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RESEARCH

Promising Algerian essential oils as natural acaricides against the honey bee mite *Varroa destructor* (Acari: Varroidae)

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Abstract

Varroosis induced by *Varroa destructor* Anderson and Trueman represents the most pathogenic and destructive disease affecting the western honey bee, *Apis mellifera.* In this study, we investigated the acaricidal activity against the *Varroa* mite using essential oils (EOs) from the aerial parts of four autochthonous Algerian herbal species, namely *Artemisia herba alba*, *Artemisia campestris*, *Artemisia judaica* and *Ruta montana*. EOs were obtained by means of hydrodistillation and their composition was characterized by gas chromatography–mass spectrometry. The toxicity of the selected EOs toward *V. destructor* and *A. mellifera* adult honey bees was evaluated using the complete exposure method. The results indicate the predominance of davanone (66.9%) in *A. herba alba*, β-pinene (19.5%) in *A. campestris*, piperitone (68.7%) in *A. judaica* and 2-undecanone (70.1%) in *R. montana* EOs. Interestingly, the LC_{50} values coupled to bee mortality rates revealed that all tested oils exhibited significant acaricidal efficiency with selectivity ratio (SR) values of 10.77, 8.78, 5.62 and 3.73 for *A. campestris*, *A. judaica*, *A. herba alba*, and *R. montana*, respectively. These values were better than that of thymol (SR = 3.65), the positive control. These findings suggest that these EOs could be used as plant-derived veterinary acaricides to control varroosis in field conditions.

Keywords Acaricidal activity · *Apis mellifera* · *Varroa destructor* · Essential oils · Complete exposure method

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Introduction

The western honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), is the most important pollinator of a wide variety of agricultural crops and the main source of bee products such as honey, propolis, pollen and royal jelly (Abd El-Wahab et al. 2021; Flores et al. 2021). In recent decades, dramatic honey bee colony losses are recorded to be linked mainly to several biotic and abiotic stressors like habitat loss, pesticides exposure, poor nutrition, beekeeping practices, climatic changes and invasive pathogens (Glenny et al. 2017; Almecija et al. 2020). Amongst its parasites, the cosmopolitan ectoparasitic mesostigmatid mite *Varroa destructor* Anderson and Trueman (Acari: Varroidae) is marked as the major pathological threat (Giacobino et al. 2014; Erban et al. 2015; Alburaki et al. 2018). This mite affects directly both adult bees and brood by feeding on their hemolymph and fat body tissue (Ramsey et al. 2019; Slowinska et al. 2019; Traynor et al. 2020; Piou et al. 2023) and acts as a viral vector, which leads to the colony's collapse within 2–3 years (Mondet et al. 2020). Besides, on an individual scale, varroosis induces very harmful effects getting to malformation, dehydration of the body and weight drop, lifespan decrease, olfactory sensation problem, immunosuppression as well as alteration of flying and foraging ability (Noël et al. 2020; Sabahi et al. 2020; Khan and Ghramh 2021).

As a consequence of these dramatic effects and losses, various synthetic acaricides have been largely used to fight *Varroa* mite such as pyrethroids (tau-fluvalinate and flumethrin), organophosphate (coumaphos), and formamidine (amitraz) (Li et al. 2017; Riva et al. 2019). Nevertheless, it should be mentioned that their high efficacy is faded by *V. destructor* resistance development, the contamination of beehive products by chemical residues of these acaricides, as well as detrimental effects on honey bee health and the environment (Riva et al. 2019; Noël et al. 2020). Recently, natural compounds, especially organic acids, herbal essential oils (EOs) and derivatives have attracted a lot of attention as potentially safe, eco-friendly and efficient alternatives to conventional acaricides (Vilarem et al. 2021). Indeed, EOs are believed to have a broad-spectrum efficacy with ovicidal, larvicidal, adulticidal and ectoparasite-repellent effects (Abbas et al. 2018). The application of EOs is reported to trigger disorders in insect and mite activities, which include antifeedant activity, growth and reproduction deficiency, breakage of cuticle as well as exuviation and respiration repression (Ben Chaaban et al. 2019). Ramzi et al. (2017) found that certain EOs tend to enhance the honey bee grooming behavior. Notably, thymol is a good example of a bioacaricide that is used by beekeepers to treat honeybee colonies infested by *V. destructor* known for its mite antifeedant and repression of reproduction and growth activities (Lindberg et al. 2000; Singh 2014).

As a source of herbal EOs, several plants have been used to extract efficient and active organic compounds worldwide and in Algeria as well. Among these Algerian plants, we can distinguish the genera *Artemisia* (Asteraceae) and *Ruta* (Rutaceae). *Artemisia* is reportedly comprising > 500 diverse species, most of which have been recognized as an important source of EOs (Hu et al. 2019). Overall, *Artemisia* species are characterized by multiple biological properties including antibacterial, antifungal, antiviral (Abad et al. 2012), antiprotozoal, anthelmintic, anti-inflammatory, cytotoxic, antitumor, insecticidal and repellent activities (Ivănescu et al. 2021). Godara et al. (2013, 2015) reported that extracts from *Artemisia absinthium* exhibit acaricidal activity against the ticks *Rhipicephalus sanguineus* and *Hyalomma anatolicum*. Furthermore, a high mortality rate was recorded by the use of

Artemisia sieberi extract to control *V. destructor* (Ashrafi Parchin et al. 2012). Field trials of extracts from *Artemisia annua* and *A. absinthium* against *V. destructor* revealed a potent miticidal effect (Rasool et al. 2017; Allabergenova et al. 2021). EOs extracted from *A. annua* and *Artemisia verlotiorum* revealed a significant repellent activity against *Varroa* under laboratory conditions (Conti et al. 2020).

The genus *Ruta* comprises 40 species, highly rich in EOs that contain mainly aliphatic ketones, e.g., 2-undecanone and 2-nonanone (Nahar et al. 2021). *Ruta* spp. have been widely identified as antiparasitic, stomachic, digestive, vermifuge and molluscicidal (Ferhat et al. 2014), antiseptic, antispasmodic, anthelmintic (Haddouchi et al. 2013), insecticidal and larvicidal agents (Boutoumi et al. 2009; Majdoub et al. 2014). Castagnino Laércio and Orsi (2012) demonstrated that *Ruta graveolens* volatile oil is effective to reduce the honey bee colonies infestation by *V. destructor* and decrease the mortality rate of parasitized honey bees.

In this study, we assessed the in vitro acute toxicity of EOs obtained from four Algerian plants – i.e., *Artemisia herba alba* Asso, *Artemisia campestris* L., *Artemisia judaica* L. and *Ruta montana* L. – against *V. destructor*. In addition, the determination of the phytochemical profile of each EO using gas chromatography–mass spectrometry (GC-MS) will help to identify their major compounds that might contribute to the pronounced acaricidal effect. To the best of our knowledge, this is the first work on the acaricidal activity of EOs from *A. campestris*, *A. judaica* and *R. montana* in the *Varroa* mite control, whereas EO isolated from *A. herba alba* from a different region has recently been tested in vitro by Alahyane et al. (2022).

Materials and methods

Plant material

Aerial parts including leaves, flowers and stems of *A. herba alba*, *A. campestris*, *A. judaica* and *R. montana* were harvested in Algeria during the flowering period (Table 1). The taxonomic identification was confirmed by Prof. Benhouhou Salima, Department of Botany, Higher National Agronomic School of Algiers (Algeria) according to the Quezel and Santa (1969) nomenclature and validated by the synonymic index of the North Africa plants. The aerial parts were dried in the dark at room temperature.

A sample of 500 g dried plant material was ground just before extraction with an electrical grinder (Moulinex AR110830) then submitted for hydrodistillation using a Clevenger-modified system for 3–4 h until total recovery of the oil. The latter was then dried over anhydrous sodium sulphate before being stored in a sealed dark glass vial and maintained at 4 °C until GC analysis and acaricidal assays. The extraction yield was expressed as a percent of the ratio between EO mass (g) per dried plant material mass (g). Experiments were performed in triplicate and the yield mean value was reported.

Gas chromatography–mass spectrometry (GC-MS) analysis

Identification of EO composition has been performed using a GC-MS system (Agilent HP 6890 Plus gas chromatograph coupled with a Agilent HP 5973 mass spectrometer). The col-

Table 1 Description of plants used in this study

Family	Plant species	Local name	Harvesting place and time	Latitude/longitude	Bioactivities	References
Asteraceae	*Artemisia herba alba*	Chih, Chiha	Taadmit, Djelfa – Oct 2021	34°16'60.00"N / 2°58'60.00"E	Antioxidant, disinfectant, antibacterial, antileishmanial, anthelmintic, nematicide, and antispasmodic properties	Abdelali et al. 2023
	A. campestris	Dgouft	Ain Roumia, Djelfa – Oct 2021	34°21'2.28"N / 3°13'43.33"E	Antioxidant, insecticidal, antitumor and antifungal activities	Rocha et al. 2021
	A. judaica	Chih, Chouihiya	Tamanrasset – Apr 2021	22°47'6"N / 5°31'22.001"E	Anti-inflammatory, antioxidant, antimicrobial, anti-helicobacter, anthelmintic, antipyretic, and analgesic properties	Amin et al. 2019
Rutaceae	*Ruta montana*	Fidjel el-djbel	Ferdjioua, Mila – May 2021	36°24'40.00"N / 5°56'15.07"E	Antioxidant, antimicrobial, pesticidal, larvicidal, insecticidal and insect-repellent properties	Nahar et al. 2021

umn used was a capillary HP-5MS (5% phenyl 95% dimethylpolysiloxane) column (30 m length, 0.25 mm internal diameter and 0.25 µm film thickness; Agilent Technologies, USA). A sample of 0.2 µl was injected in split mode (ratio 1/80) at which the GC injector temperature was set at 250 °C. Helium (He) was used as a carrier gas at 0.5 mL/min and the oven temperature program started at 60 °C, where it was held isothermally for 8 min before being increased to 250 °C (at 2 °C/ min) and held isothermally for 10 min. The ionization energy was set at 70 eV and a solvent delay of 3.5 min. The identification was achieved by matching the recorded mass spectra with those stored in Wiley and NIST library data and by comparing each compound's retention index relative to n-alkanes (C_8–C_{29}) with those in the literature (Adams 2017).

Mites and bees

Apis mellifera intermissa (belonging to the African Lineage A) workers and pupae, and adult females of *V. destructor* mites collected during the spring and summer of 2022, when mites were abundant, from hives placed in an apiary and kindly donated by the Multipurpose Agricultural Cooperative of Tizi-Ouzou (CAPTO) situated in the region of Tizi-Rached, province of Tizi-Ouzou in Algeria (36°40'42.5316''N, 4°12'28.9512''E). The same management practices were applied to all experimental bee colonies that were kept untreated against *V. destructor* for at least 6 months. Sealed brood worker or drone combs were removed from colonies at the apiary then moved to the laboratory. *Varroa destructor* adult females were collected in their reproductive stage instead of their dispersal stage in

order to avoid mites' variability in physiology and fitness. Brood cells were individually uncapped using fine forceps and inspected for adult mite's presence. Mites were picked up from larvae and non-pigmented pupae using a fine paintbrush as described previously by Dietemann et al. (2013). Collected mites were kept in Petri dishes with some bee pupae (white to pink eyes) as food substrate, to prevent mite's desiccation, and used immediately for bioassays. Mites that appeared to be newly molted, weak or abnormal were purposely discarded from selection. This protocol has been precisely chosen because it allows the collection of less traumatized mites than those obtained with other methods (Dietemann et al. 2013). Newly emerged worker honey bees were selected from healthy bee colonies (the younger population is less exposed to hive and environmental conditions than the older one). Frames of sealed worker brood showing signs of emerging bees within 1–2 days were transported into ventilated boxes to the laboratory incubator (34 °C and 60 ± 10% RH). Once young bees have emerged, they were gently inspected with plastic tweezers to confirm the absence of mites before being distributed into plastic rearing cup cages supplied with a 50% (w/v) sucrose feeding solution.

Acaricidal activity on *Varroa destructor*

Essential oil bioassays against *V. destructor* were conducted according to a modified protocol previously described by Ruffinengo et al. (2005). Briefly, six EO concentrations ranging from 0.25 to 20 µL/mL were prepared in ethanol 96%, then 1 mL of each solution was uniformly spread using a micropipette onto a filter paper that covered the full bottom surface of a Petri dish (6 cm diameter, 2 cm high). After solvent evaporation, 10 mites and five bee pupae (white to pink eyes) were placed in each Petri dish on the surface of the filter paper favoring direct contact with the solution. The dishes were sealed with parafilm and covered with a lid to prevent mite escape. The experiment was carried out at room temperature. Three replicates were realized for each treatment and dose, and the mortality percentage was expressed as a mean value. Ethanol 96% was used as the negative control and thymol as the positive control using six concentrations ranging from 0.21 to 2.06 µL/mL. All treated mites were incubated at 30 ± 1 °C and 60 ± 10% RH for 72 h. Mortality of mites was checked at 24, 48 and 72 h following the initial exposure. Mites that did not move when probed with a fine paintbrush were considered dead.

Toxicity of essential oils on *Apis mellifera* adults

The EOs toxicity to worker bees was tested using Chaimanee et al. (2021) protocol that was partly modified. Six EO concentrations ranging from 2.5 to 40 µL/mL were prepared. Then, 1 mL solution was pipetted onto filter paper covering the bottom of the plastic cup cages described by Evans et al. (2009). Ten newly emerged adult bees were placed into each cage after solvent evaporation. A 5-mL syringe filled with 50% (w/v) sucrose solution was supplied *ad libitum* on the top of the cage whereas a small amount of beebread (a mixture of polyfloral bee pollen in 50% sucrose solution) was supplied as a dietary supplement at the bottom of the cage as described by Huang et al. (2014). Ethanol and thymol with six concentrations ranging from 1.03 to 3.09 µL/mL were included as negative and positive control, respectively. Three replicates were conducted for each treatment for a total of 30 bees. The cages were placed under the same environmental conditions as those used for the acaricidal

activity against *V. destructor*. Mortality was checked by visual inspection at 24, 48 and 72 h following initial exposure. Mortality data, at the various dosages, is used as a measure of the respective medium lethal concentrations (LC_{50}, i.e., the lethal dose that causes the death of half a population) whereas selectivity ratio (SR) is used to compare efficacy among tested EOs. SR is expressed as a ratio between mite and bee toxicity:

$SR = LC_{50\ A.\ mellifera}/LC_{50\ V.\ destructor}$.

Statistical analysis

Bee and mite mortality rates were corrected for mortality against the control using Abbott's (1925) formula: $P_c = 100 \times (P_0 - P_t) / (100 - P_t)$, where P_c is the corrected mortality (%), P_t is the mortality observed in the control, and P_0 is the mortality observed in the test. Differences between groups were analyzed by two-way ANOVA and Tukey's honestly significant difference (HSD) test for the four concentrations in common (1–10 µL/mL) for *V. destructor* mortality. The average corrected mortality data were subjected to a probit analysis for calculating the LC_{50} at 95% confidence intervals (CI); normal distribution of the data of the six concentrations tested was verified using the Shapiro-Wilk test. Statistical analysis was conducted using IBM-SPSS v.28.0 (IBM, Armonk, NY, USA).

In order to establish a dose-response relationship and to estimate the highest concentration of EOs at which it did not induce bee mortality significantly higher than that observed in controls (NOAEL, no observed adverse effect level; Medrzycki et al. 2013), a two-way ANOVA followed by Tukey's post-hoc test were applied. Then, it was plotted in Graph Pad Prism v.6.01 for Windows.

For all tests the significance threshold (α) was 0.05. NOAEL was used as part of the most important steps in risk assessment methodologies.

Results

Chemical composition of essential oils

Essential oil yield was highly variable. The highest yield was recorded in *A. herba alba* (mean ± SD = 2.20 ± 0.09%), followed by *A. judaica* (0.84 ± 0.02%), *R. montana* (0.67 ± 0.06%) and *A. campestris* (0.59 ± 0.04%) on a dry-weight basis. Volatile compound identification revealed 29 compounds representing 99.9% of the total *A. campestris* EO (Table 2). Monoterpene hydrocarbons represented the major fraction including β-pinene (19.5%), *p*-cimene (8.1%), α-pinene (7.1%) and δ-terpinene (6.0%) as the major compounds. In parallel, we noted that ketones were the predominant fraction in EOs from *A. herba alba*, *A. judaica* and *R. montana* of which davanone (66.9%), piperitone (68.7%) and 2-undecanone (70.1%) were the chief constituents (Table 2).

Table 2 Chemical composition of the essential oils of *Artemisia campestris*, *A. herba alba*, *A. judaica*, and *Ruta montana*

Components	RI[a]	% Relative area[b]			
		A. campestris	*A. herba alba*	*A. judaica*	*R. montana*
Santolina triene	906	-	0.53	-	-
α-pinene	932	7.11	-	0.61	-
Camphene	946	-	1.35	-	-
Sabinene	969	1.03	-	-	1.94
β-pinene	974	19.51	1.44	-	-
α-phellandrene	1002	2.43	-	0.18	-
α-terpinene	1014	-	-	0.32	-
p-Cymene	1020	8.11	-	0.39	-
D-limonene	1024	4.91	-	-	1.95
β-phellandrene	1025	1.00	0.51	-	-
1,8-Cineole	1026	-	1.84	-	-
β-ocimene (Z)	1032	1.18	-	-	-
β-ocimene (E)	1044	1.79	-	-	-
γ-terpinene	1054	5.98	-	-	-
Cis-Linalool oxide	1067	-	-	0.33	-
2-Nonanone	1087	-	-	-	1.15
β-thujone	1112	-	0.87	-	-
Menth-2-en-1-ol (cis-p)	1118	-	-	0.16	-
Chrysanthenone	1124	-	0.38	-	-
Camphor	1141	-	9.09	-	-
Borneol	1165	-	0.86	-	-
Terpinen-4-ol	1174	1.25	0.33	-	-
2-Decanone	1190	-	-	-	1.77
Trans-piperitol	1207	-	0.29	0.64	-
Cis-carveol	1226	-	0.71	-	-
Davanone nor	1228	-	-	1.43	-
Pepiritone	1249	-	-	68.71	-
Thymol	1289	-	-	3.05	-
2-Undecanone	1293	-	-	-	70.09
2-Undecanol	1301	-	-	-	2.18
α-copaene	1374	1.00	-	-	-
2-Dodecanone	1389	-	-	-	0.97
Jasmone-E	1390	0.78	-	-	-
β-caryophyllene	1408	0.94	1.46	-	3.00
Davanafuran	1414	-	-	0.54	-
Z-β-Farnesene	1440	-	-	-	7.35
Davana ether	1450	-	5.10	-	-
Cinnamic acid	1452	-	-	1.44	-
α-humulene	1452	-	-	-	0.18
(E)-β-Farnesene	1454	-	-	0.54	-
Trans-β-caryophyllene	1464	1.02	-	0.53	-
γ-Muurolene	1478	1.56	-	-	-
α-curcumene	1479	1.41	-	-	-
γ-curcumene	1481	4.69	-	-	-
Germacrene D	1484	4.90	0.92	-	-
Bicyclogermacrene	1500	3.32	-	-	-

Table 2 (continued)

Components	RI[a]	% Relative area[b]			
		A. campestris	*A. herba alba*	*A. judaica*	*R. montana*
α-farnesene	1505	1.51	-	-	0.70
γ-cadinene	1513	1.27	-	-	-
trans-γ-bisabolene	1514	-	-	-	0.33
δ-cadinene	1522	2.89	-	6.33	-
Cadina-1,4-diene (trans)	1533	-	-	0.75	-
Maaliol	1566	-	-	-	0.14
Tridecanol	1570	-	-	-	0.87
Davanone	1587	2.68	66.94	0.67	-
Globulol	1590	7.49	-	2.78	-
Cinnamaldehyde (hydro)	1599	-	-	1.05	-
Tetradecanal	1611	-	-	-	0.25
Davanol D1	1615	-	3.02	-	-
Cedrol (epi)	1618	3.52	-	-	-
Davanol D2	1627	-	0.83	-	-
γ-Eudesmol	1630	1.19	-	-	0.52
Gossonorol	1636	-	-	1.35	-
Cadinol (epi-α)	1638	-	-	0.60	-
Muurolol (epi-α)	1640	-	0.89	-	-
Methyl jasmonate Z	1648	-	-	2.12	-
β-Eudesmol	1649	-	-	1.55	-
α-Cadinol	1652	4.31	-	-	-
α-Bisabolol	1685	-	0.78	-	-
Cedren-13-ol	1688	-	-	1.58	-
Junicedranol	1692	-	-	1.22	-
Caryophyllene acetate	1701	-	1.76	-	-
Tridecenol acetate (2E)	1703	1.10	-	-	-
Methyl tetradecanoate	1722	-	-	-	0.28
Pentadecanol	1773	-	-	-	1.02
Buccocamphor	-	-	-	1.01	-
2-Nonen-4-one	-	-	-	-	2.46
2.4-Pentanedione	-	-	-	-	2.84
Monoterpene hydrocarbons (%)		53.05	3.83	1.50	3.89
Oxygenated monoterpenes (%)		1.25	13.12	5.20	-
Sesquiterpene hydrocarbons (%)		24.51	2.38	8.17	11.56
Oxygenated sesquiterpenes (%)		16.51	10.62	9.07	1.96
Ketones (%)		3.46	68.19	70.81	79.26
Total identified compounds (%)		99.88	99.90	99.90	99.99

[a]Retention index relative to n-alkanes from literature (Adams 2017)

[b]Relative area percentage (peak area relative to the total peak area)

Acaricidal activity of essential oils on *Varroa destructor*

Artemisia herba Alba essential oil

Figure 1A shows *V. destructor* corrected mortality rates as a function of time at different concentrations of the tested EO. Mite mortality in the control was satisfactory in the range of 0-6.7%, less than the 10% threshold to reject the test. ANOVA indicates that EO concentration ($F_{3,36} = 92.7$, $P < 0.001$) and exposure time ($F_{2,36} = 3.7$, $P < 0.05$) have a strong effect on mite mortality, but not their interaction ($F_{6,36} = 0.7$, $P > 0.05$). Mortality rate after 24 h of the application of 2.5 µL/mL (65.51 ± 21.53%) was higher than that obtained after 72 h of exposure for 1 µL/mL (25 ± 10.71%). Complete mortality (100%) was registered for high concentrations, such as 5 µL/mL for 72 h and 10 µL/mL irrespective of exposure time – significantly higher than in the treatments (Fig. 1A).

Artemisia campestris essential oil

Mite mortality was affected by the *A. campestris* EO concentration ($F_{3,36} = 63.6$, $P < 0.001$) and exposure time ($F_{2,36} = 6.5$, $P < 0.05$), but not by their interaction ($F_{6,36} = 1.4$, $P > 0.05$) (Fig. 1B). For 5 µL/mL, the mortality reached 71.43 ± 6.18% after 72 h of exposure, much higher than that reached after 24 h of exposure (27.59 ± 17.92%). A high mortality rate of

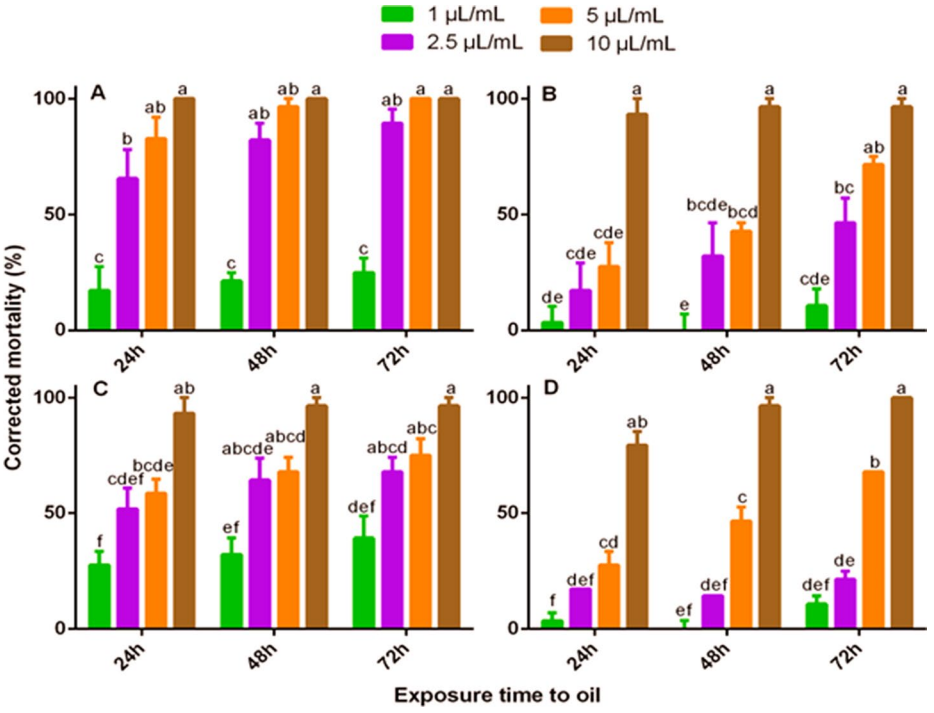

Fig. 1 Mean (+SE) corrected mortality rates of *Varroa destructor* as a function of time and of the concentration of essential oils of (**A**) *Artemisia herba alba*, (**B**) *A. campestris*, (**C**) *A. judaica*, and (**D**) *Ruta montana.* Means within a panel capped with different letters are significantly different (Tukey's HSD test: $P < 0.05$)

96.42 ± 06.19% was reached after 48 h of exposure to 10 µL/mL which differed significantly from that of the other treatments (Fig. 1B).

Artemisia Judaica essential oil

The developmental mortality rate of the mite is concentration dependent ($F_{3,36}$ = 40.1, P < 0.001) but is not affected by exposure time ($F_{2,36}$ = 2.9, P > 0.05), nor by the interaction of time and concentration ($F_{6,36}$ = 0.23, P > 0.05) (Fig. 1C). High mortality rates were recorded for all the tested concentrations. For each concentration, mortality rates at 48 and 72 h were not different compared to that after 24 h post-treatment. Mortality rate for 10 µL/mL was significantly higher than that of 1 µL/mL irrespective of exposure time, ranging from 93.10 ± 11.94 to 96.42 ± 06.19% and from 27.59 ± 10.34 to 39.28 ± 16.36%, respectively (Fig. 1C).

Ruta Montana essential oil

The *V. destructor* mortality rate is influenced by the concentration of *R. montana* EO ($F_{3,36}$ = 312.5, P < 0.001), the exposure time ($F_{2,36}$ = 23.0, P < 0.001) as well as by their interaction ($F_{6,36}$ = 5.5, P < 0.05) (Fig. 1D). The mortality recorded increased with an increase in concentration and in time of exposure. Clearly, 10 µL/mL had the highest mortality rate which differed significantly from that of the other concentrations for each exposure time (Fig. 1D).

Apis mellifera adults: toxicity evaluation

Mortality rates of adult honey bees exposed for 24, 48 and 72 h to a range of concentrations of the four essential oils were determined (Fig. 2). In addition, NOAEL, the highest concentration at which honey bee mortality is not significantly different from the control group, was determined for each EO and thymol after 72 h post treatment (Table 3). In parallel, *V. destructor* mortality rates recorded for these NOAEL values were indicated and were apparently important ranging from 96.7 to 100% (Table 3). The solvent control treatment caused negligible mortality of bees (0–3.3%). The NOAEL of *A. campestris* EO was the highest with a value of 20 µL/mL among the five tested products, indicating that it had the lowest toxicity to honey bees. On that account, all volatile oils tested had a satisfactory acaricidal effect and an acceptable toxicity to bees.

Determination of lethal concentrations and selectivity indexes

The LC_{50} values for EOs against *V. destructor* and newly-emerged worker bees were calculated based on the corrected rates of mortality using probit analysis (Table 4). The greatest toxicity of the EOs to *Varroa* mites was observed for *A. herba alba* and *A. judaica* followed by *A. campestris* and *R. montana* after 24 h (LC_{50} = 2.025–5.725 µL/mL), 48 h (LC_{50} = 1.538–4.92 µL/mL) and 72 h (LC_{50} = 1.347–3.549 µL/mL), but less toxic than the positive control (LC_{50} = 0.447–0.375 µL/mL). Note that all tested EOs had a very low toxicity to honey bees, they were even less toxic to both *V. destructor* mites and newly-emerged honey bees than the positive control (Table 4).

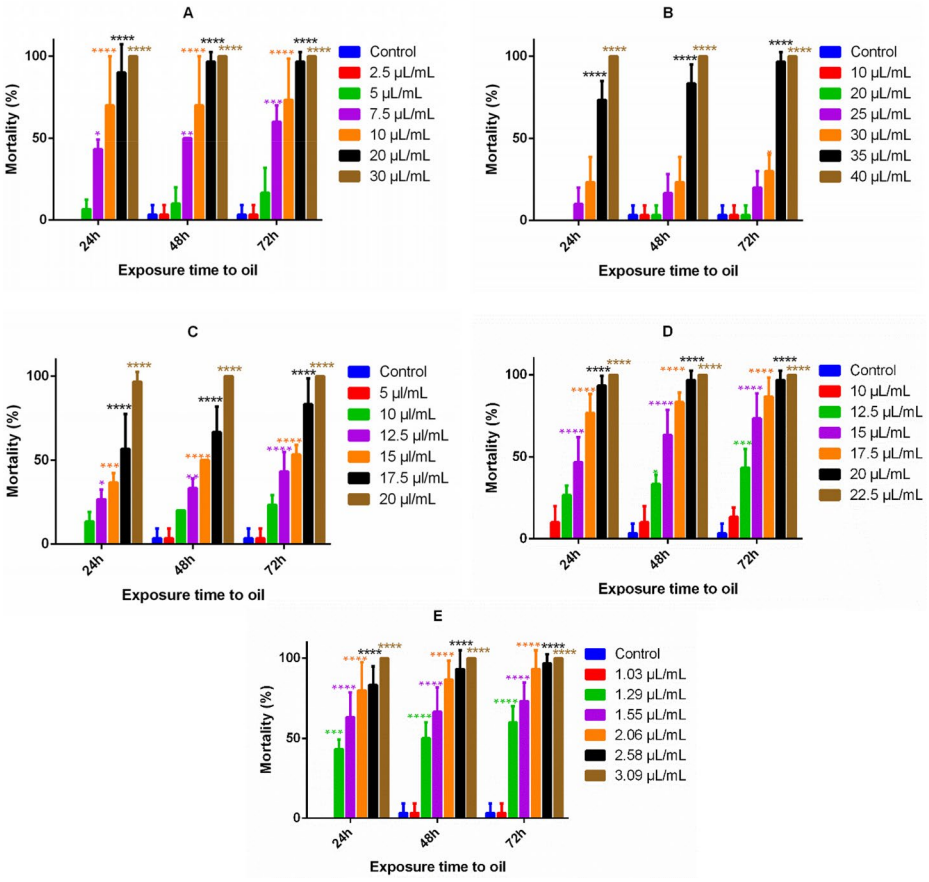

Fig. 2 Mean (+SD) mortality (%) of adult honey bees exposed to a range of concentrations of essential oils of (**A**) *Artemisia herba alba*, (**B**) *A. campestris*, (**C**) *A. judaica*, and (**D**) *Ruta montana*, and (**E**) thymol (positive control). Asterisks indicate significant differences compared to the resp. negative control (i.e., ethanol) (Tukey's HSD test: ****$p<0.0001$, ***$0.0001<p<0.001$, **$0.001<p<0.01$, *$0.01<p<0.05$)

Table 3 Estimated 'no observed adverse effect level' (NOAEL) and mean (±SD) *Varroa* mortality rates (%) after 72 h of exposure

Essential oil	NOAEL (µL/mL)	*Varroa* mortality (%)
Artemisia herba alba	5	100±0.0
A. campestris	25	100±0.0
A. judaica	10	96.66±5.77
Ruta montana	10	100±0.0
Thymol	1.03	100±0.0

Table 4 LC_{50} (µL/mL) values (+95% CI in parentheses) of essential oils and thymol (positive control) on *Varroa destructor* and *Apis mellifera* and their selectivity ratio at 24, 48 and 72 h using complete exposure method under laboratory conditions

EO	Species	24 h	48 h	72 h
Thymol	*V. destructor*	0.447 (0.299–0.594)	0.400 (0.332–0.464)	0.375 (0.301–0.445)
	A. mellifera	1.531 (1.098–1.93)	1.456 (1.164–1.731)	1.370 (1.033–1.664)
	Selectivity ratio	3.42	3.64	3.65
Artemisia herba alba	*V. destructor*	2.025 (1.809–2.265)	1.538 (1.389–1.704)	1.347 (1.226–1.483)
	A. mellifera	8.726 (7.019–10.864)	8.245 (7.327–9.306)	7.569 (6.576–8.672)
	Selectivity ratio	4.31	5.36	5.62
A. campestris	*V. destructor*	5.26 (3.082–8.261)	4.204 (2.679–5.848)	2.781 (2.466–3.102)
	A. mellifera	31.718 (29.389–34.194)	31.146 (27.917–34.66)	29.951 (26.178–34.035)
	Selectivity ratio	6.03	7.41	10.77
A. judaica	*V. destructor*	2.466 (1.196–3.777)	1.895 (0.814–2.944)	1.517 (0.819–2.183)
	A. mellifera	15.184 (12.785–18.617)	14.246 (11.976–16.668)	13.323 (11.453–14.975)
	Selectivity ratio	6.15	7.51	8.78
Ruta montana	*V. destructor*	5.725 (3.96–8.101)	4.92 (4.021–5.845)	3.549 (2.069–5.286)
	A. mellifera	14.463 (13.388–15.498)	13.931 (13.517–14.334)	13.231 (12.799–13.642)
	Selectivity ratio	2.52	2.83	3.73

By far, the highest selectivity ratio values were reached at 72 h post-treatment by *A. campestris* EO (SR = 10.77) followed by *A. judaica* EO (SR = 8.78) whereas moderate values were recorded for *A. herba alba* EO (SR = 5.62) and *R. montana* EO (SR = 3.73). Essential oils appear to be more selective than thymol (positive control), which presented the lowest SR values of 3.42 (24 h), 3.64 (48 h) and 3.65 (72 h).

Discussion

Artemisia herba alba EO yield (2.2%) appears to be in the same range as results from Spain (0.41–2.30%; Salido et al. 2004) but much higher than those reported in other studies of the MENA (Middle East and North Africa) region (0.33–1.18%; Dob and Benabdelkader 2006; Hudaib and Aburjai 2006; Lakehal et al. 2017; Amor et al. 2019; Aimad et al. 2022; Kadri et al. 2022). The literature indicated high variability of EO yields for *A. campestris* from Algeria where our yield of 0.59% is similar to that obtained by Cheraif et al. (2020) from Laghouat, Algeria (0.52%), much higher than that obtained from Djelfa, Algeria region (0.1%) (Dob et al. 2005), but much lower compared to Boutemak et al. (2017) data (0.88%).

Artemisia judaica EO yield (0.84%) was higher than that given by both Dob and Chelghoum (2006) and Abd-Elhady (2012) from Algeria (0.7%) and Egypt (0.7%), respectively. In addition, it was much lower than that obtained by Drioueche et al. (2022) from Algeria (2%) and Mohammed et al. (2022) from Saudi Arabia (1.71%). Similarly, the obtained EO yield of *R. montana* of 0.67% is low compared to previously reported yields (0.26–2.5%; Kambouche et al. 2008; Hammami et al. 2015; Bouzeraa et al. 2019; Benali et al. 2020; Zeraib et al. 2021).

According to GC-MS data, our *A. herba alba* EO belongs to the davanone chemotype (66.9%) followed by camphor (9.1%) and davana ether (5.1%). In fact, our result matched data from Abdelali et al. (2023) of *A. herba-alba* originating from the same locality, from

Cheraif et al. (2020) for the Laghouat region (Algeria) and from Salido et al. (2004) for southern Spain, which also had very high davanone contents. Other chemotypes of *A. herba alba* from Algeria, Tunisia, Libya and Morocco contain various compositions with major compounds identified as α and β-thujone, camphor, 1,8–cineole, chrysanthenone and cis-Chrysanthenyl acetate (Janaćković et al. 2015; Younsi et al. 2017; Aljaiyash et al. 2018; Bekka-Hadji et al. 2022). These compounds are present in our EO but in relatively small amounts (< 10%). *Artemisia campestris* EO revealed a high content of β-pinene (19.5%), *p*-cimene (8.1%), globulol (7.5%), α-pinene (7.1%) and δ-terpinene (6.0%). Abundance of β-pinene in this EO is in agreement with previous studies on *A. campestris* from Algeria (15.2%), Tunisia (36.4%) and Portugal (54.5%) (Abidi et al. 2018; Ammar et al. 2020; Rocha et al. 2021), whereas other studies revealed that germacrene D is the major constituent for cultivars from Tunisia and Morocco (Al Jahid et al. 2016; Younsi et al. 2017). A different EO profile is reported by Baykan Erel et al. (2012) with 1,2-dehydro acenaphthylene (20.7%), tremetone (15.8%), and capillin (10.4%) as major compounds. Piperitone (68.7%) was the major compound of *A. judaica* followed by δ-cadinene (6.3%) and thymol (3.1%), which is in agreement with the high percentage of piperitone in plants collected from both Tamanrasset (91.8%) and Ilizi (61.9%) regions in Algeria (Dob and Chelghoum 2006; Drioueche et al. 2022) and other parts of the MENA region (Abd-Elhady 2012; Abu-darwish et al. 2016; Mohammed et al. 2022). Al-Wahaibi et al. (2018) reported β-eudesmol (13.1%), hexadecanoic acid (5.7%) and spathulenol (3.7%) as major compounds in this EO from Saudi Arabia. Essential oil from *R. montana* samples is found to be dominated by the aliphatic ketone 2-undecanone (70.1%) followed by Z-β-farnesene (7.4%) and caryophyllene (3.0%). This result is in agreement with the > 78.5% of 2-undecanone in semi-arid regions compared to those from humid regions (≤ 30.3%; Mohammedi et al. 2019). Hammami et al. (2015) have reported other main components of this EO from Tunisia, namely 1-butene (38.3%), methylcyclopropane (15.5%), 2-butene (22.6%) and caryophyllene oxide (8.2%). Overall, variation and diversity in EO yields and chemical composition within the same cultivar are associated with several factors including climate and environmental conditions, soil geology, harvesting time as well as drying and extraction methods (Zeragui et al. 2019; Mohammed et al. 2022).

For the evaluation of the acute toxicity of EOs for *V. destructor*, the complete exposure method in closed Petri dishes was selected. According to Maggi et al. (2010), in complete exposure, EOs action on mites occurred by contact, fumigation and also through ingestion. An effective varroacidal agent has to present high toxicity to the mites in combination with low lethality to honey bees (Brasesco et al. 2017). Our in vitro bioassays showed that the four tested EOs overall have a potent concentration- and exposure time-dependent acaricidal effect against *V. destructor.*

On the other hand, EOs' toxicity for honey bee adults was quite low – NOAEL values were calculated for *A. campestris* (25 µL/mL), *A. judaica* (10 µL/mL), *R. montana* (10 µL/mL) and *A. herba alba* (5 µL/mL), and at these concentrations *V. destructor* lethality ranged from 96.7 to 100%. These results indicate clearly the safety of the tested EOs for *A. mellifera* honey bee workers and their efficacy to control *V. destructor*.

Selectivity index is a key parameter to evaluate the miticidal potential based on the LC_{50} values calculated for both mites and bees. Thymol, an organic treatment largely used in beekeeping practice for *Varroa* control worldwide (Damiani et al. 2009; Glavinic et al. 2022) was used as positive control. After 24 h of exposure, thymol had a SR of 3.42, lower than

that of *A. judaica* (6.15), *A. campestris* (6.03) and *A. herba alba* (4.31), and higher than that of *R. montana* (2.52). The SR value of thymol showed a slight increase to 3.65 after 72 h of exposure. Our results are in agreement with the 3.2 after 72 h of exposure found by Hýbl et al. (2021). The highest SR values at the end of the experiment were attributed to *A. campestris* (SR = 10.77) and *A. judaica* (SR = 8.78) followed by *A. herba alba* (SR = 5.62) and *R. montana* (SR = 3.73) – these values are in the same range with some reported in the literature (Conti et al. 2020; Hýbl et al. 2021).

Our findings indicated that *A. campestris* EO possessed the strongest acaricidal activity against *V. destructor*. Some studies had investigated its anthelmintic action (Abidi et al. 2018; Akkari et al. 2014), and recently, Ammar et al. (2020) explored its insecticidal effect against *Culex quinquefasciatus*, *Musca domestica*, and *Spodoptera littoralis*. Results showed larvicidal activity against *C. quinquefasciatus* and *M. domestica* with LC_{50} = 45.8 mg/L and LD_{50} = 99.8 µg/adult, respectively. *Artemisia campestris* EO was dominated by monoterpenes (53.1%) that are thought to act on the mite's capability to reproduce (Imdorf et al. 1999). Notably, several studies have confirmed the effectiveness of monoterpenes against *V. destructor* (Fassbinder et al. 2002; Gashout and Guzmán-Novoa 2009), and also against ticks such as *Hyalomma marginatum* adults (Cetin et al. 2010) and *Ixodes ricinus* nymphs (Thorsell et al. 2006). A recent study showed that β-pinene-rich EOs from *Citrus* spp. similar to our *A. campestris* EO presented a potent acaricidal activity towards *V. destructor* (Bava et al. 2021). Da Camara et al. (2020) demonstrated the toxicity of β-pinene, *p*-cimene, α-pinene and γ-terpinene against the two-spotted spider mite, *Tetranychus urticae*, both by fumigation and residual contact bioassays.

Artemisia judaica oil with high amount of ketones (70.8%) also has a good in vitro acaricidal effect towards *V. destructor*. Our study is the first reporting this activity. El-Sharabasy (2010) tested crude *A. judaica* extracts (using ethanol, acetone and petroleum ether organic solvents) against *T. urticae* and concluded strong acaricidal activity. Other properties were also attributed to its EO such as antimicrobial, insecticidal, antifeedant and antifungal action (Mohamed and Abdelgaleil 2008; Benmansour et al. 2016). High piperitone content of 68.7% in *A. judaica* EO incriminates its responsibility in the pronounced miticidal activity. Abdelgaleil et al. (2008) demonstrated insecticidal (LD_{50} = 0.68 µg/larva), and antifeedant activities of piperitone against *S. littoralis*. In the same way, piperitone from *Cymbopogon schoenanthus* expressed pronounced efficacy on the seed beetle *Callosobruchus maculatus* with LC_{50} = 1.6 µL/L (Ketoh et al. 2006).

Essential oil from *A. herba alba* achieved a noteworthy toxicity on *V. destructor* with SR = 5.62 at 72 h post treatment. Alahyane et al. (2022) also demonstrated acaricidal activity of *A. herba alba* EO on *V. destructor* in vitro, by fumigation. Still, they also reported a high fumigant toxicity of this EO to *A. mellifera* pupae (LC_{50} = 10.53 µL/L air); no toxicity data on *A. mellifera* adults were reported. Recently, *A. herba alba* EO was found to exhibit a very promising outlook as an acaricidal agent on *I. ricinus* ticks with LC_{50} = 0.3 µL/cm^2 after 2 h exposure (Elmhalli et al. 2021). El-Seedi et al. (2017) revealed that oils rich in piperitone exert a high repellent behavior up to 84.2% against *I. ricinus*.

Ruta montana EO had a relatively low efficacy in varroa control compared to the *Artemisia* EOs; however, it performed still better than thymol. Currently, no reports in literature about *R. montana* EO acaricidal activity exist. Bouzeraa et al. (2019) pointed at toxic and repellent properties against *Ephestia kuehniella* larvae of *R. montana* EO. The larvicidal effect of the hydro-methanolic extract of *R. montana* on vectors of the avian plasmodium

Culiseta longiareolata was significant (Bouabida and Dris 2022). An ethnobotanical survey by Carvalho da Silva et al. (2023) in Portugal, portrayed *R. graveolens* with ectoparasiticide power for dogs and livestock. More evidences touted insecticidal and repellent effects of EOs from various *Ruta* species (Conti et al. 2013; Akkari et al. 2014; Tampe et al. 2016; Achimón et al. 2022; Wang et al. 2022). Apparently, 2-undecanone, the major compound in *R. montana* EO, might be responsible for that miticidal effect due to its antifungal, anti-inflammatory, and insect repellent properties (Liu et al. 2015; Bailly 2023).

Our most surprising finding was that, besides monoterpenes which are well documented for their acaricidal activity against the honey bee pest mite *V. destructor*, ketones (piperitone, davanone and 2-undecanone) may be considered as promising natural candidates to fight *V. destructor* due to the good tolerance by bees and their strong varroacidal effect.

These findings must be followed up with trials in field conditions in order to optimize the delivery mode, exposure period, colony status (with or without brood) as well as the influence of climate conditions. The abundance of these plant species to beekeepers and relatively easy processing method to obtain their EOs make them practical and cost-effective parts of an integrated pest management control strategy to fight this parasitic mite and help protect the economically relevant honey bees.

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