	citral	geraniol	citronelol	cinamaldehid	thymol	eucalyptus oil	lemongrass oil	fennel oil	clove oil	cedar oil
0.5	2.2±0.2	1.9±0.3	3.2±0.5	0.9±0.1	3.6±0.6	3.0±0.8	4.1±0.7	0.7±0.2	2.9±0.3	3.1±0.6
1.25	5.9±0.6	4.6±0.4	6.3±0.8	2.8±0.6	6.1±0.8	5.7±0.6	6.0±0.3	2.7±0.6	5.5±0.5	5.9±0.7
5	3.4±0.4	3.0±0.8	4.2±0.9	1.1±0.2	3.2±0.7	3.5±0.6	4.2±0.4	1.1±0.3	3.2±0.8	3.0±0.6
7.5	2.0±0.8	2.1±0.7	3.2±0.5	0.5±0.2	2.5±0.4	2.1±0.6	3.3±0.5	0.3±0.1	2.1±0.7	2.4±0.8
Negative control (50% sugar syrup)	0.0±0.0									
Positive control (Chlorimidazole 1 mg/ml)	8.0±1.4									

Clove oil has been found to effectively inhibit the growth of many Gram-negative bacteria, several species of *Penicillium* (Azzouz and Bullerman, 1982), as well as the spore-forming *Clostridium botulinum* (Ismaiel and Pierson, 1990). Cinamaldehid exhibits activity against mycotoxigenic moulds, *Penicillium* spp. and *C. botulinum*. Thymol, a major component of thyme oil, is highly active against *Aspergillus parasiticus* (Buchanan and Shepherd, 1981), *Staphylococcus aureus* (Karapinar and Aktug,

1987) and *C. botulinum*. The literature cites references which have investigated the susceptibility of a broad range of fungi to natural products, including citral in lemon peel (Rodov *et al.,* 1995), sorbic acid and essential oils (Kubo and Lee, 1998).

Microdilution method

Recently, the scientific interest into biological properties of essential oils and natural products in general has been increased as a series of molecules with antimicrobial activity have been found in plants. Active research on the use of the biologically active secondary metabolites present in essential oils of plants such as phenols, flavonoids, alkaloids, terpenes, tannins and others, has been seen as a potential alternative to the conventionally used antifungal agents, and as means to control pathogenic fungi and fungal contamination. The response of different essential oils usually depends on the fungal species tested and may include ranges from resistant to various degrees of susceptibility (Amini et al., 2012). *In vitro* antimicrobial activity was investigated by the microdilution method and MIC and MFC was determined. Each essential oil was subjected to serial dilutions in descending concentrations starting from a concentration of 10% and finishing with a concentration of 0.08%. The data relating to these activity is summarized in Table 2. Antimicrobial activity (MIC and MFC) was observed in all essential oils included in the study. Our findings showed that citronelol has the best resultst against A. apis, with MIC of 0.3125% and MFC of 1.25%, following with thymol, with MIC of 0.625% and MFC of 1.25%.

Table 2. The antifungal assay performed by using a sterile 96-well plate and the Minimal Inhibitory Concentration (MIC) (concentration, %) and the Minimal Fungicidal Concentration (MFC) (concentration, %) of ten essential oils tested in this study.

essential oils	MICs (%)	MFCs (%)
citral	0.625	2.5
geraniol	1.25	2.5
citronelol	0.3125	1.25
cinamaldehid	1.25	5
thymol	0.625	1.25
eucalyptus oil	0.625	0.5
lemongrass oil	0.625	2.5
fennel oil	1.25	5
clove oil	1.25	2.5
cedar oil	0.625	2.5
Negative control (50% sugar syrup)	0	0
Positive control (Chlorimidazole 1 mg/ml)	0.5	1

Since ancient times, essential oils have been used for domestic and therapeutic purposes; these oils possess broad-spectrum antimicrobial properties. Essential oils have been selectively used to treat various microbial infections (Deans, 1991; Hammer et al., 1998; Hili et al., 1997). The antimicrobial properties of essential oils suggest that these substances could be used to control *A. apis*. Recently, a few studies have demonstrated the activity of essential oils against honeybee pathogens. Natural antibiotics based on essential oils may represent alternatives to chemically synthesized antibiotics. It is important to control honeybee diseases with natural antibiotics because most honeybee by-products, such as honey, must be free from contaminants. We studied the *in vitro* efficacy of ten essential oils against *A. apis.* Out of the ten selected essential oils, all were found to be effective. Our findings demonstrate that these essential oils might not only have the potential to control *A. apis* infection but may also represent possible alternatives to the use of control Chalkbrood. Our data (Table 2) clearly demonstrate that all of the essential oils used inhibited the growth of *A. apis*, which

is in contrast to the results o earlier studies on essential oils by Colin et al., 1989; Calderone et al., 1994; Larran et al., 2001.

Conclusions

All tested substances shows *in vitro* fungistatic and fungicide potential and can be considered as potential alternative active agents for prevention and control of chalkbrood disease without the use of antibiotics. In conclusion, the results presented in this paper demonstrate that most of the selected essential oils have potential antifungal activities. The use of non-toxic natural compounds could represent a natural alternative to the use of synthetic antibiotics in the control of Chalkbrood, which should therefore reduce antibiotic resistance and the levels of antibiotic residues. Further research must be conducted on these essential oils to isolate the active ingredients that kill *A. apis*.

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