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Hemp (*Cannabis sativa* cv. Kompolti) essential oil and its nanoemulsion: Prospects for insecticide development and impact on non-target microcrustaceans

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ABSTRACT

Formulation development and non-target effect studies are two crucial steps for the registration of new botanical insecticides. Previous reports put in evidence the potential of hemp (*Cannabis sativa* L.) essential oil (EO) as a new candidate ingredient for developing green insecticides. However, the validation of its target and non-target effects after proper encapsulation is missing so far. In the current work, we investigated the insecticidal efficacy of the EO obtained from the hemp variety 'Kompolti', and we optimized its formulation development into a nanoemulsion (NE). The latter was assayed against larvae of *Culex quinquefasciatus* Say, a well-known vector of *Wuchereria bancrofti* Cobbold, avian malaria, and several arboviruses, such as Western equine encephalitis virus, St. Louis encephalitis virus, West Nile virus, and Zika virus. Considering the percentage of encapsulated EO (7.5% w/w), the developed NE showed mortality on *Cx. quinquefasciatus* larvae, in terms of $LC_{50(90)}$ values [72.2 (207.2) ppm], like those of pure EO [50.8(142.3) ppm]. Non-target toxicity experiments showed limited toxicity on the aquatic microcrustacean *Daphnia magna* Straus (i.e., mortality <16% after 48 h of exposure to the EO-based NE at the LC_{90} estimated on mosquito larvae). Considering that the LC_{50} values detected in this study are lower than those belonging to many EOs, and that the application at sublethal doses caused mortality between 39.1% and 40.8% as well as a reduction in mosquito fertility resulting in a reduction in natality of 40.4% and 45.1% for the EO and EO-based NE, respectively, both products may be considered as potential environmentally friendly and sustainable insecticides.

1. Introduction

Industrial hemp (*Cannabis sativa* L.) crop represents a limitless source of different products in textile, personal care, food, nutraceutical, and pharmaceutical fields (Mark and Will, 2019). Hemp products market is progressively evolving especially in the last years, and hemp industry sales are expected to grow to $1.9 billion by 2022 in USA, led by cannabidiol (CBD)-based items (The Hemp Business Journal, 2018). In Europe, as in the rest of the world, hemp production rebounded by recently exceeding 70,000 acres, due to the increased production of seeds for food consumption, and hemp fibers for the automobile sector, along with the rising demand for CBD (European Industrial Hemp Association, 2017). In this context, hemp essential oil (EO) is achieving more and more attention and interest, because of its potential exploitation as a valuable product in the pharmaceutical, nutraceutical, cosmeceutical, and pest/vector management areas. In this latter area falls the management of *Culex* (Diptera: Culicidae) mosquitoes, which act as dangerous vectors of pathogens causing important diseases (Fonseca et al., 2004; Di Giovanni et al., 2021), including West Nile virus, lymphatic filariasis, Japanese and Saint Louis encephalitis, along with Rift Valley fever, and dengue, which seriously affect both humans and animals worldwide (Hamer et al., 2008; Kauffman and Kramer, 2017;

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Wilke et al., 2020). The frequent overuse of synthetic products for mosquito vector control is leading to insecticide resistance increasing phenomena (Lenormand et al., 1999; Raymond et al., 2001; Rivero et al., 2011). For example, in Cameroon, a significant resistance profile was recently reported for *Cx. quinquefasciatus* Say mosquitoes towards several common pesticides belonging to pyrethroid, carbamate, and organophosphate groups (Talipouo et al., 2021). In this framework, sustainable and effective vector control strategies are urgently needed (Benelli, 2015). Among promising tools, green insecticide agents appear to be worthy of investigation and exploitation, due to less harmful consequences on the environment and the human health, a limited residuality, and fewer resistance issues, with respect to those of chemical pesticides (Rincón et al., 2019; Pavela et al., 2019b). In this regard, plant EOs, generally recognized as safe products (GRAS) by Food and Drug Administration (FDA) and Flavor and Extract Manufacturers Association (FEMA), are gaining value as eco-friendly botanical insecticides (Pavoni et al., 2020a). Among them, hemp EOs were successfully tested against various arthropods of economic importance, such as aphids, mosquitoes, termites, and mites (Górski et al., 2016; Rossi et al., 2020; Tabari et al., 2020). Notably, *C. sativa* EOs from Felina 32 and Futura 75 varieties were reported to be effective against *Cx. quinquefasciatus* by Thomas et al. (2000) and Benelli et al. (2018a; b). To our knowledge, Kompolti hemp EOs were previously evaluated for antimicrobial (Novak et al., 2001), and antimycotic (Di Sotto et al., 2022) properties, while literature data on their insecticidal, acaricidal, and repellent effect are missing. To overcome the main limiting factors in the use of EOs as active ingredients for green insecticide formulations, namely the high volatility, low stability, thermal degradation, oxidative decomposition, and scarce water solubility, innovative EOs delivery systems are being recently studied (Turek and Stintzing, 2013; Pavela and Benelli, 2016). In this respect, EOs encapsulation in micro- and nanoemulsions (NEs) has turned out to be one of the most promising solutions for improving EOs physicochemical characteristics, bioavailability, and administration (Pavoni et al., 2019; Benelli et al., 2020; Ricupero et al., 2022).

Formulation development and non-target impact investigations are two key steps for the registration of new botanical pesticides. Previous studies evidenced the potential of hemp EO as a new promising ingredient for green insecticides (Górski et al., 2016; Rossi et al., 2020; Tabari et al., 2020; Thomas et al., 2000; Benelli et al., 2018a; b). However, the optimization of its proper encapsulation into nanocarriers and the validation of its target and non-target effects are missing so far. Actually, the encapsulation of EO into nanoemulsion can improve its dispersibility in stagnant water increasing the toxicity on mosquito larvae. On the other hand, we questioned: may the nanoformulation of hemp EO lead to increased toxicity to non-target aquatic microcrustaceans?

On the above, in this study the Kompolti variety fresh inflorescences were employed to produce an EO to be evaluated for its larvicidal activity against *Cx. quinquefasciatus*; then an EO-based NE was developed, and its mosquitocidal performances were compared with that of the raw EO. Furthermore, the sublethal effects triggered by being exposed to both insecticidal products on the mosquito life cycle were explored, in terms of adult emergence, fecundity, fertility, and natality. Since the ecological impact of EO-based NE is still understudied (Giunti et al., 2022), an experiment to assess the toxicity of both products was conducted on the non-target aquatic microcrustacean *Daphnia magna* Straus (Cladocera: Daphniidae).

2. Materials and methods

2.1. Crop material

The biomass of Kompolti hemp variety was cultivated by "La Biologica" farm, sited in central Italy, Marche region (Fiuminata, N 43°11'11", E 12°56'24", 318 m a.s.l.), and harvested in the first weeks of September 2019. The plant material was represented by fresh female inflorescences, that were transported to Prof. F. Maggi laboratory (University of Camerino, UNICAM), and immediately processed to obtain the EO.

2.2. Steam distillation (SD)

In this work, 2.9 kg of inflorescences were exposed for 3 h to the steam generated by inserting 2.2 L of distilled water into a 20 L stainless steel apparatus by Albrigi Luigi E0106 (Stallavena di Grezzana-Verona, Italy). The EO was produced through a steel Clevenger-type apparatus, and collected from a glass burette by separating it from water. Then, PTFE-silicon septa-sealed vials were employed to store the EO at 4 °C before the following analysis. The EO yield was calculated on dry matter (w/w), after hemp moisture content estimation on a thermo balance (Scaltec SMO 01, Germany) at 100 °C, that accounted for 58.0%.

2.3. EO GC-MS chemical characterization

Gas chromatography-mass spectrometry (GC-MS) investigation was conducted on Kompolti EO to evaluate its chemical composition. The employed GC system was an Agilent 6890 N, with a 5973 N MS spectrometer and 7863 autosampler (Agilent, Wilmington, DE). The separation of EO volatile constituents was achieved through a (5%-phenyl)-methylpolysiloxane coated capillary column (HP-5 MS, 30 m l., 0.25 mm i.d., 0.1 μm f.t., Agilent, Folsom, CA, USA). The oven was thermostated at 60 °C for 5 min, then the temperature was raised to 220 °C at 4 °C/min, afterwards until 280 °C at 11 °C/min and held for 15 min. Injector and detector temperatures were set at 280 °C. The mobile phase was represented by 99.9% He (flow of 1 mL/min). The EO was diluted in *n*-hexane 1:100, and this solution (2 μL) was introduced in the injection port in split mode (1:50 split ratio). The electron ionization mode (EI, 70 eV) was employed in full scan in the MS range 29–400 *m/z*, in order to acquire the chromatograms, that were evaluated by using the software MSD ChemStation (Agilent, Version G1701DA D.01.00). For compounds identification, Standard solutions (Sigma-Aldrich) were injected; moreover, the retention indices (RI), calculated through Van den Dool, Kratz (1963) with respect to a C_8-C_{30} mix of *n*-alkanes (Supelco, Bellefonte, CA, USA), were compared with those present in ADAMS, FFNSC2, and NIST 17 libraries (Adams, 2007; FFNSC, 2015; NIST 17, 2017). The same libraries were checked to match MS spectra of the unknown compounds with the reported ones.

2.4. EO GC-FID chemical characterization

The quantification of α-pinene, β-pinene, myrcene, limonene, 1,8-cineole, (*E*)-β-ocimene, terpinolene, (*E*)-caryophyllene, α-humulene, caryophyllene oxide, and CBD, as hemp EO marker compounds, was achieved through gas chromatography coupled to flame ionization detector (GC-FID) (Fiorini et al., 2020). An Agilent 6850 GC apparatus was employed, and after a 1:100 dilution of hemp EO in *n*-hexane of analytical grade, 0.5 μL of the solution were injected in split mode (1:30). Injector and FID temperatures were 300 °C and 360 °C, respectively. Hydrogen was the carrier gas, deriving from a PGH2–250 generator (DBS Analytical Instruments, Vigonza, Italy), with an initial flow into the column of 3.7 mL/min. A (5%-phenyl)-methylpolysiloxane coated capillary column (HP-5 MS, 30 m l., 0.25 mm i.d., 0.25 μm f.t., Agilent, Folsom, CA, USA) was used. The total run time was 15.60 min, and the oven temperature was 60 °C for 3 min, then increased to 350 °C at 25 °C/min and maintained for 1 min. Hydrogen and air flows were 40 and 400 mL/min, respectively. The calibration curves were made in 0.004–9.6 mg/mL range, by analysing analytical standards purchased by Sigma-Aldrich (Milan, Italy). β-Pinene was quantified by using α-pinene calibration curve, limonene, and 1,8-cineole by that of terpinolene, while myrcene calibration curve was employed to quantify (*E*)-β-ocimene.

2.5. EO encapsulation in nanoemulsions and their characterization

High-pressure homogenization (French pressure cell press, AMINCO USA) was used to prepare Kompolti EO-based NEs according to the reported procedure (Pavoni et al., 2020b). The composition of the prepared NEs using polysorbate 80 as surfactant and ethyl oleate as co-solvent is detailed in Table 1. Size distribution, in terms of Z-average, polydispersity index (PDI), and physical stability of NEs, stored at room temperature in tight closed vials over time up to six months, was assessed by dynamic light scattering (Malvern, UK) measurements (Kavallieratos et al., 2021).

2.6. Insecticidal assays

2.6.1. Acute toxicity trials on mosquitoes

Culex quinquefasciatus larvae (3rd instar) were mass-reared as described by Pavela (2014). Acute toxicity of the hemp EO and EO-based NE on the larvae was evaluated as mortality after 24 h. Larvicidal tests were done according to WHO (1996), with minor adjustments (Benelli et al., 2018a). The hemp EO was diluted in DMSO, while NE was used without further modification, diluted as follows, EO: 20, 40, 60, 80, 100, and 120 ppm; EO-based NE: 300, 500, 800, 1000, 1500, 2000, and 2500 ppm. For trials, the appropriate amount of EO or NE was added to 200 mL of distilled water in a 500 mL beaker, and thoroughly stirred. Larvae were moved to each bowl (25 larvae/bowl); 4 replications were run simultaneously. Test conditions were 16:8 (L:D) and 26°C. Mortality was assessed on starved larvae after 24 h.

2.6.2. Sublethal effects on mosquito development and adult reproductive parameters

According to Spinozzi et al. (2021), the effect of low concentrations (LC_{30}) on *Cx. quinquefasciatus* was assessed. Briefly, 100 3rd instar larvae of *Cx. quinquefasciatus* were moved into a plastic bowl (20 × 20 × 20 cm) filled with water (3 L). After an hour, the determined dose LC_{30} for the EO or EO-NE (i.e., 35 and 625 ppm, respectively) was added. The EO was treated with DMSO as described above, and the appropriate amount of DMSO was added to the control. 24 h-post treatment, larvae were relocated into clean water with larval food (Pavela, 2014), until adult emergence. During the experiment, mortality at 24 h, total mortality, number, and sex of newly emerged adults were observed. From each repetition, newly emerged adults were selected for the evaluation of the exposure effect to LC_{30} on the mosquito fecundity, fertility, and natality, following the method by Spinozzi et al. (2021). Ten females and ten males were introduced in breeding cages (25 × 25 × 30 cm) and fed according to Benelli et al. (2017). The oviposition was examined in a bowl (10 cm in diameter filled with water), and the number of laid eggs was evaluated every day using a Leica light microscope. Each test was repeated 4 times at 16:8 (L:D) and 25 ± 1 °C.

Table 1
Composition (%) and formulation parameters of the Kompolti hemp essential oil (EO)-based nanoemulsions (NEs).

Tested hemp NE[a]	Kompolti EO (% w/w)	Ethyl oleate (EtO) (% w/w)	Total oil phase (% w/w)	Polysorbate 80 (% w/w)	EtO/EO ratio	Surfactant/Total oil phase ratio
NE_1	3	3	6	4	1	0.66
NE_2	5	1	6	2	0.2	0.33
NE_3	4	2	6	2	0.5	0.33
NE_4	5	1	6	5	0.2	0.83
NE_5	7.5	1.5	9	2	0.2	0.33
NE_6	7.5	1.5	9	5	0.2	0.83

[a] The remaining percentage in each NE formulation is represented by ultrapure water.

2.6.3. Non-target effect of EO and EO-based NE on *Daphnia magna*

Daphnia magna adults were reared following Pavela (2014), and maintained at 25 ± 1 °C and 16:8 (L:D). Acute toxicity assays were carried out following OECD—Organization for Economic Cooperation and Development (2004), with some modifications (Pavela, 2014). *Daphnia magna* adults (2–5 days old) were exposed to LC_{90} estimated above on *Cx. quinquefasciatus* larvae. Twenty adults of *D. magna* were transferred to plastic dishes which contained 100 mL of water plus the suitable concentration of EO or EO-NE (140 and 2760 ppm, respectively). *Daphnia magna* mortality was assessed under a stereomicroscope after 24 and 48 h. For each concentration, 4 replicates (each conducted with 20 microcrustaceans) were done.

2.6.4. Statistical analysis

In mosquito acute toxicity tests, mortality data were corrected according to Abbott (1925), then probit analysis (Finney, 1971) was used to calculate the lethal concentrations. Sublethal effect and non-target toxicity experiment data were analysed through ANOVA followed by Tukey's HSD test ($p < 0.05$); before the analysis, percentages were transformed to arcsine square root values.

3. Results and discussion

3.1. Hemp essential oil semi-quantitative GC-MS profile

The Kompolti EO obtained by SD presented a yield of 0.10% w/w. A total of 44 constituents were identified, accounting for 98.1% of the whole chemical composition (Table 2). The sesquiterpene (*E*)-caryophyllene represented the most abundant compound (20.4%), although the predominant class in this EO was that of monoterpenes. Among them, the most representative were myrcene (18.9%), *α*-pinene (16.9%), terpinolene (8.1%), and *β*-pinene (6.7%) (Fig. 1). Other minor monoterpenes were limonene and (*E*)-β-ocimene, representing 4.4% and 3.2% of all chemical profile, respectively. The other components belonging to this fraction accounted for 0.3% at most. Beside (*E*)-caryophyllene, other sesquiterpenes were detected in Kompolti EO, especially *α*-humulene (6.1%), caryophyllene oxide (3.6%), selina-3,7(11)-diene (1.8%), *β*-selinene (1.4%), and *α*-selinene (1.1%). The remaining sesquiterpenes were found in negligible amounts (not more than 0.8%). Cannabinoids were almost completely missing, only a low content of cannabidiol (CBD) was indeed detected (0.2%) (Table 2).

Literature reports on Kompolti EO chemical characterization are limited, and this product was obtained by SD process in most of them. The EO yield reported in other works was not much higher than that found in the present study. Actually, it ranged from 0.21% (Di Sotto et al., 2022) to 0.71% w/w (Palmieri et al., 2021) for EO obtained by steam distilled Kompolti dry inflorescences. The composition reported by Meier and Mediavilla (1998) for the EO obtained by Kompolti fresh inflorescences harvested at the beginning of September resulted to be similar to the profile presented in the current work; again (*E*)-caryophyllene dominated over the other constituents, followed by myrcene, and *α*-pinene. Anyway, the monoterpenes fraction was the most abundant one also in this case (Meier and Mediavilla, 1998). The harvesting period affected the Kompolti EO composition, in fact from September to October the content of myrcene increased, in spite that of (*E*)-caryophyllene. Among monoterpenes, myrcene was the most significant constituent also in Kompolti EO analysed by Novak et al. (2001) and Palmieri et al. (2021). In this last work, the influence of the extraction time on Kompolti EO profile was evaluated. As a result, a longer extraction time appeared to generally enhance sesquiterpenes content and reduce monoterpenes amount, probably because the latter are more volatile compounds and are more quickly released from the plant material (Palmieri et al., 2021). The predominance of sesquiterpenes, especially caryophyllene, and caryophyllene oxide and the presence of few monoterpenes were detected in Kompolti EO analysed by Di Sotto et al. (2022). This fact was probably due to the dried status of

Table 2
GC-MS chemical characterization of the essential oil (EO) obtained by steam distillation of Kompolti hemp fresh female inflorescences.

No.	Component[a]	RI[b]	RI lit[c]	%	ID[d]
1	5,5-dimethyl-1-vinylbicyclo[2.1.1] hexane	914	920	0.2	RI,MS
2	α-thujene	918	924	tr[e]	RI,MS
3	α-pinene	923	932	16.9	RI,MS
4	camphene	939	946	0.2	Std,RI, MS
5	β-pinene	968	974	6.7	RI,MS
6	myrcene	989	988	18.9	Std,RI, MS
7	α-phellandrene	1003	1002	0.2	RI,MS
8	δ-3-carene	1005	1008	0.2	RI,MS
9	α-terpinene	1014	1014	0.2	RI,MS
10	*p*-cymene	1019	1020	tr	RI,MS
11	limonene	1024	1024	4.4	RI,MS
12	1,8-cineole	1027	1026	0.2	RI,MS
13	(*Z*)-β-ocimene	1034	1032	0.3	RI,MS
14	(*E*)-β-ocimene	1044	1044	3.2	RI,MS
15	γ-terpinene	1052	1054	0.2	RI,MS
16	*cis*-sabinene hydrate	1061	1065	tr	RI,MS
17	terpinolene	1084	1086	8.1	RI,MS
18	linalool	1097	1095	tr	RI,MS
19	endo-fenchol	1106	1114	tr	RI,MS
20	*trans*-pinene hydrate	1113	1119	tr	RI,MS
21	ipsdienol	1144	1140	tr	RI,MS
22	borneol	1157	1165	tr	RI,MS
23	terpinen-4-ol	1170	1174	0.1	RI,MS
24	*p*-cymen-8-ol	1184	1179	0.1	RI,MS
25	α-terpineol	1186	1186	0.1	RI,MS
26	ylangene	1355	1373	tr	RI,MS
27	(*Z*)-caryophyllene	1397	1408	0.4	RI,MS
28	(*E*)-caryophyllene	1410	1417	20.4	Std,RI, MS
29	α-bergamotene	1425	1432	0.5	RI,MS
30	6,9-guaiadiene	1430	1442	tr	RI,MS
31	α-humulene	1437	1452	6.1	Std,RI, MS
32	allo-aromadendrene	1443	1458	0.4	RI,MS
33	(*E*)-β-farnesene	1449	1454	0.5	RI,MS
34	β-selinene	1468	1489	1.4	RI,MS
35	α-selinene	1477	1498	1.1	RI,MS
36	δ-amorphene	1491	1511	0.1	RI,MS
37	selina-4(15),7(11)-diene	1524	1534	0.3	RI,MS
38	selina-3,7(11)-diene	1530	1538	1.8	RI,MS
39	(*E*)-α-bisabolene	1539	1544	tr	RI,MS
40	(*E*)-nerolidol	1561	1561	0.3	RI,MS
41	caryophyllene oxide	1571	1582	3.6	RI,MS
42	humulene epoxide II	1589	1608	0.8	RI,MS
43	eudesm-7(11)-en-4-ol	1683	1700	tr	RI,MS
44	CBD	2417	2430	0.2	Std,RI, MS
	Total identified (%)			98.1	

[a] Components reported according to the elution order obtained by using a HP-5MS column.
[b] Linear retention index calculated according to Van den Dool and Kratz formula (1963).
[c] Retention index obtained by ADAMS library.
[d] Identification methods: Std, comparison with analytical standards; RI, comparison of the calculated RI with those present in ADAMS and NIST 17 libraries; MS, MS matching with those reported in ADAMS, FFNSC2, and NIST 17 libraries.
[e] tr, traces (% < 0.1).

the biomass; indeed, the drying process should promote monoterpenes evaporation and sesquiterpenes higher concentration (Di Sotto et al., 2022). Regarding cannabinoids, all the cited literature papers recorded their absence, apart from the one by Palmieri et al. (2021). In this context, Zheljazkov and Maggi (2021) highlighted that SD didn't allow the disruption of hemp glandular trichomes, resulting in the production of cannabinoids-poor EO (Zheljazkov and Maggi, 2021). Notably, only CBD was detected by Palmieri et al. (2021), but in higher amounts than that found in the present study. The reason of this discrepancy could again lie in the applied drying conditions and long extraction time, which can determine the conversion of cannabinoid acids into the decarboxylated neutral forms (Palmieri et al., 2021). In conclusion, the reported variability among Kompolti EO chemical profiles found in literature can be traced to several influent factors, such as different cultivation sites and environments, harvesting periods and storage conditions, plant material characteristics, and also distillation modalities and parameters (Novak et al., 2001).

3.2. EO GC-FID profile

The quantitative GC-FID analysis, performed on the EO major terpenes and CBD (Table 3), revealed the predominance of myrcene above all the other chemical constituents (21.3 g/100 g). Secondly, (*E*)-caryophyllene was a significant compound, which was present at a concentration of 14.8 g/100 g, followed by *α*-pinene (12.8 g/100 g). In this regard, GC-FID analysis confirmed GC-MS outcomes (sect. 3.1), in having identified myrcene, (*E*)-caryophyllene, and *α*-pinene as the most abundant constituents of Kompolti EO. Other compounds, detected in lower amounts, were again terpinolene (8.6 g/100 g), *β*-pinene (5.9 g/100 g), and limonene (5.1 g/100 g) among monoterpenes andcaryophyllene oxide (8.8 g/100 g) and *α*-humulene (5.5 g/100 g) among sesquiterpenes. CBD accounted for 1.9 g/100 g. These results were consistent with those presented in other research papers regarding GC-FID analysis of hemp EO, as a confirmation that especially myrcene, (*E*)-caryophyllene, *α*-pinene, and terpinolene can be considered as marker terpenes in *C. sativa* EO (Fiorini et al., 2020; Mazzara et al., 2022a; Mazzara et al., 2022b).

3.3. Preparation and characterization of EO-based nanoemulsions

An initial screening was performed on different Kompolti EO-based NEs prepared by keeping constant at 6% w/w the percentage of total oil phase, and by varying the percentages of polysorbate 80 as a surfactant (2–5% w/w) and the ratio between ethyl oleate (as a cosolvent) and Kompolti EO (from 0.5 to 1) (NE_1, NE_2, NE_3, NE_4 Table 1). All these NEs showed a monomodal droplet size distribution (Fig. 2A), and hydrodynamic diameter (expressed as Z-average) below 200 nm (Table 4). Polydispersity index, an adimensional number representing the polydispersity of the droplets size populations, was slightly lower (< 0.250) for NE_2 and NE_3, which have the lowest surfactant/total oil phase ratio, that is 0.33 (Table 4). According to these results, the NEs having a ethyl oleate/EO ratio of 0.2, and a surfactant to oil phase ratio of 0.33 and 0.83, were also prepared at a 9% w/w of the total oil phase (NE_5 and NE_6). The calculated Z-average values were similar to those of NEs containing 6% w/w of total oil phase, while PDI values were slightly higher (between 0.280 and 0.350). Therefore, in these Kompolti EO-based NEs, PDI seems to be more sensitive than the mean droplet size diameter to the total oil payload of the formulation. Since among all tested formulations, the NE_5 has the higher percentage of Kompolti EO (7.5% w/w), and the lowest percentage of surfactant (2% w/w), it was selected to be investigated in further studies. Physical stability of NE_5 was assessed by following the variation over time of Z-average and PDI. No remarkable changes in both these parameters related to droplet size and droplet size distribution were observed up to six months of storage at room temperature. Indeed, Z-average values remained below 200 nm and PDI values between 0.2 and 0.3 (Fig. 2B).

3.4. Effect of EO and EO-based nanoemulsions on mosquitoes

To date, the existing literature on the development of new EO-based insecticides against mosquitoes has analysed more than 120 plant species, mainly belonging to the families Lamiaceae, Cupressaceae, Rutaceae, Apiaceae, and Myrtaceae (Pavela, 2015; but see also Pavela et al., 2019b). Of note, before EOs can be considered potentially suitable for use as green insecticides, their composition must be known (Isman and

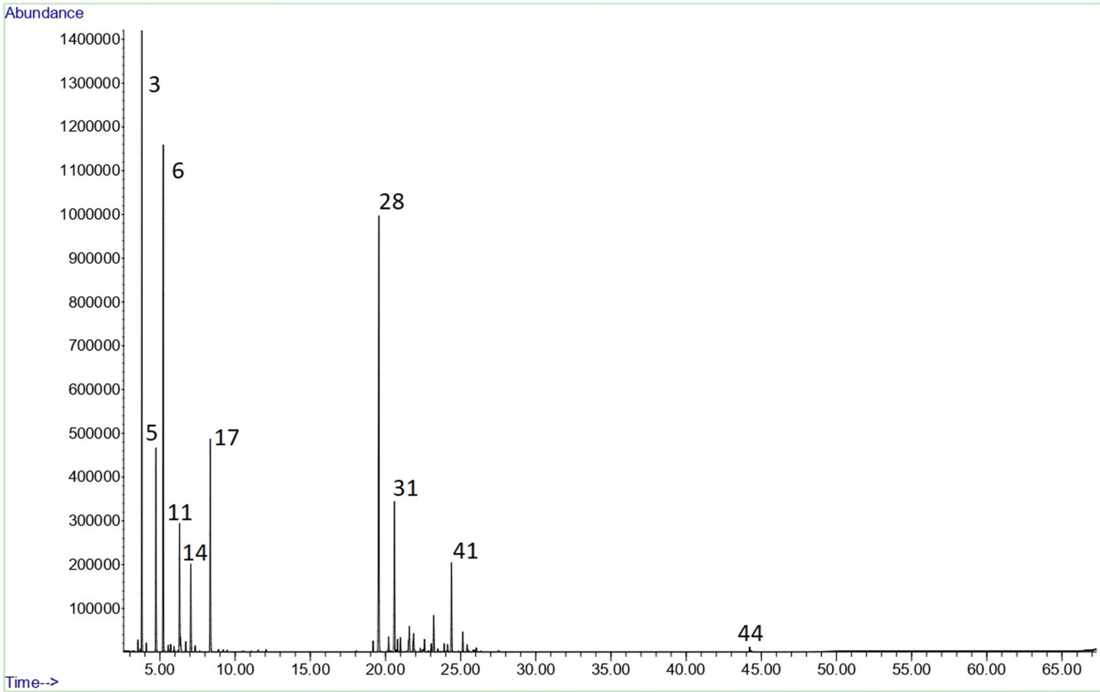

Fig. 1. Kompolti hemp essential oil (EO) GC-MS chromatogram; the peak numbering refers to Table 2.

Table 3
GC-FID chemical characterization of the essential oil (EO) obtained by steam distillation of Kompolti hemp fresh female inflorescences.

Compound	g/100 g
α-pinene	12.8
β-pinene	5.9
myrcene	21.3
limonene	5.1
1,8-cineole	0.8
(*E*)-β-ocimene	2.8
terpinolene	8.6
(*E*)-caryophyllene	14.8
α-humulene	5.5
caryophyllene oxide	8.8
CBD	1.9

Grieneisen, 2014), and their LC_{50} must be < 100 ppm (Pavela, 2015). Remarkably, the insecticidal properties of Kompolti EO and NE have not been previously described. Considering what was just mentioned, the larvicidal efficacy data of EO and EO-NE tested in this study are shown in Table 5. From the results, considering the content of EO, as the active substance of the NE, the efficacy of the NE was similar to that of pure EO. While for EO the $LC_{50}(_{90})$ was estimated to be 56.8 (142.3) ppm, for the NE the $LC_{50}(_{90})$ was estimated to be 963.7 (2763.5) ppm, equivalent to EO 72.2 (207.2) ppm. However, it should be noted that the CI_{95} overlaps in the case of LC_{50}, so this difference cannot be considered significant.

The results of these toxicity tests of hemp EO against *Cx. quinquefasciatus* differed slightly from those found in previous research that demonstrated the toxic effect of hemp EO on various mosquito species. For example, Bedini et al. (2016), testing hemp EO on *Aedes albopictus* Skuse larvae obtained an $LC_{50} = 301.56$ ppm, six-fold higher than that calculated in the current study. On the other hand, Rossi et al. (2020) tested two EOs from different hemp varieties (i.e., Felina 32 and Carmagnola CS) on *Anopheles stephensi* Liston and *Anopheles gambiae* (Giles) larvae, showing an LC_{50} between 73.5 and 78.8 ppm, much closer to that estimated in the present research. Besides the different susceptibility of diverse mosquito species to hemp EO, it is well known that the EO efficacy can vary according to several factors, such as the variety and the parts of the plant from which the EO was extracted, the plant's growing area, among others, and the EO extraction method, among others (Pavela and Benelli, 2016).

In experiments testing the impact of LC_{30} of the Kompolti hemp EO and EO-NE, an overall larval mortality of 39.1 and 40.8%, respectively, was observed (Table 6). The application of LC_{30} had no significant effect on fecundities, but mosquito fertility was significantly lower if compared with the control, and the total natality rate was reduced compared with the control by 40.4 and 45.1% for EO and EO-based NE, respectively. This fertility reduction effect has been observed in earlier research investigating the sublethal effects arising from the employ of NEs containing *Carlina acaulis* L. (Pavela et al., 2021), *Pimpinella anisum* L. EOs (Passos et al., 2022; Hategekimana and Erler, 2020), *Trachyspermum ammi* (L.) Sprague, *Crithmum maritimum* L. (Pavela et al., 2019a) and *Smyrnium olusatrum* L. EOs (Pavela et al., 2019c). The reduction in fertility and natality caused by exposing mosquito larvae to LC_{30} of the EO and EO-based NE can represent a further contribution to the insecticidal efficacy of hemp EO-based insecticides.

3.5. Effect of EO and EO-based nanoemulsions on *D. magna*

The need for an effective formulation against a target insect vector but respectful of non-target organisms is driving the development of new insecticide strategies and products (Sánchez-Gómez et al., 2022; Yeguerman et al., 2022). While several EOs have been found moderately toxic to a plethora of non-target arthropod species (see Giunti et al., 2022 for a dedicated review), a previous report outlined that the *C. sativa* EO was safe to non-target invertebrates such as *Eisenia fetida* (Savigny) earthworms and adults of the coccinellid *Harmonia axyridis* (Pallas) (Benelli et al., 2018a).

In this perspective, the Kompolti hemp EO and EO-based NE, assayed in this study for the first time for their non-target impact, caused relatively low mortality on adults of the organism *D. magna* when exposed to concentrations corresponding to the LC_{90} estimated for mosquito larvae, not exceeding 20% even after 48 h of exposure (Table 7). However, this low-to-moderate toxicity can be acceptable or not depending on the

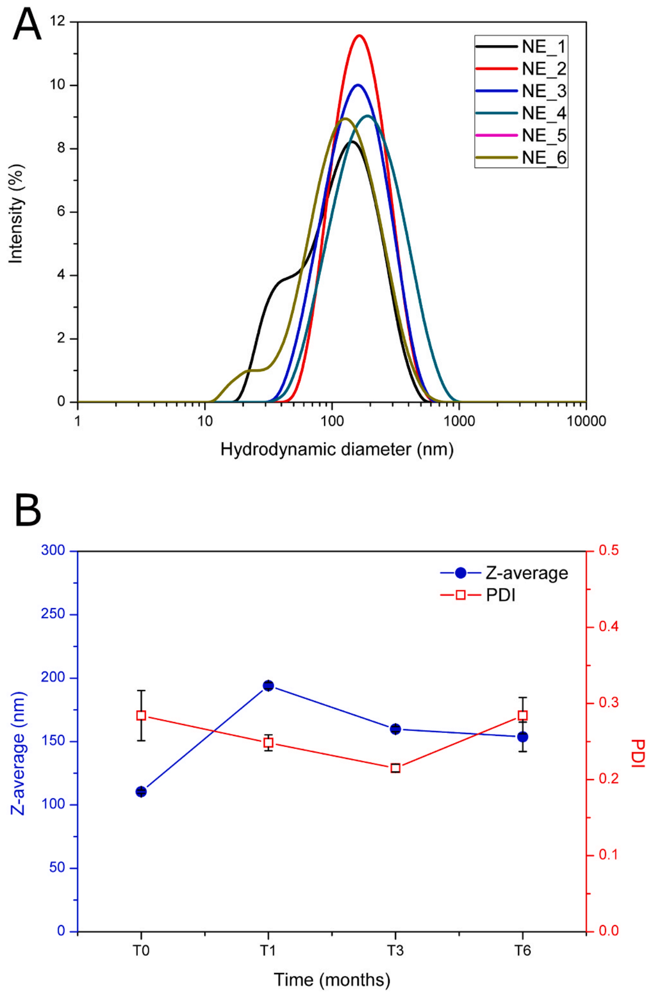

Fig. 2. Particle size distribution (intensity %) by dynamic light scattering (DLS) for all prepared Kompolti hemp essential oil (EO)-based nanoemulsions (NEs) (A); variation of Z-average and polydispersity index (PDI) values (B) over time (up to 6 months) for NE-5.

Table 4
Z-average (nm) and polydispersity index (PDI) values of Kompolti hemp essential oil (EO)-based nanoemulsions (NEs) after the preparation.

	Z-Average (nm)[a]	PDI[a]
NE_1	85.68 ± 1.05	0.377 ± 0.012
NE_2	141.46 ± 2.54	0.203 ± 0.010
NE_3	132.3 ± 1.56	0.228 ± 0.009
NE_4	83.48 ± 0.89	0.267 ± 0.011
NE_5	110.36 ± 1.29	0.284 ± 0.033
NE_6	99.73 ± 3.14	0.310 ± 0.040

[a] the reported values are mean ± standard deviation of three replicates.

contexts where this product might be used. Considering, for example, an aquaculture context, bioactive compounds in EOs if widely administered, albeit at low dosages, could have long-term biological effects on non-target organisms such as *D. magna* (Boxall, 2004; Ferraz et al., 2022; Miura et al., 2021). Finally, the results from this study, as well as earlier research on the topic (Conti et al., 2014; Miura et al., 2021), highlight the possibility of developing new effective products for the management of harmful insects such as mosquitoes, but at the same time underline the relevance of setting up safety protocols even with products from plant/natural sources.

4. Conclusions

Nowadays the need for sustainable and effective products for mosquito vector control, which can represent a valid alternative to synthetic agents leading to insecticide resistance phenomena, is urgently crucial. In this respect, green pesticides appear to be promising, due to less harmful effects on the environment and the human health, a limited residuality, and fewer resistance issues, with respect to those of the chemical products. Hemp represents one of the most available crops for industrial exploitation due to its several uses in different sectors. In particular, hemp EOs are recognized as safe, and endowed with insecticidal properties which make them exploitable as valuable eco-friendly botanical pesticides. For this purpose, EOs encapsulation in NEs has turned out to be one of the most promising strategies to improve EOs physicochemical characteristics, bioavailability, and administration. The present work demonstrated for the first time the efficacy of Kompolti EO and its nanoemulsion against *Cx. quinquefasciatus* larvae. Moreover, non-target toxicity assays showed limited impact on the aquatic microcrustacean *D. magna*, registering mortality < 16% after 48 h of exposure to the EO-based NE at the LC_{90} estimated on mosquito larvae. Notably, the LC_{50} values detected in this work are lower than those of many EOs, and the application at sublethal doses determined mortality between 39.1 and 40.8%, with a decrease in mosquito fertility, and consequently in natality of 40.4 and 45.1% for the EO and EO-based NE, respectively. As a result, the encapsulated form of this EO may be considered as an eco-friendly and sustainable mosquito larvicide deserving further research aimed at possible registration in the EU.

CRediT authorship contribution statement

Eugenia Mazzara: Investigation, Methodology, Data curation, Formal analysis, Writing – original draft. **Eleonora Spinozzi:** Investigation, Methodology, Data curation, Formal analysis, Writing – review & editing. **Filippo Maggi:** Conceptualization, Methodology, Formal analysis, Funding acquisition, Project administration, Resources, Visualization, Supervision, Writing – review & editing. **Riccardo Petrelli:** Conceptualization, Methodology, Formal analysis, Funding acquisition, Project administration, Resources, Visualization, Supervision, Writing – review & editing. **Dennis Fiorini:** Investigation, Methodology, Formal analysis, Resources, Visualization, Writing – review & editing. **Serena Scortichini:** Investigation, Methodology, Formal analysis, Resources, Visualization, Writing – review & editing. **Diego Romano Perinelli:**

Table 5
Insecticidal activity of the Kompolti essential oil (EO) and (EO)-based nanoemulsion (NE) against *Culex quinquefasciatus* 3rd[d] instar larvae.

Tested product[a]	LC_{30}[b] (ppm)	CI_{95}[c]	LC_{50} (ppm)	CI_{95}	LC_{90} (ppm)	CI_{95}	χ^2	*df*[d]	*p*-value
Hemp EO	35.5	28.7–42.8	56.8	47.9–68.5	142.3	121.7–165.9	2.856	3	0.278 ns
Hemp EO-NE	625.2	489.7–725.9	963.7	854.3–1075.1	2763.5	2297.2–3583.3	1.634	4	0.898 ns[e]

[a] EO = essential oil; NE = nanoemulsion;
[b] LC = lethal concentration killing 50% (LC_{50}) or 90% (LC_{90}) of the exposed mosquito larvae;
[c] CI_{95} = 95% confidence interval;
[d] *df* = degrees of freedom;
[e] ns= not significant ($p > 0.05$).

Table 6
Sublethal effects of Kompolti hemp essential oil (EO) and its nanoemulsion (NE) formulated at their LC_{30} on *Culex quinquefasciatus* larval mortality, as well as on the emergence, fecundity, fertility, and natality of new generation adults.

Treatment[a]	Tested concentration (ppm)	Larval mortality (%)[b]			Emergence of adults (%)[b]			Fecundity and fertility indicators[b]		Natality[b]	
		24 h	48 h	Total	Female	Male	Total	Fecundity (no. eggs/ female)	Fertility (egg hatchability %)	F_1 generation larvae (no.) out of 100 treated larvae	Natality inhibition over the control (%)
Hemp EO	35	22.2 ± 0.6[b]	25.8 ± 0.5[b]	39.1 ± 1.5[b]	29.8 ± 1.9[a]	31.1 ± 1.6[a]	60.9 ± 1.5[a]	102.8 ± 10.4	93.3 ± 1.3[b]	2847.5 ± 424.4[a]	40.4 ± 8.9
Hemp EO-NE	630	21.8 ± 0.5[b]	23.7 ± 0.8[b]	40.8 ± 2.5[b]	28.8 ± 1.3[a]	30.4 ± 1.3[a]	59.2 ± 1.8[a]	99.3 ± 8.7	91.8 ± 1.5[b]	2626.3 ± 535.1[a]	45.1 ± 11.1
Control	-	0.0 ± 0.0[a]	0.0 ± 0.0[a]	8.8 ± 5.5[a]	45.1 ± 2.1[b]	43.9 ± 2.3[b]	89.0 ± 5.5[b]	109.0 ± 12.2	97.5 ± 1.1[a]	4779.3 ± 370.5[b]	-
ANOVA $F_{2,9}$ and P-value		402.5, < 0.0001	398.7, < 0.0001	425.8, < 0.0001	285.2, < 0.0001	237.8, < 0.0001	227.8, < 0.0001	ns	16.0, 0.0011	90.9, 0.0004	ns

[a] *Culex quinquefasciatus* 3rd instar larvae were exposed to concentrations corresponding to the estimated LC_{30}
[b] Mean (%) (± SE) within a column followed by the same letter do not differ significantly according to ANOVA followed by Tukey's HSD test at $p < 0.05$ (% = arcsine transformed data); ns= not significant ($p > 0.05$)

Table 7
Effects of the Kompolti hemp essential oil (EO) and nanoemulsion (EO-NE) on non-target aquatic *Daphnia magna*.

Tested product	Mortality (%) of *Daphnia magna* adults		
	Concentration (ppm)	24 h	48 h
Hemp EO	45	5.0 ± 3.5[a]	26.3 ± 2.2[c]
Hemp EO-NE	30	7.5 ± 2.5[b]	15.3 ± 3.5[b]
Control	-	0.0 ± 0.0[a]	0.0 ± 0.0[a]
ANOVA $F_{2,9}$	-	6.98, 0.0147	89.4, $p < 0.001$

Within each column, different letters indicate significant differences (ANOVA, Tukey's HSD test, p < 0.05).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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