Stonebrood and chalkbrood in *Apis mellifera* causing fungi: *in vitro* sensitivity to some essential oils

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ABSTRACT

Aim of the present study was to evaluate the *in vitro* antimycotic activity of 17 chemically defined essential oils (EOs) both alone and as a mixture, against agents responsible for stonebrood caused by *Aspergillus flavus*, and chalkbrood caused by *Ascosphaera apis* in European honeybees. *Cinnamomum zeylanicum* yielded the lowest MIC value against *A. flavus,* but was not effective against *A.*

apis, while *Litsea cubeba* and *Pelargonium graveolens* appeared to be effective against all checked fungi. *Aspergillus niger* showed the lower sensitivity. Two mixtures composed by *L. cubeba*, *C. zeylanicum* and *Cymbopogon flexuosus* (M1) and by *L. cubeba*, *C. zeylanicum*, *P. graveolens* and *C. flexuosus* (M2), respectively, were tested, both resulting effective. The components of M1 showed a synergistic effect.

The use of mixtures allowed to decrease the total amount of EOs. The use of these products could be of interest for an alternative natural approach in honeybee disease management.

1. Introduction

Bees play an important role in agriculture, since most crop plants for global food production are pollinated in whole or part by European honeybee (*Apis mellifera* L.). Bacterial, fungal, viral and parasitic diseases can affect the productivity of honeybees and their survival. Two fungi belonging to genera *Aspergillus* and *Ascosphaera* are known to cause stonebrood and chalkbrood, respectively. These two fungal diseases are associated with serious problems in honeybee brood and have a worldwide distribution (Jensen et al. 2013). In colonies show- ing symptoms of stonebrood or chalkbrood, dead and mummified larvae (currently called 'mummies') are visible in the brood cells (Foley et al. 2014; Sarwar 2016).

Species of the genus *Aspergillus* are cosmopolitan filamentous fungi. They live as sapro- phytes in soil and can be facultative pathogens infecting plants, insects, birds and mammals, including humans (Lopes et al. 2015). *Aspergillus flavus* but also *Aspergillus fumigatus*, *Aspergillus niger* and occasionally other *Aspergillus* species are aetiological agents of stone- brood (Foley et al. 2014; Lopes et al. 2015). The infection occurs by the ingestion of conidia and through the cuticle, so adults and larvae as well as pupae can be infected (Lopes et al. 2015). Overt stonebrood symptoms are relatively common in larvae but they are a rare condition in adults (Batra et al. 1973). *Aspergillus* spp. conidia ingested by bee larvae remain in the gut until the first defecation event prior to pupation, then they hatch, and hyphae grow rapidly to form a collar-like ring near the head (Seyedmousavi et al. 2015). The larvae dye and become black, covered with powdery fungal spores, and difficult to crush, such as small stones. Hence, the name stonebrood of the disease is originated (Sarwar 2016). Worker bees are unable to remove stonebrood mummies from the cells (Seyedmousavi et al. 2015). In several countries, stonebrood is a notifiable disease that has to be reported to the author- ities if it occurs (Jensen et al. 2013; Seyedmousavi et al. 2015).

Chalkbrood is caused by the fungus *Ascosphaera apis* (Jensen et al. 2013). Chalkbrood disease develops when larvae ingest *A. apis* spores. After which mycelium growth kills the larvae and leads to new spore formation on their cuticle, eventually filling the entire cell (Vojvodic et al. 2011; Sarwar 2016). The disease is either transmitted within the colony via contaminated wax and worker bees, or between colonies via contaminated pollen on flowers, or handling by beekeepers (Vojvodic et al. 2011). Infected larvae are filled by cotton-like mycelium of *A. apis*, they dye and resemble a chunk of chalk (hence, the name chalkbrood). Alternatively, they may turn grey or pale yellow and finally black if sexual reproduction occurs. Some sponge-like chalkbrood mummies may appear at the entrance and at the bottom of the hive because hygienic worker bees can easily remove mummies from the cells and expel them from the hive (Sarwar 2016).

Essential oils (EOs) are more or less volatile substances with more or less odorous impact, produced either by steam distillation or dry distillation or by means of a mechanical treatment from one single botanic species (Schmidt 2009). They, as other plant extracts, show antibac- terial, antifungal and antiviral properties and have been screened worldwide as potential sources of novel antimicrobial compounds. EOs have been used in beekeeping for control of *Paenibacillus larvae, Varroa destructor* and *Nosema* spp. (Maggi et al. 2011; Damiani et al. 2014) and have been *in vitro* assayed against *P. larvae, V. destructor* and *A. apis* (Eguaras et al. 2005; Kloucek et al. 2012; Ansari et al. 2016a, 2016b). Even if an antagonistic effect against

A. apis has been reported by gut bacteria in *Apis mellifera carnica*, also (Omar et al. 2014), accurately selected EOs would appear to be interesting candidates to control such agents.

The aim of the present paper was to evaluate the antimycotic activity of some chemically defined EOs and of two mixtures against field isolates of *A. flavus, A. niger* and *A. apis*.

2. Results and discussion

The chemical composition of tested EOs is reported in Table S1.

Selected EOs showed a variable degree of antimycotic activity at tested dilutions against the different fungal species, with MICs ranging from >0.5 to 0.075%. In particular, *C. zeylan- icum* yielded the lower MIC (0.075%) against *A. flavus* but was not effective against *A. apis. Litsea cubeba* and *Pelargonium graveolens* appeared to be effective against all checked fungi and, in particular, they were the only effective EOs against *A. apis* yielding the lower MICs (0.025%). *A. niger* showed the lower sensitivity when assayed against all selected EOs.

C. zeylanicum and *C. flexuosus,* active versus aspergilli, yielded a MIC value >0.5% against *A. apis*. The selected mixtures resulted effective with an overall MIC of 0.06% of the composing EOs.

The fractional inhibitory concentration index (FICI) indicated that all the components of M1 had a synergistic effect for all fungi, while in M2 *L. cubeba* and *P. graveolens* showed an additive or indifferent effect (FICI = 0.6) versus *A. apis.* More detailed data about the efficacy of the selected EOs are presented in Table S2.

Even if chalkbrood is more common than stonebrood, this latter should not be underes- timated. Considered the ubiquity of causative agents, attention should be paid in control of *Aspergillus* spp. spores in the environment of hives, considered that Aspergilli can be involved in both human mycotoxicoses and allergies, also (Seyedmousavi et al. 2015).

A. flavus and *A. niger* are considered the causative agents of stonebrood, while *A. fumigatus* seems to play a minor role, failed to induce mortality in experimentally infected larvae (Foley et al. 2014). This mould was not cultured from examined fungal brood clusters used in the present work. For these reasons, this fungal species was not included in the test.

In beekeeping, the inhibitory activity of several EOs has been evaluated against *A. apis* (Colin et al. 1989; Larran et al. 2001; Dellacasa et al. 2003; Kloucek et al. 2012; Saleem et al. 2015; Ansari et al. 2016b) with different results, probably based on the composition of exam- ined EOs and on different testing procedures. In the present report, *L. cubeba* and *P. grave- olens* only showed a good efficacy against chalkbrood causing fungus, probably due to their high amounts of nerale and geraniale (*L. cubeba*) and geraniol and citronellol (*P. graveolens*) (Calderone et al. 1994; Chantawannakul et al. 2005; Ansari et al. 2016b), respectively. For *L. cubeba*, it is worthy to note how the product used by Ansari et al. (2016b) was highly effective,

even if its composition (citral 72%) was noticeably different from the *L. cubeba* EO tested by us. The same Authors also refer a good activity of a *F. vulgare* EO rich in trans-anethole and estragole against *A. apis*, while the analogous EO used in our study was rich in E-anethol and fenchone, and was not effective. Furthermore, the results of our study are not in agreement with literature data for *C. flexuosus* which did not show a strong anti *A. apis* effect (MIC > 0.5%), despite its high nerale and geraniale content.
EOs rich in thymol (*T. vulgaris*) and carvacrol (*O. vulgare*) did not yield a good antimycotic effect (Colin et al. 1989).
To the best of our knowledge, stonebrood causative agents have not been investigated in this sense. These fungal species appeared to be more sensitive to EOs. In addition to *L. cubeba* and *P. graveolens* in fact, *C. zeylanicum*, *C. flexuosus*, *I. verum*, *O. vulgare* and *T. vulgaris* EOs a good activity against tested Aspergilli. Studies on fungi associated with food spoilage demonstrated that cinnamon leaf volatile oil was found to be 100% effective against *A. niger*, *A. flavus* (Singh et al. 2007). Lemongrass resulted also active for protection of foodstuffs against storage fungi (Mishra & Dubey 1994). In particular, the antifungal action of *L. cubeba* EO has been reported against both *A. niger* (Gogoi et al. 1997) and *A. flavus* (Li et al. 2016). All the above-mentioned EOs used by us resulted active versus Aspergilli. For this fungal species, literature data about the activity of *Lamiaceae* agree with our findings. EOs from *O. vulgare* and *T. vulgaris* have been largely employed, considered their strong antimycotic action, mostly due to their high content of both carvacrol and thymol (Šegvić Klarić et al. 2007; Zabka et al. 2014; Gendy et al. 2015). However, this latter compound is integral part of acaricides and the residues represent a serious aspect since they can persist at high levels for several months in brood wax (Bogdanov et al. 1998; Carayon et al. 2014). Furthermore, these monoterpenoids can affect phototaxis in treated bees (Alayrangues et al. 2016).
For these reasons, when setting on EOs mixtures, plants belonging to families apart from Lamiaceae were chosen. These formulations would allow decreasing the total amount of EOs, minimising possible effects of toxicity for bees. The importance of using natural products non-toxic for honeybees, as an interesting alternative to synthetic drugs administration to control chalkbrood has been advanced by other Authors, also Ansari et al. (2016b). At the best of our knowledge, the present work is the first to test EOs mixtures which could be suitable in controlling both chalkbrood and stonebrood disease agents, allowing at the same time to use lower EOs amounts. This aspect would be relevant, considered that European directive relating to honey (2001/110) forbids any additions to this product that may change its natural taste.

3. Conclusions

The present paper firstly describes the use of EOs mixtures against *A. flavus, A. niger* and *A. apis,* indicating some of them as interesting candidates to control these agents.
Both mixtures showed a good efficacy against all tested fungi, in particular M1 showed a complete synergy of all the components versus selected fungal agents.
The use of these products could be of interest for an alternative approach in honeybee management, so a further step of the present work would be an *in vivo* assay to verify its practical feasibility.

Disclosure statement
No potential conflict of interest was reported by the authors.

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