Essential oils and *Beauveria bassiana* against *Dermanyssus gallinae* (Acari: Dermanyssidae): towards new natural acaricides

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ABSTRACT

Essential oils (EOs) and entomopathogenic fungi such as *Beauveria bassiana* (*Bb*) strains have the potential to be used as alternative insecticides and acaricides for controlling ectoparasites as *Dermanyssus gallinae*. These compounds have some limitations in their use: the acaricidal effect of EOs is rapid, but short-lived, whilst that of *Bb* is delayed, but long-lived. To evaluate the effect of both compounds combined against *D. gallinae*, the non-toxic dose of *Eucalyptus globulus, Eucalyptus citriodora*, *Thymus vulgaris* and *Eugenia caryophyllata* essential oils were firstly calculated for "native" strains of *Bb*. Subsequently, the effects of the combination of selected EOs

with *Bb* against nymph and adult PRMs was assessed. EO concentrations ranging from 0.0015 to 8% v/v (i.e., nine double dilutions) were used to evaluate their effect on germination, sporulation and vegetative growth rates of native strains of *Bb*. A total of 1,440 mites (720 nymphs and 720 adults) were divided into three-treated group (TGs) and one control group (CG). In TGs, mites were exposed to *Bb* in combination with the selected EO (TG1), EO alone (TG2) or *Bb* (TG3) alone. In the CG mites were exposed to 0.1% tween 80 plus EO solvent (CG). *E. globulus* and .*E. citriodora* were toxic for *Bb* in concentrations higher than 0.2% and 0.003% respectively, whilst *E. caryophyllata* and *T. vulgaris* were toxic at all concentrations tested against *Bb.* Based on the results of the toxicity assays against *Bb*, *E. globulus* was chosen to be tested as acaricide resulting non - toxic for *Bb* at concentration lower than 0.4%. Increased mortality of *D. gallinae* adults was recorded in TG1 than those in other TGs from 4 days post-infection (T+4 DPI). A 100% mortality of *D*. *gallinae* was recorded in adults at T+9 DPI and at T+10 DPI in nymphs in TG1 and later than T+11 DPI in the other TGs. Used in combination with *E. globulus*, *Bb* displayed an earlier acaricidal effect towards both haematophagous *D. gallinae* stages. The combination of *B. bassiana* and *E. globulus* at 0.2% might be used for controlling arthropods of medical and veterinary importance as *D. gallinae.*

Keywords: Eucalyptus globulus; *Beauveria bassiana*; Combined treatment effect; Acaricide effect, *Dermanyssus gallinae*

1. Introduction

Dermanyssus gallinae (De Geer, 1778) (Acari: Dermanyssidae), known as the poultry red mite (PRM), is the most economically damaging ectoparasite of laying hens worldwide (Sparagano et al., 2014). This mite species displays relative plasticity in terms of host specificity, being associated primarily with birds, but also with mammals, including humans (George et al., 2015). Heavy infestations by this pest may cause severe damage to the poultry industry, varying from decreased

53 growth rates, egg production and feed conversion to high animal mortality (Sparagano et al., 2014).
54 In addition, *D. gallinae* can be a vector of microorganisms such as *Salmonella* spp., avian
55 spirochetes, and other pathogens of poultry (Sparagano et al., 2014; George et al., 2015). In
56 humans, *D. gallinae* is responsible for severe pruritic dermatitis, representing an occupational
57 hazard for poultry workers (George et al., 2015). The control of PRM still widely relies on the use
58 of synthetic acaricides such as organophosphosphates, carbamates and pyrethroids (Sparagano et
59 al., 2014). Nonetheless, resistance phenomena to these compounds have been reported in PRM
60 (Marangi et al., 2009; Sparagano et al., 2014) and misuse/abuse of the chemicals often results in the
61 presence of pesticide residues in the organs and tissues of poultry (Marangi et al., 2012). All of
62 these disadvantages have spurred the interest of the scientific community to study alternative non-
63 chemical compounds for controlling PRM.
64 In this respect, essential oils (EOs) and entomopathogenic fungi have been investigated for their
65 potential for PRM control (Kaoud, 2010; Ellse and Wall, 2014; Immediato et al., 2015). In
66 particular, EOs like *Eucalyptus globulus*, *Eucalyptus.citriodora*, *Eugenia caryophyllata* and *Thymus*
67 *vulgaris* have been tested as acaricide against *D. gallinae* and their ovicidal, repellent and biocidal
68 activities have been widely demonstrated (Kim et al., 2004; Kim et al., 2007; George et al., 2009;
69 George et al., 2010a; George et al., 2010b; George et al., 2010c; Ellese and Wall, 2014; Nechita et
70 al., 2015). However, their volatile nature resulted in a rapid (i.e., within 24h), but short-term
71 efficacy with a mortality rate depending on the concentration (Kim et al., 2004; Kim et al., 2007;
72 George et al., 2009; George et al., 2010a).
73 Similarly, entomopathogenic fungi, including *Beauveria bassiana* (*Bb*), have been widely tested
74 against different agricultural and pasture pests (Kaaya and Hassan, 2000; Kaoud, 2010; Hussain et
75 al., 2014). Recently, it has been shown that "native" strains of *Bb* (i.e., strains isolated from the
76 environment or naturally infected hosts) were highly virulent against *D. gallinae* (Immediato et al.,
77 2015), as well as *Rhipicephalus sanguineus* (Cafarchia et al., 2015) and phlebotomine sandflies
78 (Amóra et al., 2009). However, the virulence of this fungus was not evident until 5 days post-

infection (DPI) with a mortality rate increasing according to the time of exposure, thus providing a delayed but long-term control due to *Bb* sporulation on dead mites (Maketon et al., 2008; Cafarchia et al., 2015; Immediato et al., 2015).

A combined action between entomopathogenic fungi and EOs against ectoparasites is herein tested. However, since EOs display dose-dependent fungicidal effects (Pinto et al., 2009; Tabassum and Vidyasagar, 2013), the threshold concentration value of their toxicity to entomopathogenic fungi needs to be evaluated.

Due to the lack of data on the toxicity of *E. globulus*, *E. citriodora*, *E. caryophyllata* and *T. vulgaris* for *Bb,* this study aims to evaluate the degree of toxicity of different concentrations of these essential oils against a "native" strain of *Bb,* and to assess the effect of *Bb* in combination with non-toxic dose of EOs for *Bb*, against the hematophagous *D. gallinae* life stages (i.e., nymphs and adults).

2. Materials and method

2.1. Beauveria bassiana strain origin and identification

A "native" strain of *Bb* (CD1123) was obtained from naturally infected ticks collected in a private dog shelter in Putignano (40°50'N, 17°07'E, 372 m a.s.l.), Bari, Italy and morphologically and molecularly identified as previously described (Cafarchia et al., 2015).

2.2. Essential oils

E. globulus, *E. citriodora*, *E. caryophyllata* and *T. vulgaris* (Erboristeria Magentina - Poirino, Turin, Italy) were selected on the basis of their previous defined efficacy against *D. gallinae* (Kim et al., 2004; Kim et al., 2007; George et al., 2009; George et al., 2010a; George et al., 2010c; Ellese and Wall, 2014). In particular, a stock solution in 5% in ethanol (Et-OH) was prepared for each EO and known aliquots were added to 20 ml of Potato Dextrose Agar (PDA) in order to obtain concentrations ranging from 0.0015 to 0.8% (i.e., nine double dilutions) (Maciel et al., 2010).

105 Aliquots of 5% Et-OH solution were added to 20 ml of PDA order to obtain control plates for each
106 EO concentration tested.
107 *2.3. Toxicity assays of essential oils against Beauveria bassiana*
108 The toxicity of EOs against *Bb* was assessed as previously reported for other EOs (Tamai et al.,
109 2002) and the following mathematical model was applied to evaluate the degree of toxicity:
110 $T = 20[VG] + 80[SR]/100$ (1)
111 Where:
112 T is the degree of toxicity useful for the classification of the product;
113 VG is the percentage of vegetative growth with respect to the control;
114 SR is the percentage of sporulation with respect to the control.
115 The product was classified, based on the T value, as: very toxic ($0 \leq T \leq 30$); toxic ($31 \leq T \leq 45$)
116 moderately toxic ($46 \leq T \leq 60$); non-toxic (i.e., compatible) ($T > 60$) (Tamai et al., 2002).
117 Vegetative growth (VG) was measured by placing a mycelial plug (i.e., 5 mm in diameter) onto the
118 centre of a 90 mm Petri dish containing PDA, with and without EOs solvent (control) and
119 measuring the diameter of the colonies after incubation at 25°C for 10 days (Achterman et al.,
120 2011). The sporulation was evaluated by collecting the spores from surface of fungi grown in the
121 PDA with and without the investigated EOs after 10 days incubation at 25°C.
122 Spores and mycelia were collected by scraping the surface of the plate with 4 ml of 20% tween 80
123 solution. The solution was filtered through sterile gauze to remove mycelia, and then centrifuged
124 (3,000 g × 5 min), washed twice in 1 ml of phosphate-buffered saline solution (PBS), and re-
125 suspended in 1 ml of PBS. Numbers of spores were determined by quantitative plate counts of
126 colony forming units (CFU)/ml on PDA after incubation at 25°C for 4 days and expressed as Log_{10}
127 of CFU/ml (Tamai et al., 2002; Achterman et al., 2011).
128 The effect of EOs on *Bb* germination was also measured culturing *Bb* in Sabouraud Dextrose Agar
129 (SDA) for 14 days at 25°C and collecting spores and mycelia as reported above. The obtained
130 solution was diluted in PBS in order to obtain an inoculum concentration of 10^7 conidia/ml, which

131 was evaluated by quantitative plate counts of CFU/ml in PDA. Finally, a total of 100 µl of the *B.*
132 *bassiana* spore suspension was cultured in PDA with and without different EOs concentrations and
133 incubated at 25°C for 14 days. The number of germinated spores were determined by counts of
134 CFU/ml on PDA and expressed as Log_{10} CFU/ml.
135 All the experiments were performed in duplicate and repeated three times on different days.
136
137 *2.4. Laboratory bioassay on* Dermanyssus gallinae
138 *2.4.1. Beauveria bassiana conidial infection suspension (CIS)*
139 The *Bb* strain used was maintained on PDA and kept at 4°C. The conidial infection suspension
140 (CIS) of *Bb* was obtained by culturing 15 strains on PDA for 3 weeks at 25°C. Conidia were
141 harvested by washing the plates with sterile distilled water containing 0.1% tween 80 and turbidity
142 was adjusted spectrophotometrically (Biosan DEN 1) to an optical density of 10 McFarland (1–5 ×
143 10^9 conidia/ml) as previously reported (Cafarchia et al., 2015; Immediato et al., 2015). The amount
144 of conidia was confirmed by quantitative plate counts of CFU/ml in PDA.
145
146 *2.4.2. EOs and concentrations*
147 On the basis of the results of the toxicity assay, a stock solution at 5% in ethanol (Et-OH) was
148 prepared and known aliquots were added in *Bb* CIS in order to obtain concentrations equal to its
149 non-toxic dose.
150
151 *2.4.3. Mite specimens*
152 Mites were collected from the egg-laying hen farm in Bitritto (41°03'00''N 16°50'00''E, 102 m
153 a.s.l.), Bari, southern Italy, three different times. The farm was naturally infested by the parasite and
154 no standard acaricidal treatments were conducted two months before the collection. Mites were
155 stored in sealed plastic bags and delivered to the Department of Veterinary Medicine, Unit of
156 Parasitology and Mycology, University of Bari, Italy. After morphological identification as *D.*

157 *gallinae* (Moss, 1968; Di Palma et al., 2012), mites were divided in two groups (i.e. nymphs and
158 adults) and then stored at 22±1°C to be used for the experiments within 24h of their collection.
159
160 *2.4.4. Laboratory bioassays and data analysis*
161 A total of 1,440 mites (720 for each stage) were tested. All bioassays consisted of four groups of
162 mites: three treated group (TGs) and one control group (CG). In TGs, mites were exposed to Bb
163 CIS in combination with one selected EO (TG1), with EO alone (TG2) and with *Bb* CIS alone
164 (TG3). In CG, mites were exposed to 0.1% tween 80 plus 0.2% EtOH (CG).
165 Each group was composed of three subgroups of twenty mites for reading convenience. Mites (i.e.,
166 nymph- - protonymphs and deutonymphs- and adult stages) were subjected to the same treatment
167 and placed into bioassay chambers composed of Petri dishes (60 mm diameter) containing filter
168 paper (Whatman N. 1, 10×10 mm Labor, 67 g/m2, Tecnochimica Moderna, Italy) of the same
169 diameter, soaked with 0.2 ml of treated or control solutions (see above). The mites were added soon
170 after the addition of the EOs and the control solution. The chambers were covered with a lid, sealed
171 with Parafilm® and incubated at 25°C (RH 80±5%). Mortality was evaluated daily until 100%
172 mortality was recorded in TGs. Mites were considered dead if they exhibited no movement after
173 repeated mechanical stimulation with an entomological pin by three different examiners. The dead
174 mites were not removed from the bioassay chambers except for one that was cultured on PDA to
175 verify the presence of viable fungus. All experiments were repeated in triplicate on different days.
176 *Statistical analysis*
177 The mortality rates of PRM at each time point of three independent experiments were compared
178 using Chi-square tests, with 5% significance ($p < 0.05$). Subsequently, the data was averaged and
179 the corrected mortality rate calculated using Schneider-Orelli's formula [i.e.,. Corrected mortality %
180 = (Mortality % in treated plot- Mortality% in control plot)/(100 - Mortality % in control plot) x 100]
181 (FAO, 2004). The correlation between the mortality of PRM nymphs and adults and time was

assessed using Pearson's (r) correlation coefficient. Comparisons of mortality data between TGs at each time point were performed using Chi-square or G test as appropriated.

Differences were considered significant if $p < 0.05$. The statistical analysis was performed using BioEstat (version 5.0; Mamirauá/CNPq, Belém, PA, Brazil).

3. Results

3.1. Toxicity assay of EOs against Bb

The results were expressed as mean values (± standard deviation) of six determinations representing three independent experiments (Achterman et al., 2011, Table 1). Vegetative growth (VG) was expressed as mean value (± standard deviation) of colony diameters after incubation and the sporulation and germination as mean values (± standard deviation) of Log10 CFU/ml (Tamai et al., 2002; Achterman et al., 2011- Table 1). *E. globulus* and *E. citriodora* are able to completely inhibit the germination of *Bb* by using concentrations higher than 0.4% and 0.2%, respectively, whereas *E. caryophyllata* and *T. vulgaris* inhibit the *Bb* germination at concentrations higher than 0.0015% and 0.05%, respectively. EOs affect both *Bb* vegetative growth and sporulation, being non-toxic only when *E. globulus* and *E. citriodora* are used at concentrations lower than 0.4% and 0.003%, respectively. *E. caryophyllata* and *T. vulgaris* are toxic at all concentrations tested (Table 1).

3.2. Laboratory Bioassay on D. gallinae

E. globulus 0.2% (v/v) was chosen to evaluate the effects in combination with *Bb* against *D. gallinae*, based on the results of toxicity assays against *Bb.* The *in vitro* effect against adults and nymphs of *D. gallinae* are reported in Table 2 and Figs. 1 and 2. No statistically significant differences between replicates were recorded ($p>0.05$) and the mortality rates in TGs increased according to the time of exposure, reveling a high positive correlation (Table 2. Figs. 1 and 2). In adults, a statistically higher mortality rate was recorded in TG1 than those in other TGs from 4 days post-infection (T+4 DPI) to T+ 9 DPI (Table 2, Fig.1). In nymphs, statistically significant

differences in mortality rate were recorded in TG1 than those in other TGs from T+7 to T+9 DPI (Table 2, Fig. 2). A 100% corrected mortality was recorded in adults and nymphs at T+8 and T+9 DPI, respectively, in TG1 and later than T+11 DPI in the other TGs (Figs 1-2). White fungal mycelium started to emerge on the surface of nymphs and adults of *D. gallinae* of TG1and TG3 at T+3 DPI. Fertile conidiophores appeared at T+5 DPI in TG1 and TG3, but only on the surface of dead mites (Fig. 3). Upon stereomicroscope observation and culture, no fungal growth was observed on mites of TG2 and CG.

4. Discussion

This study shows that the herein investigated EOs affect the biology of *Bb,* but their non-toxic concentrations, in combination with *Bb,* are effective against the PRM. In particular, in this study it has been shown that EOs might affect germination as well as the vegetative growth and sporulation of *Bb* according to their concentration. In the infection process, fungal germination is crucial step because the beginning of epizootics depends on the ability of the fungus to germinate on the host. In this study, the concentrations of 0.0015% *E. caryophyllata*, 0.05% *T. vulgaris,* 0.2% *E. citriodora* and 0.4% *E. globulus* might be considered suitable for testing the effect of *Bb* and EO against PRM, because they are unable to completely inhibit *Bb* germination. However, their negative effects (i.e., toxicity) on *Bb* vegetative growth and sporulation precluded their testing in the trial. In particular, although *E. globulus, E. citriodora*, and *E. caryophyllata* belong to the same plant family (i.e., Myrtaceae), *E. caryophyllata* was very toxic against *Bb* even at the lowest concentrations used (i.e., 0.0015%) because of the high concentration of eugenol (i.e., 73-83%; manufacture instruction, erboristeria Magentina - Poirino, Turin, Italy), a compound also active against *Candida* spp., dermatophytes and *Aspergillus* spp. (Chaieb et al., 2007; Pinto et al., 2009).

Similarly, the higher antifungal activity of *T. vulgaris* against *Bb* than that of .*E. citriodora* might be due to the different main compounds such as monoterpene hydrocarbons (i.e., limonene, α-pinene, *p*-cymene) and phenolic compounds (i.e., thymol) for *T. vulgaris* and oxygenated

monoterpenes (i.e., citronellal, citronellol and isopulegol) for .*E. citriodora* (Hudaib et al., 2002; Rustaiee et al., 2013; Tabassum and Vidyasagar, 2013; Ibrahim and El-Salam, 2015).

The finding that *E. globulus* was toxic for *Bb* at concentrations higher than 0.2%, compared to the other EOs, might be attributed to its major component such as 1,8-cineole, an oxygenated monoterpene, commonly called eucalyptol, which displays lower antifungal properties than phenolic compounds (Safaei-Ghomi, 2010; Hossain et al., 2016). Given that both *E. caryophyllata* and *T. vulgaris* displayed high antifungal activities neither were included in the trial. Although *E. globulus* and *E. citriodora* were non-toxic to *Bb* at concentrations lower than 0.2%, and 0.003%, respectively, only *E. globulus* was chosen to be tested in the bioassay because the acaricidal activity of EOs is dose-dependent (Kim et al., 2004; Kim et al., 2007; George et al., 2010a).

Interestingly, the results of this study suggest that *E. globulus* and *Bb* alone and/or in combination are highly active towards the nymphal and adult stages of *D. gallinae*. Previous studies have suggested that the acaricidal activity of *E. globulus* is dose-dependent and usually concentrations higher than 0.35 mg/cm² can cause a mortality of 100% in PRM within 24h (Kim et al., 2004). In this study, we used *E. globulus* at a concentration of about 0.16 mg/cm² that resulted in a mortality rate of 5% and 8% in adults and nymphs in 24h, respectively. These findings are in contrast with those previously reported, in which a PRM mortality of 15% was recorded using the same concentration of *E. globulus* (Nechita et al., 2015). *E. globulus* employed in this study being characterized by an higher 1,8-cineole content (manufacturer's instructions 85-95%, Erboristeria Magentina - Poirino, Turin, Italy) might have affected the lower acaricidal properties than that previously registered (Nechita et al., 2015). However, the proven low antifungal activity of 1,8-cineole (Safaei-Ghomi, 2010; Hossain et al., 2016) might have limited the effect on *Bb*, thus favoring the employment of this fungus in combination with the oil for the control of PRM. Since, the oil composition might vary according to the plant's geographic origin (Munoz-Bertomeu et al., 2007; Raal et al., 2007), seasonality (Flamini and Cioni, 2007), year of harvest (Chalchat et al., 2007), storage conditions (Chalchat et al., 2007) and method of oil extraction (Chiasson et al.,

2001), EOs with a high 1,8-cineole content might be employed for studying acaricide/insecticide effects in combination with entomopathogenic fungi. The employment of *Bb* alone against *D. gallinae* confirms our previous data causing 100% mortality after 12 DPI in adults and 14 DPI in nymphs (Immediato et al., 2015). In addition, the emergence of fungal mycelium after 3 DPI and conidiophores after 5 DPI on mites in TG1 and TG3 suggests that *E. globulus* does not affect the *Bb* viability and virulence, whereas 3 days is the suitable time for starting the infection process, as previously reported (Maketon et al., 2008; Immediato et al., 2015).

Compared to *E. globulus* or *Bb* alone, the association *Bb* and *E. globulus* showed a greater efficacy, mainly for PRM adult stage, which starts from T+4 DPI. On the contrary, the nymphal stage of *D. gallinae* seems to be less susceptible than adult stages to the association of *Bb* and *E. globulus* since statistically significant differences were detected ~~at~~ from T+7 DPI until at T+9 DPI among TGs. The presence of exuvium in the nymphal stage might limit the adhesion of conidia to the nymphs' cuticle, thereby prolonging the infection time as previously reported (Wu et al., 2014; Immediato et al., 2015).~~"~~ However, the combination of 0.2% *E. globulus* and *Bb* displayed an earlier acaricidal effect towards both *D. gallinae* stages. The *E. globulus* components (i.e., octopamine) affecting the nerve cord proteins of ectoparasites might inhibit their movements (Enan, 2001), thus enhancing the adhesion process of *Bb*.~~"~~ Nevertheless, the use of closed BR might have prolonged the persistence of the toxic effect of the *E. globulus* which coupled with the high virulence of *Bb* might have accelerated PRM' mortality in TG1 nymphs and adult in comparison with the other TGs (Kim et al., 2004).

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5. Conclusion

The results of the current study suggest that the combination of *Bb* 10^9 CIS and *E. globulus* at 0.2% v/v might be used against PRM's infestations in poultry houses. Nonetheless, further laboratory and field studies are required to determine the route of application and the frequency of treatment for

285 their use as bio-control agents in a pest management strategy, to reduce the hazards related to the
286 chemical products in poultry houses.
287
288 **Conflict of interests**
289 The authors declare that they have no competing interests.
290
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296
297 **References**
298 Achterman, R.R., Smith, A.R., Oliver, B.G., White, T.C., 2011. Sequenced dermatophyte strains:
299 growth rate, conidiation, drug susceptibilities, and virulence in an invertebrate model. Fungal
300 Genet. Biol. 48 (3), 335-341.
301 Amóra, S.S.A., Bevilaqua, C.M.L., Feijó, F.M.C., Silva, M.A., Pereira, R.H.M.A., Silva, S.C.,
302 Alves, N.D., Freire, F.A.M., Oliveira, D.M., 2009. Evaluation of the fungus *Beauveria*
303 *bassiana* (Deuteromycotina: Hyphomycetes), a potential biological control agent of *Lutzomyia*
304 *longipalpis* (Diptera, Psychodidae). Biol. Control. 50 (3), 329-335.
305 Cafarchia, C., Immediato, D., Iatta, R., Ramos, R.A.N., Lia, R.P., Porretta, D., Aguiar, F.L.,
306 Dantas-Torres, F., Otranto, D., 2015. Native strains of *Beauveria bassiana* for the control of
307 *Rhipicephalus sanguineus* sensu lato. Parasit. Vectors. 8, 80.
308 Chaieb, K., Hajlaoui, H., Zmantar, T., Kahla-Nakbi, A.B., Rouabhia, M., Mahdouani, K., Bakhrouf,
309 A., 2007. The chemical composition and biological activity of clove essential oil, *Eugenia*

caryophyllata (*Syzigium aromaticum L*. Myrtaceae): a short review. Phytother. Res. 21 (6), 501-506.

Chalchat, J.C., Ozcan, M.M., Dagdelden, A., Akgul, A., 2007. Variability of essential oil composition of *Echinophora tenuifolia* subsp. *sibthorpiana Tutin* by harvest location and year and oil storage. Chem. Nat. Compd. 43 (2), 225-227.

Chiasson, H., Belanger, A., Bostanian, N., Vincent, C., Poliquin, A., 2001. Acaricidal properties of *Artemisia absinthium* and *Tanacetum vulgare* (Asteraceae) essential oils obtained by three methods of extraction. J. Econ. Entomol. 94 (1), 167-171.

Di Palma, A., Giangaspero, A., Cafiero, M.A., Germinara, G.S., 2012. A gallery of the key characters to ease identification of *Dermanyssus gallinae* (Acari: Gamasida: Dermanyssidae) and allow differentiation from *Ornithonyssus sylviarum* (Acari:Gamasida: Macronyssidae). Parasit. Vectors. 5, 104.

Ellese, L., Wall, R., 2014. The use of essential oils in veterinary ectoparasite control: a review. Med. Vet. Entomol. 28 (3), 233-243.

Enan, E., 2001. Insecticidal activity of essential oils: octopaminergic sites of action. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 130 (3), 325-337.

Flamini, G., Cioni, P.L., 2007. Seasonal variation of the chemical constituents of the essential oil of *Santolina etrusca* from Italy. Chem. Biodivers. 4 (5), 1008-1019.

Food & Agriculture Organization., 2004. Resistance management and integrated parasite control in ruminants: guidelines. Module 1. Ticks: Acaricide Resistance: Diagnosis, Management and Prevention. FAO, Rome, Italy pp. 25-77.

George, D., Finn, R.D., Graham, K.M., Mul, M., Maurer, V., Moro, C.V., 2015. Should the poultry red mite *Dermanyssus gallinae* be of wider concern for veterinary and medical science? Parasit. Vectors. 8, 178.

George, D.R., Masic, D., Sparagano, O.A.E., Guy, J.H., 2009. Variation in chemical composition and acaricidal activity against *Dermanyssus gallinae* of four *Eucalyptus* essential oils. Exp. Appl. Acarol. 48 (1-2), 43-50.

George, D.R., Olatunji, G., Guy, J.H., Sparagano, O.A.E., 2010a. Effect of plant essential oils as acaricides against the poultry red mite, *Dermanyssus gallinae*, with special focus on exposure time. Vet. Parasitol. 169 (1-2), 222–225.

George, D.R., Sparagano, O.A., Port, G., Okello, E., Shiel, R.S., Guy, J.H., 2010b. Toxicity of plant essential oils to different life stages of the poultry red mite, *Dermanyssus gallinae*, and non-target invertebrates. Med. Vet. Entomol. 24 (1), 9-15.

George, D.R., Sparagano, O.A.E., Port, G., Okello, E., Shiel, R.S., Guy, J.H., 2010c. Environmental interaction with the toxicity of plant essential oils to the poultry red mite *Dermanyssus gallinae*. Med. Vet. Entomol. 24 (1), 1-8.

Hossain, F., Follett, P., Dang, V.K., Harich, M., Salmieri, S., Lacroix, M., 2016. Evidence for synergistic activity of plant-derived essential oils against fungal pathogens of food. Food Microbiol. 53 (Pt B), 24-30.

Hudaib, M., Speroni, E., Di Pietra, A.M., Cavrini, V., 2002. GC/MS evaluation of thyme (*Thymus vulgaris L.*) oil composition and variations during the vegetative cycle. J. Pharm. Biomed. Anal. 29 (4), 691-700.

Hussain, A., Rizwan-ul-Haq, M., Al-Ayedh, H., Al-Jabr, A.M., 2014. Mycoinsecticides: potential and future perspective. Recent Pat. Food Nutr. Agric. 6 (1), 45-53.

Ibrahim, S.Y., Abd El-Salam, M.M., 2015. Anti-dermatophyte efficacy and environmental safety of some essential oils commercial and *in vitro* extracted pure and combined against four keratinophilic pathogenic fungi. Environ. Health. Prev. Med. 20 (4), 279-286.

Immediato, D., Camarda, A., Iatta, R., Puttilli, M.R., Ramos, R.A.N., Di Paola, G., Giangaspero, A., Otranto, D., Cafarchia, C., 2015. Laboratory evaluation of a native strain of *Beauveria*

359 *bassiana* for controlling *Dermanyssus gallinae* (De Geer, 1778) (Acari: Dermanyssidae). Vet.
360 Parasitol. 212 (3-4), 478-482.
361 Kaaya, G.P., Hassan, S., 2000. Entomogenous fungi as promising biopesticides for tick control.
362 Exp. App. Acarol. 24 (12), 913-926.
363 Kaoud, H.A., 2010. Susceptibility of poultry red mites to entomopathogens. Int. J. Poult. Sci. 9 (3),
364 259-263.
365 Kim, S.I., Na, Y.E., Yi, J.H., Kim, B.S., Ahn, Y.J., 2007. Contact and fumigant toxicity of
366 medicinal plant extracts against *Dermanyssus gallinae* (Acari:Dermanyssidae). Vet. Parasitol.
367 145 (3-4), 377-382.
368 Kim, S.I., Yi, J.H., Tak, J.H., Ahn, Y.J., 2004. Acaricidal activity of plant essential oils against
369 *Dermanyssus gallinae* (Acari: Dermanyssidae). Vet. Parasitol. 120 (4), 297-304.
370 Maciel, M.V., Morais, S.M., Bevilaqua, C.M., Silva, R.A., Barros, R.S., Sousa, R.N., Sousa, L.C.,
371 Brito, E.S., Souza-Neto, M.A., 2010. Chemical composition of *Eucalyptus* spp. essential oils
372 and their insecticidal effects on *Lutzomyia longipalpis*. Vet. Parasitol. 167 (1), 1-7.
373 Maketon, M., Orosz-Coghlan, P., Sinprasert, J., 2008. Evaluation of *Metarhizium anisopliae*
374 (Deuteromycota: Hyphomycetes) for control of broad mite *Polyphagotarsonemus latus* (Acari:
375 Tarsonemidae) in mulberry. Exp. Appl. Acarol. 46 (1-4), 157-167.
376 Marangi, M., Cafiero, M.A., Capelli, G., Camarda, A., Sparagano, O.A.E., Giangaspero, A., 2009.
377 Evaluation of the poultry red mite, *Dermanyssus gallinae* (Acari: Dermanyssidae) susceptibility
378 to some acaricides in field populations from Italy. Exp. Appl. Acarol. 48 (1-2), 11-18.
379 Marangi, M., Morelli, V., Pati, S., Camarda, A., Cafiero, M.A., Giangaspero, A., 2012. Acaricide
380 residues in laying hens naturally infested by red mite *Dermanyssus gallinae*. PLoS One. 7 (2),
381 31795.
382 Moss, W.W., 1968. An illustrated key to the species of the acarine genus *Dermanyssus*
383 (Mesostigmata: Laelapoidea: Dermanyssidae). J. Med. Entomol. 5 (1), 67-84.

384 Munoz-Bertomeu, J., Arrillaga, I., Segura, J., 2007. Essential oil variation within and among natural
385 populations of *Lavendula latifolia* and its relation to their ecological areas. Biochem. Syst. Ecol.
386 35 (8), 479-488.
387 Nechita, I.S., Poirel, M.T., Cozma, V., Zenner, L., 2015. The repellent and persistent toxic effects
388 of essential oils against the poultry red mite, *Dermanyssus gallinae*. Vet. Parasitol. 214 (3-4),
389 348-352.
390 Pinto, E., Vale-Silva, L., Cavaleiro, C., Salgueiro, L., 2009. Antifungal activity of the clove
391 essential oil from *Syzygium aromaticum* on *Candida*, *Aspergillus* and dermatophyte species. J.
392 Med. Microbiol. 58, 1454-1462.
393 Raal, A., Orav, A., Arak, E., 2007. Composition of the essential oil of *Salvia officinalis* L. from
394 various European countries. Nat. Prod. Res. 21 (5), 406-411.
395 Rustaiee, A.R., Yavari, A., Nazeri, V., Shokrpour, M., Sefidkon, F., Rasouli, M., 2013. Genetic
396 diversity and chemical polymorphism of some *Thymus* species. Chem. Biodivers. 10 (6), 1088-
397 1098.
398 Safaei-Ghomi, J., Ahd, A.A., 2010. Antimicrobial and antifungal properties of the essential oil and
399 methanol extracts of *Eucalyptus largiflorens* and *Eucalyptus intertexta*. Pharmacogn. Mag. 6
400 (23), 172-175.
401 Sampaio, I.B.M., (Ed.), 2002. Estatística aplicada à experimentação animal. FEPMVZ, Belo
402 Horizonte, 265 pp.
403 Sparagano, O.A.E., George, D.R., Harrington, D.W.J., Giangaspero, A., 2014. Significance and
404 control of the poultry red mite, *Dermanyssus gallinae*. Annu. Rev. Entomol. 59, 447-466.
405 Tabassum, N., Vidyasagar, G.M., 2013. Antifungal investigations on plant essential oils: a review.
406 Int. J. Pharm. Pharm. Sci. 5 (2), 19-28.
407 Tamai, M.A., Alves, S.B., Lopes, R.B., Faion, M., Padulla, L.F.L., 2002. Toxicidade de produtos
408 fitossanitários para *Beauveria bassiana* (Bals.) Vuill. Arq. Inst. Biol. 69 (3), 89-96.

Wu, S., Gao, Y., Zhang, Y., Wang, E., Xu, X., Lei, Z., 2014. An entomopthogenic strain of *Beauveria bassiana* agaisnt *Frankliniella occidentalis* with no detrimental effect on the predatory mite *Neoseiulus barkeri*: evidence from laboratory bioassay and scanning electron microscopic observation. PLoS One. 9, e84732.

FIGURE

Fig. 1. Corrected mortality rates of *Dermanyssus gallinae* adults treated with 0.2% *Eucalyptus globulus* essential oil (EgEO) and *Beauveria bassiana* (*Bb*) alone and in combination (*Bb*+EgEO) at different days post-infection (DPI). Statistically significant differences are marked with the same letters.

Fig. 2. Corrected mortality rates of *Dermanyssus gallinae* nymphs treated with 0.2% *Eucalyptus globulus* essential oil (EgEO) and *Beauveria bassiana* (*Bb*) alone and in combination (*Bb*+EgEO) at different days post-infection (DPI). Statistically significant differences are marked with the same letters

Fig. 3. Adult *Dermanyssus gallinae* dead specimen at T+5 DPI from TG1 with the presence of fertile conidiophores on the surface of mite's body.