Microemulsions for delivery of Apiaceae essential oils – towards highly effective and eco-friendly mosquito larvicides?

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Abstract

The development of effective and eco-friendly pesticides to manage mosquito vector larvae is a timely and crucial challenge nowadays. Insecticide resistance is on the rise, therefore plant-borne insecticides may represent promising candidates to control insect vectors. In the present work, we encapsulated selected essential oils (EOs) from *Pimpinella anisum*, *Trachyspermum ammi* and *Crithmum maritimum* into highly stable microemulsions (MEs) with the aim to develop effective and eco-friendly larvicidal formulations. MEs made with both single EOs and their mixtures were tested against 3rd instar larvae of *Culex quinquefasciatus,* assessing acute and chronic toxicity, emergence of adults, and their impact on non-target invertebrates, i.e., *Daphnia magna*, *Tubifex tubifex* and *Eisenia fetida*. All MEs were able to exert toxicity against mosquito larvae, with LC_{50} values in the ranges 1.45-4.01 $ml.L^{-1}$, along with high larval mortality and low percentage of hatched adults following short-term exposure to sublethal concentrations. Low or none mortality was observed on *D. magna* and *E. fetida.* Taken together, these results give new insights for the exploitation of plant-borne EOs as active ingredients of novel and reliable larvicidal products.

Keywords: *Pimpinella anisum*, *Trachyspermum ammi*; *Crithmum maritimum; Culex quinquefasciatus; Daphnia magna*; *Eisenia fetida*; sub-lethal effects

1. Introduction

Arthropod-borne diseases are on the rise (Buckingham, 2015, Benelli and Duggan, 2018; Rosenberg et al., 2018), as recently evidenced by the recent Zika virus outbreaks (Yakob and Walker, 2016; Fernandes et al., 2018). Therefore, the effective management of bloodsucking arthropods, with special reference to mosquitoes and ticks, is a crucial challenge nowadays (Benelli and Beier, 2017; Benelli and Mehlhorn, 2016; Wilke et al., 2018). Nowadays, the preference of the global market of insecticides is expected to move toward more healthy and eco-friendly products. In this regard, the industry of essential oils (EOs) is growing by 5% per year (CBI, 2009a), being capable to provide the raw material for the manufacture of effective botanical insecticides which are expected to gain 7% of the global pesticide market by 2025 (Isman, 2015).

EOs are liquid mixtures made up of volatile, lipophilic and low molecular weight compounds formed by distinct biogenetic pathways, with terpenoids and phenylpropanoids, as the most common constituents (Morshedloo et al., 2017). Their use is particularly welcome since many of them are GRAS (Generally Recognized as Safe) by both the US FDA (Food and Drug Administration) and the EPA (Environmental Protection Agency), thus avoiding hazards for public health and environment (Miresmailli and Isman, 2014).

After the introduction of EO-based insecticides on the market at the beginning of 21th century, the scientific research on their insecticidal activity has seen an unpredictable progression in the last decade (Isman and Grieneisen, 2014). Overall, most of EOs are currently marketed and used as repellent agents against mosquitoes (Benelli et al., 2013; Giatropoulos et al., 2013) although many of them could be suitable

78 as ingredients of ovicidal, larvicidal and adulticidal formulations (Pavela et al., 2009;
79 Pavela, 2009; Benelli, 2015a,b). Uses in the protection of stored foodstuffs have also
80 been documented (Hashem et al., 2018).
81 Recently, the market registration of EO-based insecticides is being simplified,
82 e.g., EFSA (European Food Safety Authority) is shortening the marketing authorization
83 for those botanicals classified as 'LRASs' (low-risk active substances) (EC Regulation,
84 2009). On an industrial scale, the most promising EOs to be used as botanical
85 insecticides should fulfil the following requirements: (*i*) availability and cultivation on a
86 large scale of the plant source; (*ii*) high EO yield; (*iii*) low prices of EO (generally
87 correlated with the yield) and raw material from which EOs are obtained. Thus, to
88 warrantee a sustainable production on a global scale, the agrochemical companies
89 should select those EOs for which a global production of at least 50 tons can be assured
90 (CBI 2009b, Shrinivas and Kudli, 2008).
91 On this basis, in the present study we selected three EOs for which we
92 previously obtained very promising results for a possible use in botanical insecticides,
93 namely *Pimpinella anisum* L., *Trachyspermum ammi* (L.) Sprague and *Crithmum*
94 *maritimum* L, all from the Apiaceae family (Benelli et al., 2017; Pavela et al., 2017;
95 Pavela et al., 2017). Notably, these EOs showed a LC_{50} value on the filariasis vector
96 *Culex quinquefasciatus* Say third instar larvae below 50 ppm. Such value is a threshold
97 to screen potentially useful ingredients for insecticidal formulations (Pavela, 2015).
98 *P. anisum* is a Mediterranean annual herb widely cultivated and employed as flavouring
99 of sauces, liqueurs, confectionery and bakery products (Iannarelli et al., 2017). In the
100 folk medicine, it has been employed to cure gastrointestinal problems, as galactugoge
101 and expectorant (Iannarelli et al., 2018). The EO is obtained from the fruits (schizocarp)

102 with an average yield in the range 2-6% and a price on the market estimated between 7
103 and 9 €/kg (Lubbe and Verpoorte, 2011).
104 *T. ammi*, known as ajowain, is an annual plant occurring in the arid regions of Egypt,
105 Iran, Iraq, Afghanistan, Pakistan and India (Vitali et al., 2016). The fruits (schizocarps)
106 are used as flavourings of foodstuffs and as preservatives; they are also employed in the
107 folk medicine in the cure of flatulence, gastrointestinal diseases and respiratory
108 problems (Bairwa et al., 2012).
109 *C. maritimum*, called sea fennel, is a halophytic plant occurring in rocky coastal
110 areas of the Mediterranean basin (Pavela et al., 2017). This herb is particularly enjoyed
111 in kitchen, to make salads or as pickled vegetable (Cornara et al., 2009; Bremness,
112 2004). In the traditional medicine sea fennel aerial parts have been employed as an
113 antiscorbutic, digestive, diuretic, antitussive and anti-inflammatory agent (Carrió and
114 Vallès, 2012; Savo et al., 2011; Cornara et al., 2009).
115 As new eco-friendly insecticides, EOs enjoy several advantages such as the wide
116 spectrum of efficacy against mosquito vectors and other pests of medical and
117 agricultural relevance (Benelli and Pavela, 2018a,b), the multiple mode of actions (e.g.
118 different molecular targets in insects are addressed), the unlikely insurgence of
119 resistance in insects, and the low environmental impact (Pavela and Benelli, 2016).
120 However, these advantages are counterbalanced by several drawbacks that are currently
121 limiting their spread and marketing. These weaknesses are linked to the nature of EOs.
122 Indeed, the volatility of EO constituents limits their persistence in the environment so
123 that frequent reapplication is required when used in the field. The lipophilicity of the
124 molecules limits their applicability in wet environments where mosquitoes are usually
125 breeding. Many EO constituents are highly instable under light, air and high

126 temperature exposures so that they give raise to degradation products devoid of
127 efficacy. Being very sensitive to genetic and environmental factors, EOs may exhibit a
128 significant intra-specific variability giving raise to several 'chemotypes' which can
129 influence the whole biological efficacy. Moreover, as EOs get old, their overall quality
130 tends to decrease because of alteration of their organoleptic attributes (e.g., odor, flavor,
131 color and consistency). All the above disadvantages highlight the need for efficient
132 stabilization processes. The latter rely on the so-called encapsulation process through
133 the development of an appropriate formulation. Currently, the preparation of aqueous
134 dispersions through the nanoencapsulation technology appears to be the most suitable
135 approach, in particular the development of microemulsions (MEs) (Bilia et al., 2014).
136 MEs are homogeneous and isotropic nanodispersions endowed with low viscosity,
137 optical transparency, thermodynamic stability and an internal (dispersed) phase having
138 typical sizes of 10 ~ 200 nm. They are made stable trough the combination of a co-
139 surfactant and an interfacial film of surface active molecules (Bonacucina et al., 2009).
140 MEs are excellent candidates for novel delivery systems enjoying an extended shelf life,
141 ease of preparation and scalability with reduced external energy input (McClements,
142 2012). They improve the solubilization of poorly water-soluble compounds for better
143 bioavailability, and high possibility of enhanced absorption behaviour (Bonacucina et
144 al., 2009; Gupta and Moulik, 2007).
145 Nano- and MEs have been also broadly developed to improve solubility and
146 spreading capacity of pesticide EOs by their dispersion into an aqueous phase (Hashem
147 et al., 2018), allowing their evaluation as promising mosquito insecticides (Ghosh et al.,
148 2013; Sugumar et al., 2014; Duarte et al., 2015) and repellents (Nuchuchua et al., 2009;
149 Sakulku et al., 2009). Microemulsion formulation of EOs demonstrated to offer several

advantages such as increase in water solubility, dissolution rate, dispersion uniformity, stability and easiness of preparation (Cespi et al., 2017). Moreover, they prevent the degradation of active ingredients and extend their bioavailability for long time (Song et al., 2009; Tadros et al., 2004). Besides, the small size of the droplets allows them to be deposited uniformly on plant leaves; wetting, spreading and permeating may also be enhanced because of the low surface tension of the whole system (Du et al., 2016).

Culex quinquefasciatus Say (Diptera: Culicidae), also known as the southern house mosquito, is a major vector of lympatic filariasis (Jambulingam et al., 2016; Vadivalagan et al., 2017). Besides, it has been recently investigated as a vector of Zika virus (Benelli and Romano, 2017; Guedes et al., 2017; van den Hurk et al., 2017). In this framework, mosquito management is being challenging due to mosquito growing resistance to synthetic pesticides largely overused worldwide (Naqqash et al., 2016; Fotakis et al., 2017; Mastrantonio et al., 2017; Bharati and Saha, 2018).

In the attempt to develop effective and safer mosquito control tools, herein we presented a method to fabricate eco-friendly microemulsions containing EOs from *P. anisum*, *T. ammi* and *C. maritimum* and their binary and ternary mixtures, evaluating their acute and chronic toxicity against *C. quinquefasciatus*. Furthermore, we analysed the impact of these microemulsions on non-target invertebrates, including aquatic species such as *Daphnia magna* Straus and *Tubifex tubifex* (Müller), as well as the earthworm *Eisenia fetida* (Savigny).

2. Materials and methods

2.1. Plant materials

174

175 Schizocarps of anise were collected from a cultivated field placed in Castignano
176 (central Italy, N 42°56′25″08, E 13°37′29″64, 475 m a.s.l.), in September 2017.
177 Schizocarps of ajwoain were harvested from plants grown in the research garden of
178 University of Maragheh, Iran (N 37°23′, E 46°16′, 1485 m a.s.l), in August 2017.
179 Flowering aerial parts of sea fennel were harvested in Le Conquet, Finistere, Bretagne,
180 France, N 48°20'33.20", O 4°46'17.81", 7 m s.l.m.) in September 2017. Herbarium
181 specimens were stored in the Herbarium Universitatis Camerinensis, Camerino, Italy,
182 Herbarium of Agricultural and Natural Resources Research and Education Center,
183 Kohgiluyeh and Boyer-Ahmad, Iran, and in the Herbarium of Géoarchitecture,
184 Université de Bretagne Occidentale, Brest, France, under the voucher codes CAME
185 28168, No 4526 and BRECK9, respectively. Polysorbate 80 (TEGO® SMO 80; Evonik
186 Industries, Essen, DE), ethyl oleate (CrodamolTM EO; Croda, Goole, UK) and
187 vegetable glycerol were supplied by ACEF (Fiorenzuola d'Arda, IT). Deionized water
188 and ethanol 96% were standard reagent grade.
189

190 *2.2. Hydrodistillation*
191

192 Schizocarps of anise and ajowain (1000 g) and flowering aerial parts of sea
193 fennel (870 g) were roughly crushed in a mortar to facilitate the release of the essential
194 oils from the secretory channels, then immersed in a 10 L flask filled with 6 L of
195 deionized water and heated into a mantle system Falc MA (Falc Instruments, Treviglio,
196 Italy). Condensation of the EOs was achieved in a Clevenger-type apparatus for 3 h.
197 Once obtained, the oils were decanted for 30 min, separated from the aqueous layer and

198 dehydrated with anhydrous Na_2SO_4, finally collected in amber vials of 30 mL sealed
199 with PTFE-silicon septa which were stored at + 4°C until use. The oil yields, calculated
200 on a dry weight basis, were 2.4, 2.5 and 0.8%, respectively.
201
202 *2.3. GC/EIMS analysis*
203
204 The analyses were done by an Agilent 6890N gas chromatograph equipped with
205 a 5973N single quadrupole mass spectrometer and an auto-sampler 7863 (Agilent,
206 Wilmingotn, DE). Following our previous procedure with slight modifications
207 (Quassinti et al., 2014), separation of essential oil components was obtained by using
208 two different coated capillary columns, i.e. the HP-5MS (5%
209 phenylmethylpolysiloxane, 30 m x 0.25 mm i.d., 0.1 μm film thickness; J and W
210 Scientific, Folsom, CA), and the DB-WAX (polyethylene glycol, 30 m x 0.25 mm i.d.,
211 0.25 μm film thickness; J & W Scientific, Folsom). The analytical conditions employed
212 were the same as those reported by Benelli et al. (2018a).
213
214 *2.4. Identification of essential oil components*
215
216 Chromatograms were analysed by the MSD ChemStation software (Agilent,
217 Version G1701DA D.01.00) and the NIST Mass Spectral Search Program for the
218 NIST/EPA/NIH EI and NIST Tandem Mass Spectral Library v. 2.3. For α-pinene,
219 camphene, sabinene, β-pinene, myrcene, α-phellandrene, *n*-octanal, δ-3-carene, α-
220 terpinene, *p*-cymene, (*Z*)-β-ocimene, (*E*)-β-ocimene, γ-terpinene, terpinolene, linalool,
221 terpinen-4-ol, α-terpineol, bornyl acetate, (*E*)-anethole, thymol, carvacrol, (*E*)-

caryophyllene and myristicin, the peak assignment was based on the comparison with the respective analytical standard purchased from Sigma-Aldrich (Milan, Italy). Otherwise, we adopted the interactive combination of the coherence of temperature-programmed arithmetic index (AI) (Van den Dool and Kratz, 1963) with the MS fragmentation pattern obtained for each peak with respect to those stored in ADAMS, FFNSC2 (2012), NIST17 (2017) and WILEY275 MS libraries.

2.5. Preparation of EO microemulsions

All the MEs were formulated and prepared following the procedure reported by Cespi et al. for the *S. olusatrum* EO (Cespi et al, 2017). Distilled water drops were added to the oil phase under agitation. The oil phase was given by EO or EO-ethyl oleate mixture, Polysorbate 80, glycerol and ethanol. EOs were added singularly and in binary or ternary mixtures. In addition, controls without EOs were prepared. The composition of samples and controls are reported in Table 1.

2.6. Characterization of EO microemulsions

Visual inspection of formulations was done by a polarizing optical microscope (MT9000, Meiji Techno Co Ltd, JP) endowed with a 3-megapixel CMOS camera (Invenio 3S, DeltaPix, DK). Transparent and isotropic samples, were further characterized using a dynamic light scattering (DLS) using backscattered light detector working at 173° (Zetasizer nanoS, Malvern Instrument, UK). Samples (1 mL) were inserted into disposable cuvettes and examined at 25°C, following temperature

246 equilibration (180s). The analysis was performed at different time points: 0 day (t0), 15
247 days (t15), 1 month (t30), 3 months (t90) and 6 months (t180).
248 Moreover, to predict the stability of the MEs, they were exposed to an accelerating
249 centrifugal force of 2000 x g for 60 min using an ultracentrifuge (Biofuge pico, Heraeus
250 Instruments, Germany) (Jumaa and Müller, 2002).
251
252 *2.7. Mosquitocidal activity*
253
254 *2.7.1. Acute toxicity*
255 *C. quinquefasciatus* larvae were reared as detailed by Benelli et al. (2018a,b). To
256 evaluate the toxicity of the MEs we followed WHO (1996) with slight changes detailed
257 by Pavela et al. (2017). ME toxicity at 10.0, 8.0; 6.0, 5.0, 3.0, 2.0, 1.5, 1.0, 0.6 and 0.3
258 mL L^{-1} was evaluated on 3rd instar larvae. Four replicates were done for each
259 concentration. Distilled water was the negative control. α-cypermethrin (Vaztak®)
260 tested at concentrations from 0.1 to 3.0 µg L^{-1} was the positive control (from 0.1 to 3.0
261 µg L^{-1}). Larval mortality was noted after 24 h. Assays were done at 25±1 °C, 70±5 %
262 R.H., 16:8 h (L:D).
263
264 *2.7.2. Chronic toxicity of sublethal doses*
265 To assess the impact of ME sublethal concentrations in terms of chronic larval
266 toxicity and adult emergence post-treatment at sub-lethal concentrations, *C.*
267 *quinquefasciatus* larvae were exposed to ME LC_{30} for 24 h. Tested ME concentrations
268 were summarized in Table 4 (uniform concentration 4.0 ml.L^{-1} was used for MEs where
269 no larval mortality was found). After 24 h, surviving larvae were moved to clean water

and fed on dog food (Pedigree, USA) until pupation. Larval mortality was evaluated daily during for 7 days. Total larval mortality and the count and sex of hatched adults were determined according to Benelli et al. (2017). Experimental conditions were 25±1 °C, 70±5 % R.H., 16:8 h (L:D).

2.8. Toxicity on non-target aquatic species

Herein we evaluated the impact of MEs and α-cypermethrin two important non-target aquatic species, i.e., *D. magna* and *T. tubifex*. In our tests, adults (2-5 days old) of both invertebrates were exposed to LC_{90} concentrations estimated on mosquito larvae. 20 *D. magna* or *T. tubifex* were transferred to plastic dishes containing 100 mL of water plus the proper concentration of the MEs or α-cypermethrin (detailed in Table 6). Mortality was determined after 24 h. Experimental conditions were 25 ± 1 °C, 70 ± 5% R.H., 16:8 h (L:D).

2.9. Toxicity on earthworms

Following OECD (1984), we tested the toxicity of MEs on *E. fetida* adults. The earthworms were reared following Pavela (2018). The artificial soil was composed as in Benelli et al., (2018a). MEs were added to the soil at 1 mL kg^{-1}. Furthermore, α-cypermethrin at 25 mg kg^{-1} of dry soil [equivalent = Vaztak® at 500 mg kg^{-1}] was the positive control. Distilled water served as negative control. In the assays, selected ME, only water, or α-cypermethrin diluted in water, was mixed into the soil composed as detailed above and 10 earthworm adults were added. The samples (650 g) were stored in

294 glass pots (1 L) covered with gauze (Benelli et al., 2018a). Mortality of *E. fetida* was
295 noted after 5 and 10 days of exposure at 20±1 °C, R.H. 80–85 %, 16:8 (L:D) and 600
296 lux.
297
298 *2.10. Statistical analysis*
299
300 Control mortality exceeding 20% was used to correct the observed mortality
301 relying to Abbott's formula (Abbott, 1925). Thus, probit analysis was used to calculate
302 $LC_{50(90)}$ values and related parameters (Finney, 1971). Mosquito toxicity data over time
303 post-exposure to ME LC_{30} as well as non-target species mortality data (%) were
304 transformed by arcsine√ then analysed by ANOVA followed by Tukey's HSD test
305 ($P \leq 0.05$).
306
307 **3. Results and discussion**
308
309 *3.1. Chemical profile of incapsulated EOs*
310
311 Chemical composition of *P. anisum*, *T. ammi* and *C. maritimum* EOs is reported
312 in Table 2. Overall, the yields obtained from hydrodistillation from the three species,
313 i.e. 2.4, 2.5 and 0.8%, respectively, along with the relatively low cost of raw material,
314 support their usage on an industrial scale.
315 Anise EO was of high quality, being characterized by very high levels of (*E*)-
316 anethole (relative abundance 96.7%), with little amounts of methyl chavicol (1.6%).
317 Other components occurring in this sample were geijerene (traces), (*Z*)-anethole (0.1%),

γ-himachalene (0.5%), α-zingiberene (0.1%) and (*E*)-pseudoisoeugenyl 2-methylbutyrate (0.4%). This composition was perfectly consistent with those reported in our previous studies (Iannarelli et al., 2017,2018; Benelli et al., 2017), attesting a high-quality product obtained from cultivated fields of central Italy that fulfills the requirements of European Pharmacopoeia (2005).

Ajowain EO was mainly composed of three monoterpenes, with thymol as the most abundant (62.6%), followed by *p*-cymene (18.7%) and γ-terpinene (15.8%). Other minor compounds occurring in this EO were β-pinene (0.7%), α-thujene (0.3%), α-pinene (0.1%), myrcene (0.3%), δ-3-carene (0.3%), α-terpinene (0.3%), terpinen-4-ol (0.2%), α-terpineol (0.1%), carvacrol (0.3%) and others at trace levels. This composition was consistent with those previously found in other ajowain batches from Iran and India (Pavela et al., 2018; Vitali et al., 2016; Kamte et al., 2018).

Sea fennel EO showed a more complex profile, with a total of thirty-nine compounds identified. The monoterpene fraction (80.9%) was the most abundant in the oil followed by that of phenylpropanoids (18.0%). γ-Terpinene (33.0%), thymol methyl ether (22.0%) and dillapiole (17.5%) were the main EO constituents. Other components occurring at noteworthy levels were *p*-cymene (8.7%), α-pinene (6.4%) and sabinene (6.0%). This composition was qualitatively consistent with that previously found in sea fennel growing in the same area (Pavela et al., 2017). Main differences in the current study consisted in a higher content of monoterpenes, namely γ-terpinene and thymol methyl ether, and a lower content of phenylpropanoids including dillapiole and myristicin.

Previous studies conducted by our group showed that the main constituents of these EOs such as (*E*)-anethole, thymol and γ-terpinene have LC_{50} values below 50 ppm

342 which is considered an important threshold to select the most promising ingredients to
343 be used in green biopesticides (Pavela et al., 2017; Pavela et al., 2018). Their
344 mechanisms of action include inhibition of detoxicant enzymes (e.g. anethole) as well
345 as interaction with cholinergic (thymol), GABA and octopaminergic systems
346 (monoterpenes) (Afshar et al., 2017; Benelli et al., 2017; López et al., 2018). Regarding
347 acute toxicity, some of these constituents, namely (*E*)-anethole, thymol and γ-terpinene
348 exhibited LD_{50} values of 2090, 1680 and 980 mg kg^{-1}, respectively, in rats following
349 oral administration (Isman and Machial, 2006). These values are far higher than that of
350 some commercial insecticides of synthetic nature and are promising for the future
351 development and market authorization by regulatory agencies. In this regard, an EO-
352 based formulation should have an LD_{50} above 5 g/kg in rats (Isman and Machial 2006).
353
354 *3.2. Preparation and characterization of EO microemulsions*
355
356 MEs were prepared using a composition already tested after a wide screening of
357 samples and which demonstrated to give stable *Smyrnium olusatrum* EO-MEs (Cespi et
358 al., 2017). Herein, *T. ammi* and *C. maritimum* EOs were added at the concentration of
359 1.5% w/w (sample S1 and S2). Instead, a dilution in another oil phase was necessary for
360 *P. anisum* EO due to issues related to recrystallization of the pure sample. Ethyl oleate
361 was selected since it is commonly used for preparation of emulsions and MEs (Date and
362 Nagarsenker, 2008) and showed good solvent properties for *P. anisum* EO crystals.
363 After a preliminary screening, ethyl oleate-EO 1:3 ratio was selected as the minimum
364 ratio able to avoid recrystallization. Thus, in this specific case the total amount of oil
365 phase was 2% w/w, keeping constant *P. anisum* EO at 1.5% and adding 0.5% of ethyl

366 oleate (sample S3). Anyway, adding a total oil amount of 2% it was not possible to
367 obtain a proper ME. In fact, even if polarized microscopy image showed the presence of
368 an isotropic system (Fig. 1A), DLS analysis highlighted a multimodal size distribution
369 centred around 1 μm (Fig. 1B). Therefore, we decreased the EO phase at the total
370 concentration of 1.5% w/w, keeping constant 1:3 ethyl oleate-*P. anisum* EO ratio
371 (sample S4).

372 Figure 2A showed DLS traces of *T*. *ammi* EO (S1), *C*. *maritimum* EO (S2) and
373 *P*. *anisum* EO (S4) microemulsions. S1 and S2 had a bimodal size distribution centred
374 at 58 and 700 nm and at 50 and 500 nm, respectively. The fraction of the populations
375 with the smaller droplet size was predominant. The presence of a second particles
376 population with a larger diameter in the intensity plot corresponds to a marginal fraction
377 of the whole population. In fact, the intensity of the scattered light is proportional to the
378 power of six of the particle diameter, therefore only a small number of particles
379 possessed a size over 100 nm.

380 S4 showed a single particles population below 100 nm (medium diameter at
381 around 40 nm). This could be explained by the fact that the presence of ethyl oleate
382 (0.375% w/w) could promote the formation of smallest droplets of the oil phase.

383 Afterwards, we prepared binary and ternary mixtures of *T*. *ammi*, *C*. *maritimum*
384 and *P*. *anisum* EOs (S5-S10), as reported in Table 1. For the MEs containing *P*. *anisum*
385 EO, the addition of ethyl oleate was not necessary since the solubilizing action on the
386 crystals was carried out by the presence of the other EOs. Here again, DLS analysis
387 showed the presence of two different populations referred to all the samples; the
388 principal size distribution is around 50 nm while the second one is over 100 nm (Fig.
389 2B).

After the storage at room temperature, the samples were analysed using polarized light microscopy (data not shown) at different time points to test their physical stability. All the samples were isotropic under polarized light inspection; thus they were further analysed by DLS. The DLS results were expressed in terms of hydrodynamic diameter (Fig. 3A) and distribution width (Fig. 3B), both related to the main population that, in number, represented almost the 100% of the oil phase. No relevant differences in terms of hydrodynamic diameter and width of the size distribution of the oil phase over a period of 6 months were observed. It could be noted that sample S1, S2 and S4 (containing only one EO) showed a slight reduction of the distribution width, suggesting a stabilizing effect: MEs possess a dynamic equilibrium that over time could lead to an increase in the uniformity of the oil phase size. The same considerations could be done for the results obtained by the centrifugal stability test. The value related both to hydrodynamic diameter and width distribution showed only a slight difference pre- and post-centrifugation (Fig. 4A and 4B). This means that the mechanical stress applied to the samples trough the centrifugation process did not affect the physical properties of the MEs and the samples could be considered stable over time (Majekodunmi, 2015; McClements, 2007).

3.3. Toxicity on Culex quinquefasciatus and non-target invertebrates

The efficacy of MEs on *C. quinquefasciatus* larval mortality is reported in Table 3. LC_{50} ranging from 1.45 to 4.01 $ml.L^{-1}$ and LC_{90} ranging from 1.81 to 6.48 $ml.L^{-1}$ were estimated for our tested MEs. However, although all our MEs caused acute

toxicity in mosquito larvae. Notably, no significant differences concerning the larvicidal activity were found among MEs.

ME containing *T. ammi* EO could be determined as most efficient among the three MEs containing only one EO as their active substance. $LC_{50(90)}$ of 1.57(2.53) $ml.L^{-1}$ was estimated for this ME, matching approximately 23.5(37.9) $\mu l.L^{-1}$ of the EO content with the major share of thymol (62.6%). Thymol is known for its very good insecticidal and acaricidal efficacy (Tabari et al., 2017), oftentimes significantly better compared with some other EO components, including (*E*)-anethole, the major constituent of the EO from *P. anisum*, or γ-terpinene contained in the EO from *C. maritimum* (Pavela, 2015b).

A significant synergistic rise in efficacy was observed in some MEs containing binary EO mixtures. The highest synergistic effect was seen in the mixture of EOs from *T. ammi* and *P. anisum*, contained in the MEs in the ratios 1:1 (S5) or 1:2 (S8). LC_{50} was estimated as 1.45 or 1.59, respectively and LC_{90} as 1.81 or 2.08 $ml.L^{-1}$, respectively, for these MEs (Table 3). These values correspond to the EO mixture content of approximately 21.7 or 23.8 and 27.1 or 31.2 $\mu l.L^{-1}$ for S5 or S8, respectively. The observed phenomenon of the synergistic increase in biological efficacy of the mixture of EOs from *T. ammi* and *P. anisum* may be attributed to the combination of two major substances (i.e., thymol and (*E*)-anethole) contained in this mixture, whose mutual synergistic action on mosquito larval mortality has already been described (Pavela 2015b). This improvement may be due to the concurrent action at different target sites in the insect, with (*E*)-anethole neutralizing the detoxification system of insect (Hashem et al., 2018) and thymol being able to modulate the GABA-gated

436 chloride channels and to inhibit the octopamine receptors (Rattan, 2010; Pavela and
437 Benelli, 2016). Synergistic increase in efficacy was not significant in other MEs.
438 All the tested MEs can be considered as highly efficient given that upon
439 conversion of the lethal concentrations to the contents of EOs as active substances in the
440 MEs, LC_{90} was estimated as less than 100 $\mu l.L^{-1}$, and for the most efficient formulation,
441 LC_{90} was even less than 25 $\mu l.L^{-1}$. At the same time, EOs with LC_{90} lower than 100
442 $\mu l.L^{-1}$ are generally considered as highly promising to develop plant-borne larvicides
443 (Pavela, 2015a). Nowadays, a rather limited number of studies tested EO-based
444 nanoemulsions on mosquito species, including *Aedes aegypti* L. (Ghosh et al., 2013;
445 Duarte et al., 2015) and *Cx. quinquefasciatus* (Sugumar et al., 2014). Duarte et al.,
446 (2015) showed that rather high concentrations of *Rosmarinus officinalis* L.
447 nanoemulsions, i.e., 250 ppm, are needed to achieve 90% mortality of *Ae. aegypti* larvae
448 after 48 h of exposure. A similar achievement was reported by Sugumar et al., (2014)
449 showing 98% mortality of *Cx. quinquefasciatus* larvae within 4 h post-treatment with
450 250 ppm of eucalyptus EO nanoemulsions.
451 Besides, nanoemulsions of citronella, hairy basil, and vetiver EOs showing a
452 mean diameter lower than 250 nm, were successfully tested as stable and long lasting
453 mosquito repellents, achieving a protection time of 4.7 h when tested at EO
454 concentration of 10%, 5%, and 5% respectively (Nuchuchua et al., 2009). In addition,
455 Sakulku et al. (2009) focused on the improvement of nanoemulsion repellent activity,
456 pointing out that the nanoemulsion stability can be boosted adding glycerol, due to its
457 co-solvent and highly viscous properties.
458 The sub-lethal effects of EO MEs against mosquito species have scarcely
459 studied. In the present research, the MEs had a significant impact on further larval

development and on subsequent hatching of *C. quinquefasciatus* adults (Tables 4 and 5). Even a relatively short-term exposure of the larvae (24 h) to water contaminated with LC_{30} of MEs was found to have the potential of causing subsequent high larval mortality (Table 4). In this respect, ME (S6) showed the highest efficacy, causing 100% larval mortality. This ME contained EOs from *C. maritimum* and *P. anisum* at the ratio 1:1. Mortality over 90% was also caused by MEs S9, S4 and S3, all containing the EO from *P. anisum*.

The high larval mortality was the cause of a subsequent low percentage of hatched adults (Table 5), while almost all MEs containing the anise EO (except S5 and S10) caused less than 10% adults finally hatched. It is thus very likely that (*E*)-anethole contained in the anise EO at a relative percentage of about 97% was responsible for this phenomenon (see also Hashem et al., 2018). This is the first report describing the effect of sublethal concentrations on larval development and on the hatching of adult mosquitoes for this EO. It is therefore difficult to compare our results with the results of other authors. However, as previously found, sublethal doses or concentrations of some other EOs may have a significant negative effect on larval development and on the reproductive parameters in adults (Pavela 2013a,b; Benelli et al., 2018b).

Although the MEs were highly toxic to the mosquito larvae, at the same time they were friendly to some non-target organisms (Table 6). The selectivity of EOs to non-target organisms such as natural predators of the pests, including fish and some aquatic invertebrates, has also been confirmed by other authors (Pavela, 2014 and 2018; Pavela and Govindarajan, 2017; Castilhos et al., 2018; Ribeiro et al., 2018). As presented herein, the lethal concentration resulting in 90% mortality of the *C. quinquefasciatus* larvae caused only very low mortality of adult microcrustaceans *D.*

484 *magna* and no mortality of adult earthworms *E. fetida* (Table 6), unlike α-cypermethrin
485 which, even in much lower concentrations, caused almost 100% mortality in all the
486 tested non-target species. However, relatively high mortality was still observed in a
487 representative of aquatic invertebrate species, i.e., *T. tubifex* (Table 6), caused by all the
488 tested MEs. The lowest sensitivity was seen in *T. tubifex* adults to S5 and S8 MEs,
489 which were also evaluated as providing the highest efficacy against the mosquito larvae.
490 *T. tubifex* is one of the most sensitive aquatic organisms to various pesticides, and this
491 species is therefore considered as an important element in eco-toxicological tests
492 (Bettinetti et al., 2005).
493
494 **4. Conclusions**
495
496 The formulation of EOs to improve their stability and mosquito larvicidal
497 efficacy is an important challenge in the field of applied entomology aimed to vector
498 control (Benelli, 2018). The present work showed that some Apiaceae food plants such
499 as anise, ajowain and sea fennel are promising sources of botanical insecticides. Their
500 EOs are highly effective as mosquito larvicides giving acute and chronic toxicity as well
501 as reduction of adult emergence in *C. quinquefasciatus,* an important filariasis vector.
502 These effects are maintained after encapsulation in stable MEs. MEs encapsulating EOs
503 had no negative impact on two non-target species, despite a certain mortality was
504 observed in *T. tubifex;* thus, they can be therefore considered as relatively safe for the
505 environment. Additional tests will be needed to study the effect of sublethal
506 concentrations of the MEs on developmental and reproductive parameters of the non-
507 target species, as well as the determination of their LD_{50} in mammals.

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Conflict of Interest

The authors declare no competing interests.

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Table

Table 1. Composition (%) of essential oil microemulsions (S1-S10) and controls (C1-C4).

Sample	Polysorbate 80	Alcoholic phase*	Essential oils			Ethyl oleate	H_2O
			Trachyspermum ammi	*Crithmum maritimum*	*Pimpinella anisum*		
S1	13	35	1.5	-	-	-	50.5
S2	13	35	-	1.5	-	-	50.5
S3	13	35	-	-	1.5	0.5	50
S4	13	35	-	-	1.125	0.375	50.5
S5	13	35	0.75	-	0.75	-	50.5
S6	13	35	-	0.75	0.75	-	50.5
S7	13	35	0.75	0.75	-	-	50.5
S8	13	35	0.5	-	1	-	50.5
S9	13	35	-	0.5	1	-	50.5
S10	13	35	0.25	0.25	1	-	50.5
C1	-	35	-	-	-	-	65
C2	13	-	-	-	-	-	87
C3	13	35	-	-	-	-	52
C4	13	35	-	-	-	1.5	50.5

* The alcoholic phase is composed of 30% glycerol and 5% ethanol 96%.

Table 2. Chemical composition of the incapsulated essential oils from *Pimpinella anisum*, *Trachyspermum ammi* and *Crithmum maritimum*.

No.	Component[a]	AI HP-5MS[b]	AI Lit. ADAMS[c]	AI DB-WAX[d]	RI Lit. NIST 17[e]	Relative abundance %[f]			ID[g]
						Pimpinella anisum	*Trachyspermum ammi*	*Crithmum maritimum*	
1	α-thujene	921	924	1026	1025		0.3±0.0	0.4±0.1	AI,MS
2	α-pinene	926	932	1023	1010		0.1±0.0	6.4±1.4	Std,AI,MS
3	Camphene	939	946					tr[h]	Std,AI,MS
4	Sabinene	965	969	1122	1122		tr	6.0±1.2	Std,AI,MS
5	β-pinene	968	974	1110	1110		0.7±0.2	0.3±0.0	Std,AI,MS
6	myrcene	989	988	1160	1160		0.3±0.0	0.5±0.1	Std,AI,MS
7	α-phellandrene	1003	1003	1158	1122		tr	tr	Std,AI,MS
8	*n*-octanal	1005	998	1179	1178			tr	Std,AI,MS
9	δ-3-carene	1008	1008	1121	1122		0.3±0.0	tr	Std,AI,MS
10	1,4-cineole	1012	1012	1209	1208				AI,MS
11	α-terpinene	1014	1014	1179	1177		0.3±0.1	0.7±0.2	Std,AI,MS
12	*p*-cymene	1022	1020	1270	1270		18.7±2.7	8.7±1.9	Std,AI,MS
13	β-phellandrene	1025	1025	1209	1208		0.3±0.1	0.2±0.0	AI,MS
14	(*Z*)-β-ocimene	1037	1032	1232	1238			0.9±0.2	Std,AI,MS
15	benzene acetaldehyde	1044	1036	1610	1604			tr	AI,MS
16	(*E*)-β-ocimene	1047	1044					tr	Std,AI,MS
17	γ-terpinene	1057	1054	1235	1241		15.8±2.4	33.0±3.4	Std,AI,MS
18	*cis*-sabinene hydrate	1066	1065	1289	1289			tr	AI,MS
19	terpinolene	1085	1086	1282	1280		tr	0.2±0.0	Std,AI,MS
20	*trans*-sabinene hydrate	1098	1098	1473	1176		tr	tr	AI,MS
21	linalool	1102	1119				tr		Std,AI,MS
22	*cis*-*p*-menth-2-en1-ol	1120	1118	1623	1620			tr	AI,MS
23	*allo*-ocimene	1129	1128	1371	1370		tr		AI,MS
24	geijerene	1136	1138			tr			AI,MS
25	terpinen-4-ol	1173	1174	1610	1604		0.2±0.0	1.2±0.3	Std,AI,MS
26	*p*-cymen-8-ol	1185	1179	1853	1853		tr	tr	AI,MS
27	α-terpineol	1189	1193	1705	1707		0.1±0.0	0.1±0.0	AI,MS

28	*cis*-piperitol	1193	1195						AI,MS
29	methyl chavicol	1195	1195	1671	1671	1.6±0.4			AI,MS
30	*trans*-piperitol	1206	1207						AI,MS
31	thymol, methyl ether	1230	1232	1592	1594			22.0±2.9	AI,MS
32	carvacrol, methyl ether	1235	1241	1603	1604			tr	AI,MS
33	(*Z*)-anethole	1250	1249	1758	1758	0.1±0.0			AI,MS
34	bornyl acetate	1282	1287					0.1±0.0	Std,AI,MS
35	(*E*)-anethole	1287	1282	1831	1834	96.7±2.1			Std,AI,MS
36	thymol	1295	1289	2182	2185		62.6±3.9	0.1±0.0	Std,AI,MS
37	carvacrol	1303	1298	2182	2182		0.3±0.1	tr	Std,AI,MS
38	(*E*)-caryophyllene	1413	1417						Std,AI,MS
39	α-*trans*-bergamotene	1427	1432					tr	AI,MS
40	γ-himachalene	1468	1481	1701	1700	0.5±0.1			AI,MS
41	*ar*-curcumene	1481	1479					tr	AI,MS
42	bicyclogermacrene	1487	1500					tr	AI,MS
43	α-zingiberene	1492	1493	1717	1718	0.1±0.0		tr	AI,MS
44	β-bisabolene	1505	1505					tr	AI,MS
45	myristicin	1518	1517	2271	2272			0.5±0.1	Std,AI,MS
46	β-sesquiphellandrene	1523	1521	1773	1173			tr	AI,MS
47	germacrene B	1546	1559					tr	AI,MS
48	elemicin	1559	1555	2231	2232			0.1±0.0	AI,MS
49	dillapiole	1623	1620	2368	2370			17.5±2.8	AI,MS
50	(*E*)-pseudoisoeugenyl 2-methylbutyrate	1839	1841			0.4±0.1			AI,MS
	Oil yield (%, w/w)					2.4	2.5	0.8	
	Total identified (%)					99.4	99.6	99.1	

[a]The elution order of components is from a HP-5MS capillary column. [b] Arithmetic retention index (AI) according to Van den Dool and Kratz (1963) calculated using a standard mixture of *n*-alkanes (C_8-C_{30}) on a HP-5MS column. [c]Literature AI value according to Adams (Adams, 2007). [a]Arithmetic retention index (AI) according to Van den Dool and Kratz (1963) calculated using a standard mixture of *n*-alkanes (C_8-C_{30}) on a DB-WAX column. [e] Literature retention index (RI) value according to NIST 17 (NIST, 2017). [f] Values are means of three measurements ± SD. [g] Identification procedure: Std, by RT, AI and MS overlapping with those of analytical standard; AI, coherence of calculated arithmetic index with respect to those reported in Adams (2017) and NIST 17 (2017); MS, matching of MS fragmentation with respect to Adams, NIST 17, Wiley 275 and FFNSC 2 libraries.[h]tr, traces, % below 0.1.

Table 3. Probit analysis showing the LC_{50} and LC_{90} values of selected essential oil microemulsions (in $ml.L^{-1}$) and α-cypermethrin (in $\mu g.L^{-1}$) tested on 3rd instar larvae of *Culex quinquefasciatus*.

Treatment	LC_{50}	CI_{95}	LC_{90}	CI_{95}	Chi
S1	1.57	1.46-1.69	2.53	2.29-2.92	0.681 n.s.
S2	2.23	2.10-2.38	3.21	2.93-3.64	1.917 n.s.
S3	2.39	2.12-3.33	4.13	3.96-4.82	1.822 n.s.
S4	4.01	3.71-4.33	6.48	5.83-7.47	2.281 n.s.
S5	1.45	1.41-1.52	1.81	1.73-1.92	0.384 n.s.
S6	1.63	1.56-1.71	2.18	2.04-2.42	2.427 n.s.
S7	1.83	1.09-2.09	3.06	2.86-3.12	3.252 n.s.
S8	1.59	1.52-1.65	2.08	1.95-2.27	3.166 n.s.
S9	1.94	1.83-2.07	2.76	2.53-3.12	2.425 n.s.
S10	1.91	1.01-2.12	3.19	2.97-3.28	1.009 n.s.
C1	not effective (no mortality at 10 $ml.L^{-1}$)				
C2	not effective (no mortality at 10 $ml.L^{-1}$)				
C3	not effective (no mortality at 10 $ml.L^{-1}$)				
C4	not effective (no mortality at 10 $ml.L^{-1}$)				
α-Cypermethrin	0.52	0.38-0.62	1.6	1.51-1.78	2.235 n.s.

n.s.= not significant ($P>0.05$)

Table 4. Toxicity over time of essential oil microemulsions tested at sub-lethal concentration (LC_{30}) on 3rd instar larvae of *Culex quinquefasciatus*.

Treatment	Applied concentrations LC_{30} (ml.L^{-1})	Larval mortality (%)*							
		24 h	48 h	72 h	96 h	120 h	144 h	168 h	Total
S1	1.3	35.1±7.1c	36.7±2.4c	48.3±4.7cd	51.7±2.4c	51.7±2.4bc	51.7±2.4c	53.3±2.4cd	53.3±2.4cd
S2	1.8	11.7±2.4b	23.3±4.7bc	51.7±6.2d	55.1±3.5c	55.1±2.4c	56.7±2.4c	61.7±4.1d	65.7±6.2d
S3	1.7	43.8±4.1c	63.3±4.7d	75.1±2.4e	78.3±2.4d	79.5±2.9d	80.7±7.2de	80.7±7.2ef	89.5±3.8e
S4	3.1	46.7±6.2cd	66.7±8.2d	78.3±6.2e	79.5±5.3d	79.5±5.3de	80.5±2.4de	83.7±5.5f	88.2±5.3e
S5	1.1	26.7±10.3c	33.3±2.4c	33.3±2.4c	35.5±5.4b	35.5±5.4b	35.5±5.4b	36.7±2.4b	42.5±6.2c
S6	1.2	48.3±2.8d	65.1±4.7d	85.2±7.1e	95.3±2.8e	100.0±0.0f	100.0±0.0f	100.0±0.0g	100.0±0.0f
S7	0.8	6.7±2.4b	6.7±2.4a	8.3±2.4ab	10.1±5.1a	11.7±2.4a	13.3±1.5a	13.3±1.5a	15.2±3.8b
S8	1.3	43.1±4.1cd	66.7±2.4d	68.3±2.4d	68.3±2.4cd	70.5±4.1cd	70.5±4.1d	70.5±4.1e	75.8±5.2de
S9	1.5	50.0±10.8d	60.0±4.1d	73.3±6.2e	80.1±2.4d	85.8±5.4e	85.8±5.4e	90.5±2.4f	95.3±2.4ef
S10	0.8	15.0±8.2b	16.7±2.4b	31.7±7.1c	35.0±4.7b	36.7±2.4b	38.3±2.4b	40.2±4.1b	45.8±3.9c
C1	4.0	0.0±0.0a	5.0±2.4a	5.0±2.4a	5.0±2.4a	5.0±2.4a	5.2±2.5a	8.3±4.7a	8.3±4.7a
C2	4.0	0.0±0.0a	5.5±2.7a	5.5±2.7a	5.5±2.7a	5.5±2.7a	8.3±4.7a	8.3±4.7a	8.3±4.7a
C3	4.0	8.7±2.1b	10.7±3.2ab	10.7±3.2b	10.7±4.2a	10.7±4.2a	10.7±3.2a	10.7±3.2a	10.7±3.2a
C4	4.0	0.0±0.0a	5.0±2.4a	5.0±2.4a	5.0±2.4a	5.0±2.4a	8.3±4.7a	8.3±4.7a	8.3±4.7a
Negative control	0	1.7±2.4a	1.7±2.4a	5.0±2.4a	8.3±4.7a	8.3±4.7a	8.3±4.7a	8.3±4.7a	8.3±4.7a
ANOVA *F*, *P*		428.53, *P*<0.001	332.87, *P*<0.001	539.56, *P*<0.001	298.58, *P*<0.001	312.55, *P*<0.001	265.18, *P*<0.001	356.19, *P*<0.001	431.75, *P*<0.001

* The average mortality (in % ± SD) of larvae post-treatment with sublethal concentrations, LC_{30} (in ml.L^{-1}) **. Means ± SD within a column followed by the same letter do not differ significantly (Tukey's HSD test, P < 0.05) % = arcsine square root transformed data. Negative control = water.

Table 5. Impact of essential oil microemulsions tested at sub-lethal concentration (LC_{30}) on 3rd instar larvae of *Culex quinquefasciatus* on adult emergence.

Treatment	Applied concentrations LC_{30} (ml.L^{-1}) **	Adult emergence (%)*		
		Female	Male	Total adults
S1	1.3	18.9±5.2cd	26.3±3.5d	45.2±2.9d
S2	1.8	12.2±2.4c	15.5±3.1c	27.7±4.2c
S3	1.7	4.3±0.2b	5.4±0.5b	9.5±0.7b
S4	3.1	2.1±0.3ab	5.1±0.1b	7.2±0.2b
S5	1.1	26.1±3.9d	28.1±8.5d	54.2±6.5d
S6	1.2	0.0±0.0a	0.0±0.0a	0.0±0.0a
S7	0.8	42.3±6.4e	39.2±5.2df	81.5±5.8e
S8	1.3	12.5±4.2c	8.6±0.9bc	21.1±2.1c
S9	1.5	0.0±0.0a	2.2±0.1a	2.2±0.1a
S10	0.8	31.3±4.5e	21.3±3.9d	52.6±4.1d
C1	4.0	42.5±8.2e	48.3±7.2f	90.8±5.5e
C2	4.0	43.8±5.9e	42.1±6.5ef	85.9±12.2e
C3	4.0	49.5±4.2e	35.4±4.3e	84,7±4.9e
C4	4.0	45.3±7.1e	45.1±7.2f	90.4±6.5e
Negative control	0	48.9±6.4e	40.9±8.5ef	89.8±6.7e
ANOVA *F*, *P*		428.76, $P<0.001$	452.17, $P<0.001$	418.33, $P<0.001$

* The average emergence of mosquito adults (in % ± SD) from larvae treated with sublethal concentrations - LC_{30} (in ml.L^{-1}) **. Means ± SD within a column followed by the same letter do not differ significantly (Tukey's HSD test, $P < 0.05$) % = arcsine square root transformed data. Negative control = water.

Table 6. Toxicity of essential oil microemulsions on three non-target invertebrates.

Treatment	Applied concentrations LC_{90} ($ml.L^{-1}$)	*Daphnia magna* *	*Tubifex tubifex* *	*Eisenia fetida* * ($1\ ml.kg^{-1}$ soil – for microemulsion $25\ mg.kg^{-1}$ for α-cypermethin)	
				5th day	10th day
S1	2.53	21.7±4.7c	95.3±4.1d	0.0±0.0a	0.0±0.0a
S2	3.21	0.0±0.0a	93.3±2.1d	0.0±0.0a	0.0±0.0a
S3	4.13	16.7±8.5bc	100.0±0.0d	0.0±0.0a	0.0±0.0a
S4	6.48	6.7±2.4b	96.7±2.4d	0.0±0.0a	0.0±0.0a
S5	1.81	9.5±4.7bc	73.3±8.5c	0.0±0.0a	0.0±0.0a
S6	2.18	20.1±11.5c	95.5±3.5d	0.0±0.0a	0.0±0.0a
S7	3.06	0.0±0.0a	98.1±3.3d	0.0±0.0a	0.0±0.0a
S8	2.08	13.3±2.4bc	70.1±10.8c	0.0±0.0a	0.0±0.0a
S9	2.76	18.3±6.2bc	83.5±6.8cd	0.0±0.0a	0.0±0.0a
S10	3.19	16.7±8.5bc	96.5±2.4d	0.0±0.0a	0.0±0.0a
C1	4.0	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
C2	4.0	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
C3	4.0	15.2±5.2bc	6.7±2.4b	0.0±0.0a	0.0±0.0a
C4	4.0	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
Negative control	-	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
Positive control	$1.6\ \mu g.L^{-1}$	100.0±0.0d	100.0±0.0d	99.0±1.0b	99.0±1.0b
ANOVA *F*, *P*		758.35, $P<0.001$	936.11, $P<0.001$	9,801.000, $P<0.001$	9,801.000, $P<0.001$

*Average mortality of *D. magna*, *T. tubifex* and *E. fetida* adults (± SD) achieved after application of LC_{90} estimated for mosquito larvae. Means ± SD within a column followed by the same letter do not differ significantly (Tukey's HSD test, $P < 0.05$) % = arcsine square root transformed data Negative control = water.

Figure

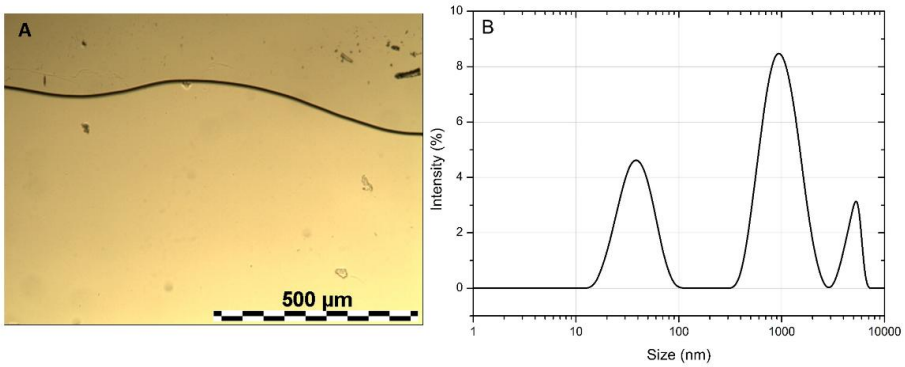

Figure 1. Image from the polarized light microscopy (A) and dynamic light scattering (DLS) traces (B) of EOs microemulsion S3.

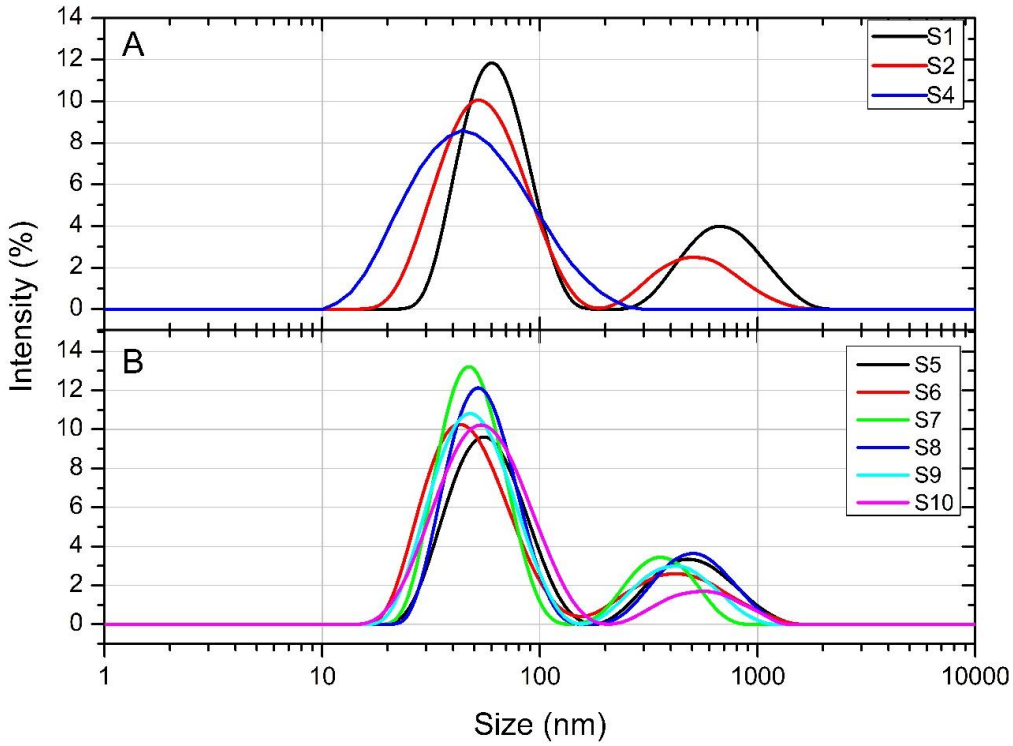

Figure 2. Dynamic light scattering (DLS) traces of singles EOs (A) and binary and ternary mixtures (B).

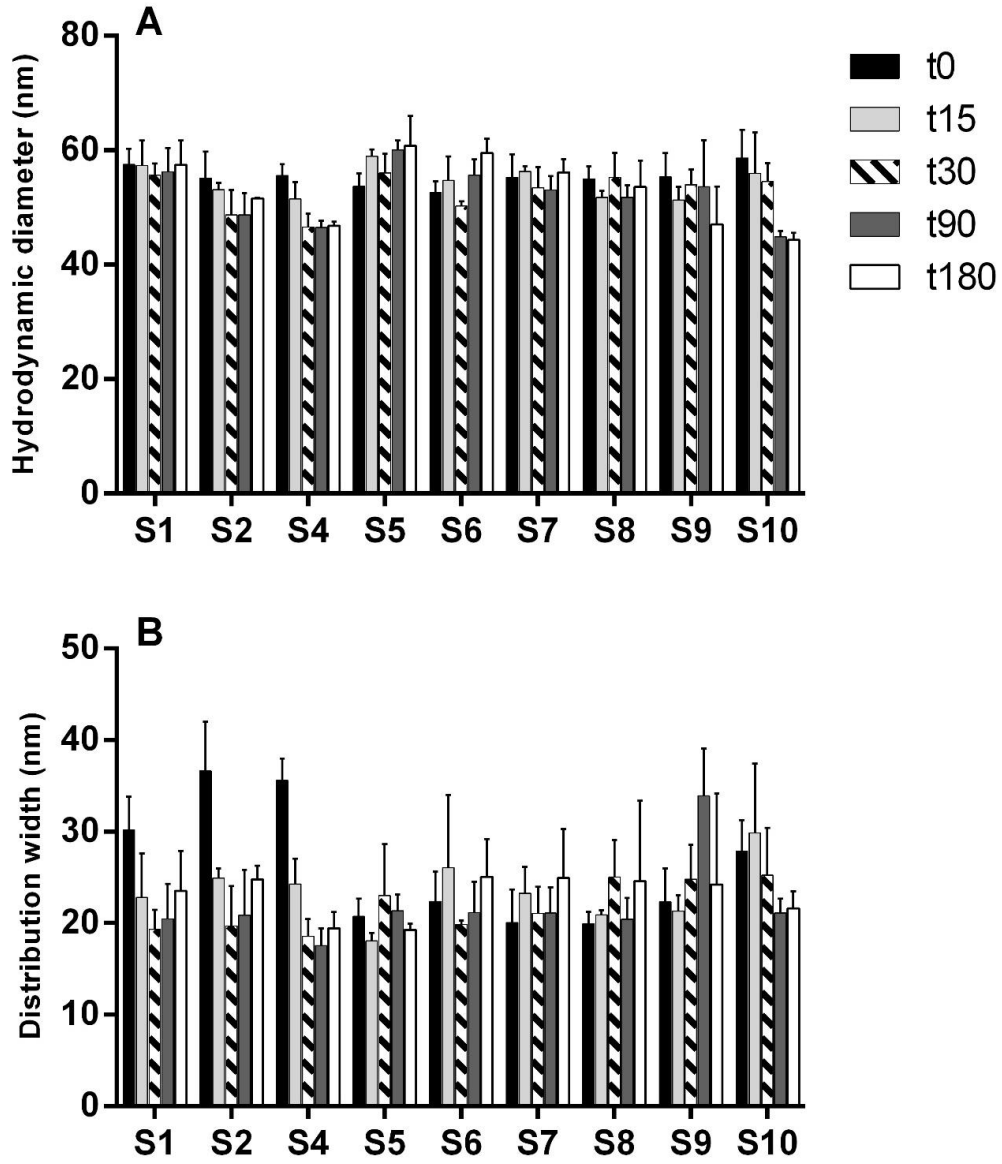

Figure 3. Hydrodynamic diameter (A) and distribution width (B) of essential oil microemulsions at different timepoints (t0, t15, t30, t90, t180), expressed in Size Distribution by Intensity.

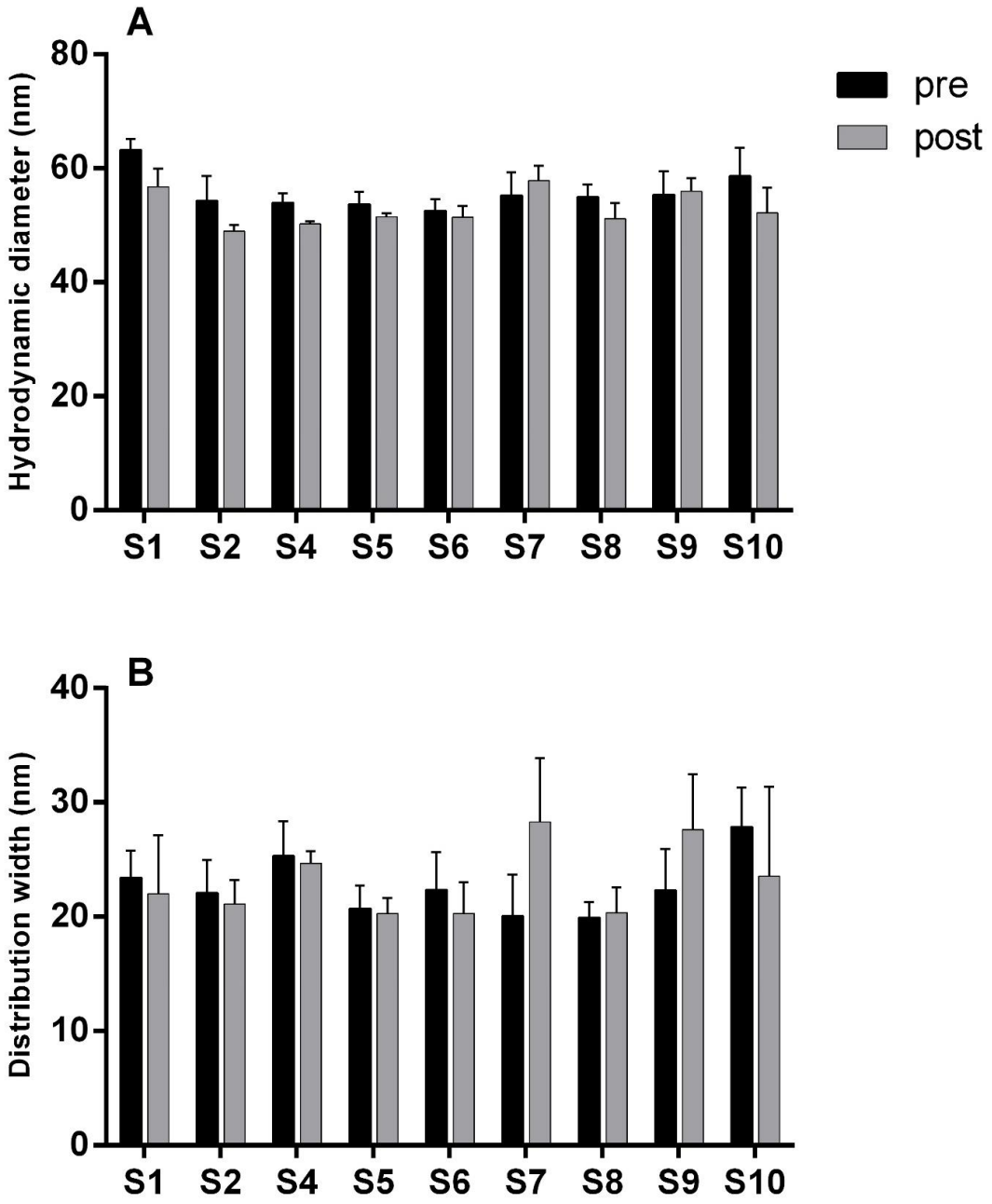

Figure 4. Hydrodynamic diameter (A) and distribution width (B) of essential oil microemulsions before and after centrifugation (accelerate stability test) at 6000 rpm for 1 h, expressed in Size Distribution by Intensity.