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Short communication

Bay laurel (*Laurus nobilis*) as potential antiviral treatment in naturally BQCV infected honeybees

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ABSTRACT

Viral diseases are one of the multiple factors associated with honeybee colony losses. Apart from their innate immune system, including the RNAi machinery, honeybees can use secondary plant metabolites to reduce or fully cure pathogen infections. Here, we tested the antiviral potential of *Laurus nobilis* leaf ethanolic extracts on forager honeybees naturally infected with BQCV (Black queen cell virus). Total viral loads were reduced even at the lowest concentration tested (1 mg/ml). Higher extract concentrations (≥5 mg/ml) significantly reduced virus replication. Measuring *vitellogenin* gene expression as an indicator for transcript homeostasis revealed constant RNA levels before and after treatment, suggesting that its expression was not impacted by the *L. nobilis* treatment. In conclusion, plant secondary metabolites can reduce virus loads and virus replication in naturally infected honeybees.

Honeybee colony losses are driven by multilevel interactions of biotic and abiotic factors including environment, diseases and pesticides (Potts et al., 2010). Among the biotic factors, viruses (*e.g.*, Acute bee paralysis virus – ABPV, Black queen cell virus – BQCV, Deformed wing virus – DWV and Israel acute paralysis virus – IAPV) have been shown to be important contributors, especially when multiple stress factors act cumulatively (Berthoud et al., 2010; Chen et al., 2014; Cox-Foster et al., 2007; Dainat et al., 2012). Black queen cell virus (BQCV) is a positive single-stranded RNA virus belonging to the family of *Dicistroviridae* (Aubert et al., 2008). It infects honeybees worldwide and is a common infectious agent in larvae, pupae and adult bees (Chen et al., 2004). The main site for BQCV infection is the gut of the honeybee, but also includes queen ovaries (Chen et al., 2006). Although it usually occurs as an asymptomatic infection (Anderson and Gibbs, 1988), its genome copy number can be significantly elevated by sublethal doses of pesticides (Doublet et al., 2015; Locke et al., 2012), co-infections with *Nosema ceranae* (Doublet et al., 2015) or during winter (Dainat et al., 2012), thus reducing honeybee survival. Moreover, BQCV was detected with higher viral load in CCD (Colony Collapse Disorder) affected bees (Johnson et al., 2009) and was also associated with colony death by preventing queen replacement after queen loss (DeGrandi-Hoffman and Chen, 2015). BQCV is transmitted by vertical and horizontal routes and is readily detected in nurse and forager bees (Chen et al., 2006; Tentcheva et al., 2004). However, no clear disease-specific clinical symptoms have been described for adult honeybees (Aubert et al., 2008). BQCV infection of queen larvae may result in larval death.

Currently, no chemotherapeutic agent exists that cures honeybee viral diseases. However the ribonucleic analog ribavirin, used as an antiviral drug in human viral infections, showed some efficiency against DWV, BQCV, and Sacbrood virus (SBV), though further studies are necessary to establish if ribavirin is safe and can improve colony health (Freiberg, 2012). Fedorova et al. (2011) focused on the effects of artificial ribonucleases on the Acute bee paralysis virus (APBV), as they can inactivate the virus *via* genomic degradation. This study promotes the use of honeybees and their viruses as a new *in vivo* test system suitable for the general screening of antivirals.

Several plant extracts (isolated plant compounds or propolis extracts) are effective against certain bee pathogens such as *e.g.*, *Paenibacillus larvae* (Antúnez et al., 2008; Boligon et al., 2013; Damiani et al., 2014; González et al., 2015; Hernández-López et al., 2014; Mihai et al., 2012) and *Nosema* sp. (Damiani et al., 2014; Porrini et al., 2011), but none of them have been tested on honeybee viruses (reviewed in Erler and Moritz, 2015). A few of these

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studies already aimed at assessing the antibiotic effect of plant-derived compounds extracted from propolis, *Artemisia absinthium* and *Laurus nobilis*, directly in honeybees (Antúnez et al., 2008; Damiani et al., 2014; Erler and Moritz, 2015, Porrini et al., 2011). *Laurus nobilis* extracts proved to have a high antibiotic potential at well tolerated *in vivo* doses and were highly efficient in reducing the development of *N. ceranae* spores in the honeybees' midgut. Furthermore, the extracts exhibited antimicrobial activity against *P. larvae* (Damiani et al., 2014; Porrini et al., 2011) and were proposed to be a source of potential antivirals (Ertürk et al., 2000; Loizzo et al., 2008), although the effects on honeybee viruses were never directly tested. The chemical composition of *Laurus* leaves is only partly discovered (Conforti et al., 2006; El et al., 2014). Nonetheless, the biological effects (*e.g.*, antioxidant, antimicrobial and antitumor activity) of some of its compounds, including polyphenols (Vardapetyan et al., 2014), essential oils (Chmit et al., 2014) and polysaccharides (Chmit et al., 2014) have already been identified. High amounts of bioactive compounds (*e.g.*, antimicrobial, antioxidative) were extracted in alcohol (ethanol or methanol) (Dai and Mumper, 2010; Damiani et al., 2014; Muñiz-Márquez et al., 2014). The ethanolic extraction has the advantage of offering products which are safe and stable for *in vivo* treatments (Dai and Mumper, 2010).

Here, we aim to test the antiviral potential of *L. nobilis* ethanolic extracts using naturally infected honeybees. Measuring the antiviral pharmacodynamics using naturally BQCV-infected honeybees presents a time-saving and low-stress tool (artificial infections induce stress in bees) for testing bioactive compounds in honeybee diseases.

Dry *Laurus nobilis* leaves of Greek origin (Fuchs, Dissen a.T.W., Germany) were ground to powder. 40 g of powdered leaves were macerated in the dark in 100 ml 80% ethanol at room temperature under continuous agitation. After five days, the ethanol was removed using a rotary evaporator (Rotavapor® R-215, Büchi, Germany) (Porrini et al., 2011). The remaining extract (10.92 g, color: dark green, consistency: creamy) was kept at 4 °C until further usage. The content of bioactive compounds of the *L. nobilis* extract was characterized by total polyphenols, total flavonoids and flavone/flavonol determination (Online Resource Material and Methods).

Naturally infected honeybees have a range of BQCV abundances. As we wanted to select a colony with high BQCV abundance, bees from several colonies had to be screened for high BQCV genome copy numbers. Natural BQCV infection was verified by screening adult worker bees (nurses collected from the brood nest of the test colonies and forager bees) of four colonies (free of *Nosema* sp., *Varroa destructor* and DWV, qPCR verified; Paldi et al., 2010) at the university apiary (USAMV Cluj-Napoca), using BQCV-specific quantitative real-time PCR (qPCR). Bees were treated against *Varroa* using Varachet Forte (containing Amitraz and Tau-fluvalinate) fumigation according to the manufacturer's instructions. Dilution series from plasmid clones, containing the target BQCV genome fragment, were used to establish standard curves (range: $1 \times 10^3 - 1 \times 10^7$ BQCV genome copies, $r^2 = 0.94$) to estimate virus genome copy number per 4 μg RNA for each single bee (for qPCR details see below and Locke et al., 2012). BQCV genome copy numbers of the tested individuals were in the range estimated for adult bees in Locke et al. (2012). All tested individuals were naturally infected with colony-specific and worker type-specific BQCV quantities, with forager bees showing the highest BQCV values (Online Resource Fig. S1). From the four tested colonies, the colony (Colony 3) with the highest BQCV load and lowest BQCV load variance was selected for collecting the experimental forager bees (Online Resource Fig. S1). Moreover, the BQCV prevalence in Romania was 100% in screened adult bees, during a two year country-wide survey monitoring the health status of honeybees (C.O. Coroian, person. comm.) concurrent to our study.

For the experiment, pollen-carrying forager bees (*Apis mellifera carnica*, approx. age: 14–21 days) were caught at the hive entrance of the queen-right colony. Twenty summer (first week of August) forager bees were randomly selected for each treatment group and fed *ad libitum* for 10 days with 1, 5 or 10 mg *Laurus* extract in 1 ml sucrose solution (50%) in hoarding cages (size: 14.5 × 8 × 10.5 cm), kept in an incubator at 34 °C, 60% relative humidity and full darkness (according to Williams et al., 2013). Total dispersion of the extracts in the sugar solution was obtained by brief sonication, avoiding problems of solubility. The mixture was freshly supplied every day and weighed before and after 24 h feeding to estimate the average amount of food intake (extract ingested/honeybee/day). Control bees were kept under the same conditions (20 bees/cage) and fed *ad libitum* with sucrose solution (50%). At the end of the feeding experiment, all bees were frozen in liquid nitrogen and kept at −80 °C until further processing.

Viability tests were used to estimate the toxicity of the differently concentrated extracts on BQCV infected bees. 9–10 bees were placed in sterile plastic cups (100 ml) and fed *ad libitum* for 10 days with 1, 5 or 10 mg *Laurus* extract in 1 ml sucrose solution (50%). Each treatment group was analyzed in quintuplets. Experimental design was mainly based on the methodology of Damiani et al. (2014).

The extent of each viral infection was assessed by qPCR using 5–6 forager bees per treatment group. RNA extraction, cDNA synthesis (Tetro cDNA Synthesis Kit, Bioline), qPCR, estimation of PCR efficiencies (range: 1.83–1.98) and determination of relative gene expression were done according to methods described in Aurori et al. (2014) with some slight modifications of the amplification protocol (initial denaturation: 10 min at 95 °C; 40 amplification cycles: 95 °C for 10 s, 56 °C for 10 s, 72 °C for 20 s). The relative overall BQCV virus load (no strand-specific reverse transcription, including negative and positive strand) was assessed using the primers described by Locke et al. (2012). Negative strand quantification, an indicator of the replication extent, was measured performing strand-specific cDNA synthesis (30 min at 45 °C), using Tag-BQCV-sense primers: Tag-BQCV-qF-7893 (5′-AGCCTGCGCACCGTGGAGTGGCGGAGATGTATGC-3′), Tag-BQCV-qR-8150 (5′-AGCCTGCGCACCGTGGGGAGGTGAA-GTGGCTATATC-3′) (Locke et al., 2012; Yue and Genersch, 2005). *Vitellogenin* gene expression (Simone et al., 2009) was measured as an indicator of the general fitness of the tested honeybees (Amdam and Omholt, 2002), as vitellogenin is the main protein of the honeybee haemolymph and a key protein, being involved in stress resistance, immunity, hormonal regulation, and promoting longevity in honeybees (Amdam and Omholt, 2002; Nunes et al., 2013). As such, *vitellogenin* is an excellent marker to test if the bees' metabolism/transcription might be affected by such antiviral treatments and to ensure that the tested concentrations are safe for honeybees. *Ribosomal protein S5* (*RPS5*) was used to standardize *vitellogenin* gene expression levels and virus loads among individuals and feeding groups (Evans, 2006): *RPS5* was the only stable reference gene among those tested (β-*actin, GAPDH, peptidylprolyl isomerase A*), fulfilling the criteria of a reference gene according to Pfaffl et al. (2004). BQCV virus genome copy number per bee and treatment group are given in Table S2.

Extract feeding and qPCR data were tested for normality with Shapiro-Wilk's *W* test and Kolmogorov-Smirnov test, and log-transformed if they did not match normality. *L. nobilis* treatment effects between groups were tested using One-way, or Kruskal-Wallis ANOVAs depending on data distribution. Viability tests were analyzed using the Kaplan-Meier survival function. All replicates per treatment group and control were pooled to get a sufficient amount of individuals (in total 195 bees) to perform the Kaplan-Meier tests, as in some groups no individual died during