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SHORT COMMUNICATION

In vitro Evaluation of Acaricidal Efficacy of Selected Essential Oils against *Dermanyssus gallinae*

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A B S T R A C T

Intensification of poultry production has been associated with an increase in parasite prevalence and adaptation of an invasive external parasitic species, such as the poultry red mite (*Dermanyssus gallinae*). The studies of biological efficacy (contact and fumigant) and level of toxicity to mites indicated that the external application of essential oils (EOs) can be an alternative to acaricides. In this study, the results of acaricidal efficacy of eight selected EOs - *Lavandula angustifolia* Mill., *Laurus nobilus* L., *Mentha x piperita* L., *Mentha spicata* L., *Ocimum basilicum* L., *Salvia officinalis* L., *Satureja montana* L. and *Thymus vulgaris* L. are presented. Their chemical profiles were analyzed by gas chromatography coupled with mass spectrometry (GC–MS). The acaricidal efficacy of EOs (6% concentration) was tested on adult mites over 10 days in laboratory conditions using the Petri-dish method, through direct exposure for 1 min (contact toxicity) and subsequent exposure for 1 h (residual toxicity). The most effective EO in direct exposure-contact, after 48 h of observation, was *S. montana* (100% toxicity), while the greatest residual effect was observed in *T. vulgaris* (11% toxicity). The obtained results showed high efficacy of the EOs against the mites through direct contact and thus their great acaricidal potential. However, the activity was lost with subsequent exposure, indicating the absence of prolonged effect. Possible ways to overcome this problem are discussed further in this paper. In any case, the present study confirmed the acaricidal potential of herbal medicines that can be used in the integrated control of poultry red mite.

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INTRODUCTION

Dermanissus gallinae De Geer (1778), commonly known as the poultry red mite, is a haematophagous ectoparasite of poultry that can cause high mortality among fledglings as well as adult birds. The accelerated growth of poultry farming in recent decades has led to an escalation of *D. gallinae* prevalence, which has been accompanied by a remarkable adaptive potential of the parasite. Nowadays it represents one of the most significant problems in the egg production industry, especially in Europe. High-level infestations can lead to large economic losses and clinical manifestations of diseases (anaemia, chronic stress and death due to blood loss) (Kilpinen, 2015; George *et al.*, 2015). In addition, mites can serve as vectors of poultry pathogens of both bacterial (*Erysipelothrik rhusiopathiae, Salmonella subsp. enterica* ser. Gallinarum) and viral (Avian influenza virus, Fowlpox virus) origin (Sparagano *et al*., 2014; Schiavone *et al*., 2022).

To address this problem, various control measures are being implemented by poultry farmers. So far, mite control is primarily based on the application of synthetic acaricides (phoxim, cyfluthrin, fluralaner), followed by silica-based products, inert oils, and high temperatures. Since all these measures have some limitations, a combination of two or more measures in the form of integrated pest control is most commonly used (Quilicot *et al*., 2020). The use of some chemical acaricides has recently been banned due to increased resistance of mites, safety issues, side effects on non-target species, and the presence of residues in food and environmental contamination (Marangi *et al*., 2012; Sparagano *et al*., 2014; George *et al*., 2015). These

motivated researchers to look further for alternatives such as the utilization of plant-derived acaricides, exploitation of biological resources (bacteria, fungi, and predatory mites) and development of vaccines (Decru *et al*., 2020; Imran and Alsayeqh, 2022). Although these scientific advances offer promising opportunities to mitigate the effect of *D. gallinae* infestations, only some solutions are present on the market, while others have remained at the pre-commercial stage (Sparagano *et al*., 2014).

Plants produce a variety of natural products (secondary metabolites). These compounds have important biological functions, providing protection for plants from herbivores or pathological microbes, and serving as attractants for pollinators and seed-dispersing animals. Many plant-derived products (PDPs), such as essential oils and extracts, are generally harmless to non-target organisms. Although they have a long history of safe application in human medicine (Isman 2016), the first study focused specifically on poultry mites was conducted by Kim *et al*. (2004) followed by extensive further investigations by various researchers, whereby acaricidal potential has been demonstrated for several classes of PDPs. Furthermore, their contact and fumigant toxicity and repellent activity have been investigated, either as an individual treatment or in combination with other control measures.

Essential oils (EOs) are complex mixtures of volatile compounds obtained from aromatic plants (Štrbac *et al*., 2021). In previous studies, many EOs have shown significant acaricidal activity. Their pharmacological properties origins from chemical composition, i.e., the bioactive compounds they contain, and which may belong to various chemical groups (hydrocarbon terpenes, terpenoids, phenylpropanoids etc). However, the presence and percentage representation of certain compounds of EOs is highly variable depending on plant variety, climate, and environmental conditions, which leads to the differences in their biological activities (Fokou *et al*., 2020; de Sousa *et al*., 2023). The aim of this study was to investigate the acaricidal activity of eight EOs from the Balkan Peninsula (Southern Europe) and to evaluate their potential and limitations to be included in formulations for rational control of *D. gallinae* in the poultry industry.

MATERIALS AND METHODS

Essential oils and chemical analyses: The EOs of *Lavandula angustifolia* Mill. (lavender), *Laurus nobilus* L. (laurel), *Mentha x piperita* L. (peppermint), *Mentha spicata* L. (spearmint), *Ocimum basilicum* L. (basil), *Salvia officinalis* L. (sage), *Satureja montana* L. (winter savory) and *Thymus vulgaris* L (thyme) were obtained from Institute of Field and Vegetable Crops, Novi Sad, Republic of Serbia. The oils were extracted from dry plant material by steam distillation. Plant material of *L. angustifolia*, *S. officinalis* and *L. nobilus* originated from Montenegro, and other species from Serbia. Qualitative and semi-quantitative chemical characterization of EOs was performed using Agilent Technologies 6890N gas chromatograph coupled with Agilent Technologies 5975B electron ionization mass-selective detector (GC-MS analyses), as described by Štrbac *et al*. (2022).

Acquisition of mites: Biological tests were performed on adult female *D. gallinae*, obtained by mechanical removal from cages of highly infested farms located in Vojvodina (northern Serbia), and which had not received any treatment two months before the experiment. Mites were identified using the identification keys (morphological characteristics) provided by Moss (1968) and Di Palma *et al*. (2012). A quantity of *D. gallinae*, approximately 4-5 cm^3 per sample, was placed in a five-litre nylon bag. The bag was first expanded to allow air in, closed by tying and then delivered to the laboratory on the same day. The bags were then opened and placed on insulated trays. After an adaptation period of 24 h, *D. gallinae* mites were used for the experiments.

Evaluation of essential oils toxicity *in vitro:* Toxicity bioassay was carried out using the Petri-dish method (Pavlićević *et al*., 2017) with some change. 100 (experimental groups, five replicates, n=20/repeat) plus 20 (negative control groups) live newly fed adult female mites of *D. gallinae* for each EO examination were used. The mites were treated with one dilution of EOs (6%) diluted in water with 0.1 % Tween 80. Water with 0.1 % Tween 80 was used as negative control.

The contact toxicity test was performed by directly spraying mites in Petri-dishes (Ø 55 mm) with the tested aqueous emulsion of EOs, for one minute. After exposure, the mites were transferred to clean Petri-dishes, where they remained until the end of the test. The dishes were equipped with filter paper to remove excess liquid from the specimens and prevent suffocation, simulating the effect of impurities and absorbent surfaces in poultry farms.

The residual toxicity test was performed by subsequently exposition of mites during one-hour. The inner surface of the Petri-dishes was sanded and degreased, after which the tested EOs were applied and distributed evenly. The dishes were strained at room temperature and the mites were introduced into the test containers after 24 hours of drying. After the one-hour exposure, the mites were transferred to clean Petri-dishes for observation.

Subsequently, all Petri dishes were covered with their lids and sealed with layer of Parafilm and finally kept at 23±1 ºC and humidity 54±5%, with a 16:8 light: dark cycle. Mite mortality in dishes was assessed after 48 h, to monitor the acaricidal activity of EOs. Mites were classed as dead if they exhibited no movement after repeated agitation with an entomological pin under a mounted lens at 4× magnification.

Statistical analyses: The results represent the cumulative number of dead mites for 48 h after treatment. The percentage of mortality (dead mites/total mites × 100) with Standard Deviation (±SD) was calculated for each treatment. Data in percentages were subjected to arcsine transformation and analysed by t-test and ANOVA using Microsoft Excel Spreadsheet Software 2010. The Student t-test was used to compare mortality in the control and treatment groups. For comparison of mortality between treated groups, One-way ANOVA with Tukey-Kramer as a post-hoc test was used with a p-value threshold of 0.05.

RESULTS

Analyses of the chemical composition of Eos: The GC-MS analyses clearly show the complex chemical composition of the EOs tested (Table 1). The identified compounds, their representative percentages and total number varied among investigated samples. The identified compounds were present in different percentages and belong to different chemical classes such as monoterpene hydrocarbons, alcohols, phenols, ketones, esters and acetates, whereby sesquiterpenes were present in significant amounts only in *O. basilicum* EO. Due to the large number of identified compounds, only those with abundance that exceeding 1% in at least one oil are shown.

Acaricidal activity of the EOs: The comparison of acaricidal activity of 6% aqueous emulsions of the EOs against *D. gallinae*, at different exposure times - 1 min (contact toxicity) and 60 min (residual toxicity) after 48 h, is shown in Fig. 1. After 1 min of exposure, all EOs showed significant ($P<0.05$) contact toxicity to *D. gallinae* except *O. basilicum* oil. After 60 min of exposure, only *T. vulgaris* showed significant ($P<0.05$) residual toxicity to *D. gallinae*.

Due to the weak acaricidal effect after exposure of 60 min, these results were excluded from further statistical analysis. Exposure of one min to the investigated EOs led to a statistically significant acaricidal effect (ANOVA, $P<0.05$). Tukey Kramer post-hoc analysis showed that *S. montana*, *T. vulgaris* and *S. officinalis* expressed equal activity ($P>0.05$), which is higher than the activity of other EOs ($P<0.05$). *L. nobilis* showed moderate acaricidal activity, while *M. spicata*, *M. x piperita* and *L. angustifolia* showed similar ($P<0.05$) but lower activity compared to the other samples. *O. basilicum* showed no acaricidal effect on mites considering that there was no statistically significant difference in comparison with the control group ($P>0.05$) (Table 2).

DISCUSSION

A various *in vitro*, and a smaller number of *in vivo* studies conducted to date suggest that EOs possess acaricidal activity against *D. gallinae* (Kim *et al*., 2004; George *et al*., 2010a; Magdaş *et al.*, 2010; Soares *et al*., 2015; Tabari *et al*., 2020; Roczeń-Karczmarz *et al*., 2022). Despite important differences in the research methodology, *in vitro* assays are generally focused on simulating farm conditions and how the information obtained can be useful in poultry production. In the present study, one-minute exposure (contact toxicity) simulates the situation in which mites are directly exposed with their entire bodies to the external application of EO. Absorbent surfaces (e.g., floors) and ambient impurities have the ability to absorb the applied aqueous emulsion, allowing *D. gallinae* to leave the treated surface. Exposure for 60 min (residual toxicity) simulates the situation in which the mites are subsequently exposed to the formulation by coming out of hiding and contact with the treated surfaces. The exposure time is limited to 1 h because the mites try to escape from these areas due to the nature of their movement and the possible irritation caused by the EO.

During the examination of the EOs acaricidal properties, researchers used different exposures (1 min - 72 h) and concentrations or doses per unit of area or volume (mg/cm^2; mg/cm^3). In the present study, the concentration of the aqueous EO emulsions, expressed in %, was used instead of the dose per unit area, since this is the simplest way to prepare the solutions under practical conditions of use. The concentration of 6%, which is higher than the concentrations usually used in other studies, was chosen to reliably evaluate the residual effect on subsequently exposed mites. Therefore, *in vitro* methodology used in the present study is novel, and it is performed in the attempt to simulate in the best possible way the conditions in the field.

Other studies have also demonstrated acaricidal efficacy of selected EOs against *D. gallinae*. Kim *et al*. (2004) demonstrated 100% (thyme and mint) and 89% (lavender) efficacy at a dose of 0.07 mg/cm^2 after 24-h exposure (by contact). At a dose of 0.21 mg/cm^2, efficacy was 97.39% (peppermint) and 32.05% (sage), while a dose of 0.35 mg/cm^2 gave efficacy of 100% (lavender), 67% (sage), 62% (peppermint), and 56% (basil). George *et al*. (2010a) also demonstrated efficacy after 24-h exposure as follows: 100% (thyme), 77.80% (winter savory), 64.74-89.96% (lavender), 32.05% (sage) and 20.88% (spearmint). Roczeń-Karczmarz *et al.* (2022) found that pure EOs of thyme and lavender, applied at maximum (100%) concentration at a dose of 0.28 mg/cm^2 and an exposure of 48 h, had an efficacy of over 90%. EOs of lavender and peppermint, applied at a dose of 0.6 mg/cm^2 and an exposure of 72 h, had an efficacy of 100% and 65%, respectively (Magdaş *et al*., 2010). These results suggest that acaricidal activity depends mainly on the plant species from which the EO was obtained, i.e., its chemical composition. From this point of view, compounds such as p-cymene, carvacrol, thymol, α-thujone and 1,8-cineole were highly represented in the most effective oils (thyme, winter savory, sage, and laurel).

Botanical formulations offer a strong alternative to chemicals such as organophosphates and pyrethroids in the control of ectoparasites such as *D. gallinae* (Abbas *et al*., 2018; Puvača *et al*., 2019; Radsetoulalova *et al*., 2020). Thus, the results of the present and other studies suggest a strong direct effect of most tested EOs against *D. gallinae*, although the exact mechanism of action should be further investigated. In addition, plant-based formulations are most likely less susceptible to resistance development (due to active ingredients from various chemical groups) and more acceptable in terms of the residues in animal products and the environment in comparison with chemicals (Borges and Borges, 2016; Abbas *et al*., 2018; Puvača *et al*., 2019).

However, the use of EOs in the treatment of ectoparasites has some limitations. As shown in the present study, the residual effect is still significantly lower than contact (direct) effect. This can be explained by the unstable nature of EOs, as their active ingredients are prone to evaporation and destabilization (Maes *et al*., 2019; Nehme *et al*., 2021). Direct action (alone) can only achieve short-term results which is most likely not sufficient, suggesting that efficacy and duration of prolonged action are important factors for successful control of *D. gallinae*. On the other hand, some EOs such as thyme can still be effective acaricides despite low residual toxicity, due to their fast effect (George *et al*., 2010b). Moreover, EOs of

Table 1: Chemical composition (% of total peak area) of tested essential oils determined by gas chromatography-mass spectrometry.

AI*	Compound	% of total peak area							
		LA	LN	MP	MS	OB	SM	SO	TV
932	α-Pinene	**1.03**[ab]	**6.20**	0.90	0.54	0.10	**1.16**	**3.16**	**2.47**
946	Camphene	0.58	0.49	0.03	-	0.03	0.44	**5.36**	0.62
971	Sabinene	0.18	**9.40**	0.36	0.16	0.04	-	0.02	-
976	β-Pinene	**1.09**	**5.06**	**1.15**	0.67	0.19	0.66	**1.16**	0.18
1016	α-Terpinene	0.07	-	0.46	0.16	-	**2.32**	0.25	-
1023	*p*-Cymene	0.54	**1.61**	0.11	0.35	0.19	**42.83**	0.90	**41.72**
1027	Sylvestrene	-	**1.66**	-	-	-	-	-	-
1028	Limonene	**1.06**	-	**1.17**	**4.37**	0.18	**1.52**	**2.22**	**1.26**
1029	1,8-Cineole	**17.29**	**55.30**	**5.22**	0.53	**3.52**	0.73	**8.40**	0.66
1035	*cis*-Ocimen	**3.20**	-	0.19	-	-	-	-	-
1056	γ-Terpinene	0.27	-	0.67	0.32	0.02	**14.59**	0.36	-
1100	Linalool	**37.52**	**4.32**	-	-	**62.80**	**1.20**	0.20	**4.37**
1106	α-Thujone	-	-	-	-	-	-	**38.8**	-
1116	β-Thujone	-	-	-	-	-	-	**5.07**	-
1143	Camphor	**4.76**	-	-	-	0.73	-	**19.75**	0.22
1152	Menthone	-	-	**22.00**	**1.28**	-	-	-	-
1163	Isomenthone	-	-	**9.39**	0.50	-	-	-	-
1164	Borneol	**14.2**	-	-	-	-	**1.27**	**1.89**	0.69
1171	Menthol	-	-	**32.55**	**4.20**	-	-	-	-
1176	Terpinen-4-ol	**6.71**	**1.89**	**3.26**	**1.16**	0.46	0.78	0.34	-
1182	*izo*-Menthol	-	-	**1.29**	0.13	-	-	-	-
1191	α-Terpineol	**1.96**	**1.56**	0.46	0.12	0.51	-	-	**11.71**
1193	Dihydro carveol	-	-	-	**1.73**	-	-	-	-
1196	*trans*-4-Caranone	-	-	-	**8.67**	-	-	-	-
1197	Methyl chavicol	-	-	-	-	**3.78**	-	-	-
1204	*trans*-Dihydrocarvone	-	-	-	**1.49**	-	-	-	-
1238	Pulegone	-	-	**4.45**	0.10	-	-	-	-
1244	Carvone	-	-	-	**64.44**	-	-	-	-
1255	Linalyl acetate	**6.11**	-	-	-	-	-	-	-
1284	Bornyl acetate	-	-	-	-	0.74	-	**1.86**	-
1292	Thymol	-	-	-	-	-	-	-	**31.59**
1293	Menthyl acetate	-	-	**10.03**	**1.01**	-	-	-	
1303	Carvacrol	-	-	-	-	-	**28.11**	-	-
1327	*izo*-Dihydro carveol acetate	-	-	-	**3.11**	-	-	-	-
1348	α-Terpinyl acetate	-	**11.55**	-	-	-	-	-	-
1383	β-Bourbonene	-	-	0.18	**1.76**	-	-	-	-
1390	β-Elemene	-	-	-	-	**1.72**	-	-	-
1418	β-Caryophyllene	0.53	-	**2.78**	0.93	0.92	**2.46**	**3.95**	0.82
1434	*α-trans*-Bergamotene	-	-	-	-	**2.11**	-	-	-
1436	γ-Elemene	-	-	-	-	**1.42**	-	-	-
1452	α-Humulene	-	-	-	-	**1.13**	-	**4.15**	-
1479	γ-Muurolene	-	-	-	-	**4.45**	-	-	-
1503	α-Bulnesene	-	-	-	-	**3.66**	-	-	-
1512	γ-Cadinene	-	-	-	-	**2.98**	-	-	-
1639	α-*epi*-Cadinol	-	-	-	-	**2.17**	-	-	-
	% of total identified compounds	97.1	99.0	96.6	97.7	93.8	98.1	97.8	96.3
	Total number of identified compounds	27	14	31	33	42	17	27	15

*AI - arithmetic retention index; LA – *Lavandula angustifolia*; LN - *Laurus nobilus*; MP - *Mentha x piperita*; MS –*Mentha spicata*; OB - *Ocimum basilicum*; SM - *Satureja montana*; SO – *Salvia officinalis*; TV - *Thymus vulgaris*; [a]Only compounds that are prevalent by more than 1% in at least one essential oil are shown in the table; [b]Compounds present in more than 1% are shown in bold.

Table 2: Tukey-Kramer post-hoc analysis of mortality after 1 min exposure to EOs

Essential oils	Essential oils						
	MP	LN	LA	TV	MS	OB	SM
LN	0.2193						
LA	1	0.2647					
TV	2.98E-05	0.03087	4.04E-05				
MS	0.4676	0.001552	0.4038	9.87E-08			
OB	0.1501	0.000227	0.1209	1.62E-08	0.9969		
SM	6.83E-07	0.000881	9.18E-07	0.8768	3.01E-09	5.58E-10	
SO	0.002489	0.5755	0.003334	0.7665	7.53E-06	1.11E-06	0.1052

* LA – *Lavandula angustifolia*; LN - *Laurus nobilus*; MP - *Mentha x piperita*; MS –*Mentha spicata*; OB - *Ocimum basilicum*; SM - *Satureja montana*; SO – *Salvia officinalis*; TV - *Thymus vulgaris*

thyme and lavender showed high persistent activity against *D. gallinae* 30 days after application on filter paper, with the effects at 72h of 100% and nearly 80%, respectively (Nechita *et al*., 2015). In a study of Tabari *et al*. (2020), EOs of *Litchi chinensis* and *Syzygium aromaticum* showed promising toxic effects up to 4 days after application.

In the context of the residual efficacy, the results of previous study conducted for banned acaricide carbaryl, in a 0.1% aqueous solution, showed its high efficacy for 70 days (Pavlićević *et al*., 2016). The SiO_2 formulation had a prolonged efficacy of 80% even after 60 days. The residual effect of a 20% aqueous emulsion P 547/17, on a galvanized sheet substrate and in an inclined position, was 57% after one month and remained at the same level in the following month (Pavlićević *et al*., 2017, 2018), In practice, a facility treated twice with selected inert oils, will

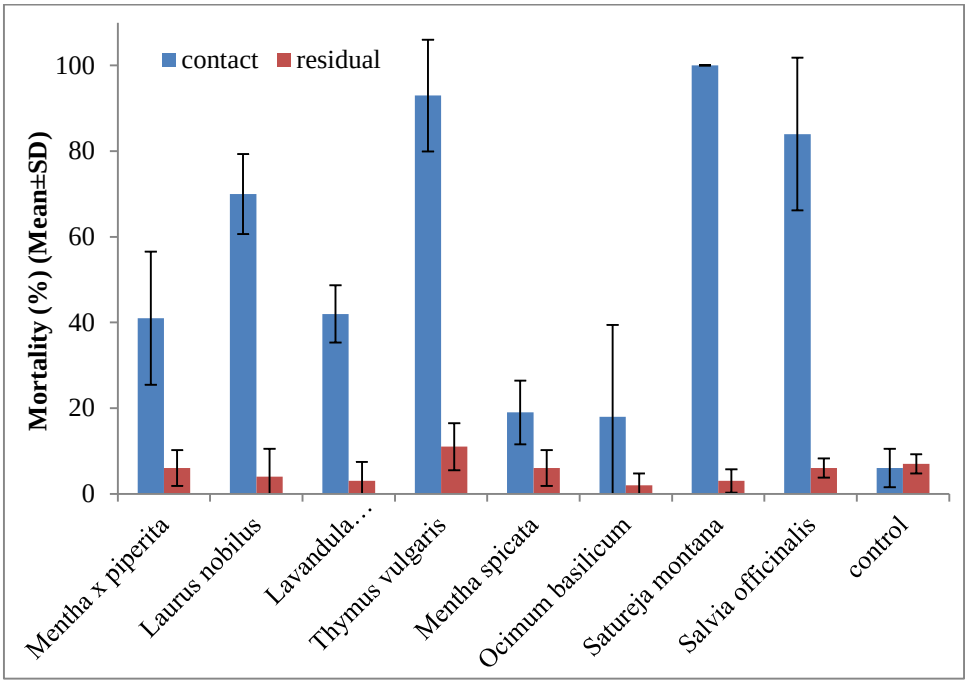

Fig. 1: Average mortality rate (%) of *Dermanyssus gallinae* adult mites after exposure to examined essential oils (± SD).

require one or two more treatments depending on the conditions. If the conditions are fully met, no treatment at all is required during the one-year exploitation of a new flock (Pavlićević *et al*., 2016).

One of the possible solutions to prolong the effect of EOs against *D. gallinae* is to combine different EOs or their ingredients to obtain a synergistic effect. Thus, in a study of Amer *et al.* (2020), a formulation containing garlic oil (2.5%), rosehip oil (4.2%), rapeseed oil (4.8%) and polysorbate (14.0%) showed a rapid and strong acaricide effect in contact sprays *in vitro*, while there was a significant improvement in the groups treated with plant oils *in vivo*. Another option is to use multiple applications over several consecutive days, as in the study performed by Chun *et al*., (2018), where five external treatments with *Chamaecyparis obtusa* (Siebold & Zucc.) Endl. EO resulted in a control effect of about 80% for 18 days. Three treatments with RP03™, a formulation consisting of 20% neem oil, were effective in controlling *D. gallinae* for slightly more than 2 months in facilities inhabited by laying hens (Camarda *et al*., 2018).

Given the unstable and volatile properties of EOs, encapsulation techniques can effectively reduce the degradation of EO active ingredients, evaporation, smell and odour, whereby they can provide a slow and continuous release of active substances (Maes *et al*., 2019; Linh *et al*., 2022). For example, encapsulated carvacrol with yeast cell walls showed high acaricidal activity against common tick in domestic animals, *Rhipicephalus microplus* Canestrini (Acari: Ixodidae), while exhibiting lower volatilization (Lima *et al*., 2017). A microencapsulated formulation of *Cymbopogan winterianus* Jowitt ex Bor EO with gelatin: acacia showed promise for mosquito repellency due to its relatively slow release rate (Songkro *et al*., 2018). These examples suggest the usefulness of encapsulation in controlling ectoparasites including *D. gallinae*, although further studies should confirm these considerations.

In the end, EOs may play an important role in environmentally friendly integrated pest management, which implies the use of safe, non-chemical methods and measures in the prevention and treatment of diseases caused by pest species, and the use of chemical acaricides only after these measures have failed (Decru *et al*., 2020). Moreover, it may also refer to the combination of different methods. Thus, the combination of ivermectin (0.25 mg/ml) and allicin (a compound commonly found in garlic EO) (1 mg/ml) exhibited very high *in vitro* acaricide rates of 98.7%, 98.4%, 99.4% and 99.9% on days 7, 13, 21 and 28 after treatment, respectively (Kang *et al*., 2023). EOs can also be combined with other alternative methods, especially those that have a delayed, but long-term effect as in the case of entomopathogenic fungi (Gay *et al*., 2020). For example, *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreale: Cordycipitaceae) has been combined with different EOs, whereby the combination with *E. globulus* Labill at very low concentration (0.2%) proved to be a highly promising option for the control of *D. gallinae* (Immediato *et al*., 2016).

Conclusions: The present study demonstrated that EOs have high activity against *D. gallinae*, as the results of the contact toxicity tests showed. However, their unstable and volatile nature allows only short-term effect. Therefore, methods and strategies to increase their residual effect are needed before their widespread individual use in practice is possible. Alternatively, EOs can still be an important part of an integrated pest management of *D. gallinae* designed to achieve sustainable, rational control of this important parasite.

Authors contribution: RR, AP, JP and IS made substantial contributions to the basic idea, while the conduct of the research was made possible by RR and AP. NS and DO are responsible for the procurement of EOs and the GC-MS analyses, while the experiment (*in vitro* acaricidal effect) was designed and conducted by RR and AP with great advisory assistance from JP and IS. For interpreting the results and drawing conclusions are responsible RR, FŠ and AP, while the final version of the

manuscript was drafted by AP and FŠ with the assistance of all co-authors who revised the manuscript.

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