*Manuscript
Click here to view linked References

1 **The essential oil from industrial hemp (*Cannabis sativa* L.) by-products as an effective

2 tool for insect pest management in organic crops**

3

4 Giovanni Benelli[1,2] *, Roman Pavela[3], Riccardo Petrelli[4], Loredana Cappellacci[4], Giuseppe

5 Santini[4], Dennis Fiorini[5], Stefania Sut[6], Stefano Dall'Acqua[6], Angelo Canale[1], Filippo Maggi[4]

6

7 [1] Department of Agriculture, Food and Environment, University of Pisa, via del Borghetto 80,

8 56124 Pisa, Italy

9 [2] The BioRobotics Institute, Sant'Anna School of Advanced Studies, viale Rinaldo Piaggio

10 34, 56025 Pontedera, Pisa, Italy

11 [3] Crop Research Institute, Drnovska 507, 161 06 Prague, Czech Republic

12 [4] School of Pharmacy, University of Camerino, Camerino, Italy

13 [5] School of Sciences and Technology, University of Camerino, Camerino, Italy

14 [6] Department of Pharmaceutical and Pharmacological Sciences, Natural Product Laboratory,

15 University of Padova, Padova, Italy

16

17 * Corresponding author. Phone: +39 0502216141, Fax: +39 0502216087; e-mail:

18 benelli.giovanni@gmail.com (G. Benelli)

19

20 **Abstract**

21

22 The inflorescences of industrial hemp (*Cannabis sativa* L.) represent a consistent by-
23 product that is underutilized. Moving from the concept that this plant part has evolved as a
24 natural weapon against phytophagous insects, secreting important secondary metabolites such
25 as cannabinoids and volatile terpenes, herein we assayed the potential of its essential oil as a
26 botanical insecticide. For the purpose, the essential oil was obtained by fresh inflorescences of
27 hemp (monoecious cv. Felina 32) by steam-distillation and analysed by gas chromatography
28 (GC-FID) and gas chromatography-mass spectrometry (GC-MS). The oil was tested against
29 the filariasis vector *Culex quinquefasciatus*, the peach-potato aphid *Myzus persicae*, the
30 housefly *Musca domestica* and the tobacco cutworm *Spodoptera littoralis.* To prove its
31 harmlessness on non-target invertebrates, it was tested on the multicolored Asian lady beetle,
32 *Harmonia axyridis,* and *Eisenia foetida* earthworms and compared with α-cypermethrin as the
33 positive control. The essential oil composition was dominated by monoterpene and
34 sesquiterpene hydrocarbons, with (*E*)-caryophyllene (45.4%), myrcene (25.0 %) and α-pinene
35 (17.9%) as the most abundant compounds. Results from insecticidal tests showed that the
36 essential oil from inflorescences of industrial hemp cv Felina 32 was highly toxic to *M.*
37 *persicae* aphids (LC_{50} of 3.5 $mL.L^{-1}$) and *M. domestica* flies (43.3 µg $adult^{-1}$), while toxicity
38 was moderate towards *S. littoralis* larvae (152.3 µg $larva^{-1}$), and scarce against *C.*
39 *quinquefasciatus* larvae (LC_{50} of 252.5 $mL.L^{-1}$) and adults (LC_{50} > 500 $\mu g.cm^{-2}$). Contrary to
40 α-cypermethrin, the hemp cv Felina 32 essential oil was not toxic to non-target invertebrate
41 species, including 3rd instar larvae and adults of *H. axyridis* ladybugs and adults of *E. foetida*
42 earthworms. Taken together our results shed light on the possible utilization of the crop
43 residue of industrial hemp as a source of environmental-friendly botanical insecticides to be

used in Integrated Pest Management and organic agriculture, particularly to manage aphid and housefly populations.

Keywords: hemp; *Cannabis sativa*; essential oil; aphids; earthworms; mosquito vectors

49 **1. Introduction**

50

51 In the last years, the market of conventional agrochemical products to combat insects
52 and agricultural pests has experienced a significant decrement due to the explosion of
53 botanical pesticides that have conquered the trust of agricultural labourers and have been
54 increasingly employed in Integrated Pest Management (IPM) programmes (Isman and
55 Machial, 2006; Thakore, 2006; Benelli et al., 2017a, 2018a,b). In this regard, botanical
56 insecticides are favourable accepted by consumers due to their recognized efficacy, the eco-
57 friendly impact, the low toxicity on mammals and beneficial organisms (Desneux et al., 2007;
58 Benelli et al., 2016; Pavela and Benelli, 2016; Stevenson et al., 2017), and the low or none
59 possibility to cause resistance in arthropod pests. Thus, this trend is expected to still go up in
60 the next years because of marketing of new products (Isman, 2015) and of the streamlining
61 regulation operated by authorities.

62 Among various crops having the potential to be employed in IPM programmes, here
63 we focused on industrial hemp (*Cannabis sativa* L.).

64 Indeed, a hallmark of hemp (in both var. *indica* and *sativa*) is the presence of
65 glandular hairs concentrated on leaves and, to a major extent, on inflorescences that secrete a
66 sort of oleoresin functioning as a barrier entrapping and killing plant enemies (Potter, 2009).
67 These parts are normally discharged during the conventional hemp processing, thus
68 representing an underutilized biomass for further application. In particular, they are a rich
69 source of essential oil containing mainly monoterpene and sesquiterpene hydrocarbons
70 (Bertoli et al., 2010).

71 The exploitation of hemp by-products as a source of botanical insecticides is a matter
72 of interest for farmers, allowing them to maximise the commercial value of hemp cultivation.
73 Our idea is to obtain bioactive essential oils from the inflorescences of industrial hemp that

74 usually remain underutilized to manufacture natural insecticides to be employed in organic
75 agriculture and IPM programmes. Indeed, research in this topic area is still poor.
76 Cultivation of industrial hemp to produce insecticides displays the following strengths:
77 (i) lack of similar products (i.e. hemp-based insecticides); (ii) low costs of raw material and
78 availability of agricultural lands for its cultivation; (iii) increasing demand for eco-friendly
79 and safe products; (iv) possibility to split the end products in other fields (e.g., cosmetics and
80 pharmaceutics). Supporting literature comes from the recent investigations by Benelli et al.
81 (2018a) and Bedini et al. (2016) who found that the hemp essential oil is effective against
82 larvae of mosquito vectors and moth pests, as well as against flies and snails.
83 In the present work, we used GC-MS analysis to investigate the chemical composition
84 of the essential oil from the inflorescences of industrial hemp cv. Felina 32 cultivated in
85 central Italy. The quantification of the marker compounds α-pinene, myrcene, terpinolene,
86 (*E*)-caryophyllene and cannabidiol in the essential oil was performed by GC-FID.
87 Furthermore, we explored the insecticidal effects of industrial hemp cultivated in
88 central Italy on a panel of economically important target insects including two vectors of
89 public health importance, i.e., the mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae),
90 and the house fly *Musca domestica* L. (Diptera: Muscidae) (Benelli & Mehlhorn 2016;
91 Davies et al., 2016), and two insect pests attacking crops of high economic interest, i.e., the
92 aphid *Myzus persicae* (Sulzer) (Rhyncota: Aphididae), and the tobacco cutworm *Spodoptera*
93 *littoralis* (Boisduval) (Lepidoptera: Noctuidae). In particular, *C. quinquefasciatus* is
94 recognized as a vector of lymphatic filariasis, West Nile and Zika virus (Benelli and Romano,
95 2017), while *M. persicae* and *S. littoralis* are able to feed on more than 400 and 80 plant
96 species, respectively (Bass et al., 2014; OEPP/EPPO, 2015), with severe economic damages
97 for farmers.

98 To prove the safety of hemp essential oil, its toxicity on beneficial organisms such as
99 the multicolored Asian lady beetle *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae)
100 and the earthworm *Eisenia foetida* (Savigny) (Oligochaeta, Lumbricidae) has been evaluated
101 and compared with α-cypermethrin as positive control. Based on our results, a future
102 application of this multi-purpose crop as a source of botanical insecticides to combat
103 agricultural pests and vectors of public importance may be possible.
104
105 **2. Materials and methods**
106
107 *2.1. Plant material*
108 The inflorescences of industrial hemp cv. Felina 32 (Assocanapa, Torino, Italy) were
109 collected from a cultivated field placed in Fiuminata, central Italy (N 43°11'11", E
110 12°56'24", 318 m a.s.l.) in August 2017. The crop utilized was normally employed to produce
111 seed oil. A voucher specimen was archived and deposited in the Herbarium of the Centro
112 Ricerche Floristiche dell'Appennino (APP), Barisciano, L'Aquila, Italy, under the codex APP
113 No. 57789.
114
115 *2.2. Steam distillation*
116 Fresh inflorescences of hemp (2500 g) were inserted in an Albrigi Luigi E0106
117 (Stallavena di Grezzana-Verona, Italy) stainless steel apparatus (capacity 20 L) and subjected
118 to steam-distillation for 3 h. Steam was produced from 2 L of water at the bottom of
119 apparatus. Once obtained the yellowish oil was decanted, then collected using a funnel and
120 dehydrated with anhydrous Na_2SO_4. The yield was calculated on a dry weight basis, by
121 calculating the water content of inflorescences prior to distillation. The oil was stored in
122 amber glass vials at +4 °C before insecticidal assays.

123

124 *2.3. GC-MS analysis*

125 Hemp essential oil, diluted 1:100 in *n*-hexane injected into an Agilent 6890N gas

126 chromatograph equipped with a 5973N mass spectrometer. Separation was achieved on a HP-

127 5 MS (5 % phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 µm film thickness; J & W

128 Scientific, Folsom) column. As oven temperature programme we used the following operative

129 conditions: 5 min at 60°C then increase up to 220 °C with a gradient of 4°C/min, then

130 increase up to 280 °C at 4 °C/min, held for 15 min. The temperature of injector and detector

131 was 280 °C; the carrier gas was helium (He) with a flow rate of 1 mL/min and using a split

132 ratio of 1:50.

133 The chromatograms were obtained in full scan using electron-impact (EI, 70 eV)

134 mode. The mass range scanned was 29–400 *m/z*. Data were elaborated by using the MSD

135 ChemStation software (Agilent, Version G1701DA D.01.00) and the NIST Mass Spectral

136 Search Program for the NIST/EPA/NIH Mass Spectral Library v. 2.0. The analysis was

137 repeated three times and the mean values reported. The peak assignment was achieved by

138 comparison with analytical standards bought from Sigma-Aldrich (Milan, Italy) (see Table 1).

139 In addition, the combination of the correspondence of the linear retention indices,

140 calculated using a mixture of C*8*-C*30* *n*-alkanes (Supelco, Bellefonte, CA, USA) according to

141 the Van den Dool and Kratz formula (Van den Dool & Kratz, 1963), and mass spectra with

142 respect to those reported in ADAMS, NIST 08 and FFNSC2 libraries (Adams, 2007; NIST

143 08, 2008; FFNSC2, 2012) was used as an additional parameter for peak assignment. The

144 percentage values were obtained from the peak areas without calculating the response factors.

145

146 *2.4. Quantification of the marker compounds by GC-FID*

147 Quantification of α-pinene, (*E*)-caryophyllene, terpinolene, cannabidiol, myrcene in
148 the essential oil was performed by means of gas-chromatography coupled with flame
149 ionization detection (GC-FID) using a GC 6850 from Agilent Technologies. The analytical
150 standards of the above compounds were purchased from Sigma-Aldrich (Milan, Italy). The oil
151 was diluted with chloroform (10 mg in 1 mL of chloroform) and 0.5 µL injected in split mode
152 (split ratio 1:30) into the GC. The injector temperature was 300°C. The carrier gas was
153 hydrogen produced by a generator (PGH2-250 from DBS Analytical Instruments, Vigonza,
154 Italy). The initial gas flow in the column was 3.7 mL min^{-1}. Chromatographic column coating
155 was a (5%-Phenyl)-methylpolysiloxane (HP-5, 30 m, 0.32 mm i.d., 0.25 µm film thickness,
156 from Agilent Technologies). The oven temperature was held at 60°C for 3 min, then raised at
157 25°C min^{-1} until 350°C and held at 350°C for 1 min, for a total run time of 15.60 min. The
158 FID temperature was set at 360°C and hydrogen flow was 40 mL $min^{-1}$and air flow was 400
159 mL min^{-1}. The quantification was performed by using the calibration curves obtained for
160 analytes investigated which were built by preparing stock standard solutions at 6 different
161 concentrations in the range 0.225-14 mg mL^{-1}. Correlation coefficients ranged from 0.9944 to
162 0.9999.
163
164 *2.5. Insect and earthworm rearing*
165 The method by Benelli et al., (2017a) was used to rear third instar larvae and non-
166 blood-fed females of *C. quinquefasciatus* tested in the experiments described below. In assays
167 testing houseflies (*M. domestica*), adult females were obtained as described by Benelli et al.
168 (2018a). An artificial insect diet (Stonefly Industries, Bryan, TX, USA) as reported by Sut et
169 al. (2017) was used to rear early 3rd instar larvae of *S. littoralis*. *M. persicae* adults were
170 reared on greenhouse potted cabbage *Brassica oleracea* convar. *capitata* (L.) ALEF.
171 (Brassicaceae), according to the procedure detailed by Stepanycheva et al. (2014), while *H.*

172 *axyridis* larvae and adults were obtained as described by Pavela et al. (2013). All the tested
173 insects were maintained at 25±1 °C, 70±3% R.H. and 16:8 h (L:D).
174 Adult earthworms (*E. fetida,* weight 350–500 mg) with well-developed clitella were
175 reared in laboratory (>20 generations; out-crossed once), while keeping the earthworms in
176 artificial soil according to OECD (1984), room temperature was 20±1 °C. The artificial soil
177 was as described by Pavela (2018). Maximum water-holding capacity (35%) of the soil was
178 checked weekly.
179
180 *2.6. Toxicity on Culex quinquefasciatus larvae*
181 We used a WHO method *(WHO, 1996)* with slight adjustments as described by Pavela
182 et al. (2017) to test the acute toxicity of *C. sativa* cv Felina 32 essential oil diluted in dimethyl
183 sulfoxide (DMSO) on *C. quinquefasciatus* 3rd instar larvae (tested concentrations: 40, 60, 70,
184 100, and 150 µl L^{-1}, concentrations were calculated considering the pure essential oil as 100
185 % concentration). The tests provided mortality values ranging from 10% and 90%. Four
186 duplicate trials were performed for each concentration. Distilled water with the same amount
187 of DMSO used to test *C. sativa* cv Felina 32 essential oil was used as the control. Larval
188 mortality was noted after 24 h. α-Cypermethrin was used as the positive control since its
189 insecticidal efficacy, as well as that of many essential oils, is based on neurotoxicity.
190
191 *2.7. Toxicity on Culex quinquefasciatus adults*
192
193 According to WHO reports (WHO, 1996), the most common method of protection
194 against adults is by means of impregnated nets or treated residential walls. Therefore, tarsal
195 tests for the development of botanical insecticides against adults are highly important. The
196 tarsal contact test was conducted to evaluate the acute toxicity of *C. sativa* cv Felina 32

197 essential oil for adult females of *C. quinquefasciatus*. The method by WHO (1996) with
198 minor changes by Benelli et al. (2017b) was used for this test, while the *C. sativa* cv Felina 32
199 oil was diluted with acetone (2 mL, Sigma-Aldrich, Germany) plus silicon oil (3.6 mg.cm^{-2}).
200 The obtained mixture was carefully applied onto filter paper (Whatman No. 1, size: 12×15
201 cm). The essential oils were tested at 5 doses ranging from 100 to 500 µg.cm^{-2}. After a drying
202 phase of 24 h at 25 °C the treated filter paper pieces were inserted in test tubes where 20
203 female mosquitoes, 2-5 days old, were exposed for 1 h. After this period the females were put
204 in plastic cages (20×20×20 cm, 26±1 °C 16:9 L:D) and kept there for 24 h; a sucrose solution
205 was supplied to them as food. Mortality was determined at 24 h from their exposure. *α*-
206 Cypermethrin was used as the positive control.
207
208 *2.8. Toxicity on Musca domestica adults*
209 Topical application tests were performed to test the acute toxicity of *C. sativa* cv
210 Felina 32 essential oil against *M. domestica* adult females (3–6 days old). We used the
211 method reported by Pavela et al. (2018), i.e. a microelectric applicator on the pronotum of
212 houseflies anesthetized using CO_2 was used to apply 1 µL of acetone (Sigma-Aldrich,
213 Germany) carrying the *C. sativa* cv Felina 32 essential oil at concentrations ranging from 30
214 to 250 µg adult^{-1} (each replicated at least 4 times). Acetone without the *C. sativa* cv Felina 32
215 essential oil was used as the control. Subsequently, the houseflies were put into a recovery
216 box (10×10×12 cm, 26±1 °C 16:9 L:D) and kept there for 24 h before determining their
217 mortality. *α*-Cypermethrin was used as the positive control.
218
219 *2.9. Toxicity on Myzus persicae*
220 Toxicity assays were conducted to test the toxicity of *C. sativa* cv Felina 32 essential
221 oil on *M. persicae* aphids. The toxicity was tested in adults on potted cabbage plants with 3-4

222 true leaves, as detailed by Stepanycheva et al. (2014). The *C. sativa* cv Felina 32 essential oil

223 was co-formulated with Tween 80 (1:1, v:v); the concentrations of 15, 10, 5.0, 2.5 and 1.2

224 ml.L^{-1} were used in the assays, equivalent to 7.5, 5.5, 2.5, 1.25 and 0.6 ml.L^{-1} of the *C. sativa*

225 cv Felina 32 essential oil. A manual sprayer was used to apply this mixture on the cabbage

226 plants, at 50 ml m^{-2} (corresponding to approx. 500 L.ha^{-1}), while water plus Tween 80 at 7.5

227 mL.L^{-1} was tested as the negative control (50 mL.m^{-2}). For each replicate, 50 *M. persicae*

228 adults (4 replicates per tested concentration) were tested at 25±1 °C, 70±5% R.H., and using

229 the photoperiod 16:8 h (L:D). The mortality rate of *M. persicae* was noted at 48 h from

230 spraying. α-Cypermethrin was used as the positive control

231

232 *2.10. Toxicity on Spodoptera littoralis larvae*

233 In this case, the toxicity of the *C. sativa* cv Felina 32 essential oil on 3rd instar larvae

234 of the tobacco cutworm, *S. littoralis*, was evaluated using a method recently described by Sut

235 et al. (2017), i.e. through topical application of the essential oil diluted in acetone on the

236 larvae. The larvae were treated on the dorsum with 1 µL of acetone, which contained five

237 different concentrations (ranging from 100 to 400 µg larva^{-1}) of the *C. sativa* cv Felina 32

238 essential oil. Four replicates (n=20 larvae per replicate) were used for each tested oil

239 concentration. Acetone (without any *C. sativa* cv Felina 32 essential oil) served as the control.

240 The moth larvae were then moved to a recovery box (10×10×7 cm) with thin holes on each

241 wall to avoid fumigation effects, and kept there at 26±1 °C, 70±3 % R.H., and 16:8 L:D for

242 24 h. Subsequently, the larval mortality was determined. α-Cypermethrin was used as the

243 positive control.

244

245 *2.11. Toxicity on the non-target species Harmonia axyridis*

246 Using the method described by Pavela et al. (2013), 3rd instar larvae and adults (3-7
247 days old) of the ladybug *H. axyridis* – a non-target organism – were tested to assess the acute
248 toxicity of the *C. sativa* cv Felina 32 oil. The tested concentrations (5.5, 2.5, 1.25 and 0.6
249 $mL.L^{-1}$), as well as the testing procedure were the same for both the (ladybug) larvae and
250 adults as described in "Toxicity on *M. persicae*", since the latter is a common prey of *H.*
251 *axyridis*, which means that they share the same ecological niche. Only one difference was
252 implemented – the *C. sativa* cv Felina 32 essential oil was applied on ladybug larvae and
253 adults in open Petri dishes (9 cm in diameter; ten insects tested per replicate; 4 replicates per
254 tested concentration). The commercial insecticide Vaztak® was applied in the concentration
255 recommended for its use against aphids – 0.1% (v/v), equivalent to 0.005% (w/v) of α-
256 cypermethrin, the active substance. Subsequently, 20 mL of the application liquid was applied
257 per m^2, equivalent to about 200 $L.ha^{-1}$, while the negative control was treated only with water
258 containing the appropriate equivalent (11 $ml.L^{-1}$) of Tween 85, and a 50 mL dose of the
259 application liquid was applied per m^2, equivalent to about 500 $L.ha^{-1}$. The individuals of *H.*
260 *axyridis* were moved to clean Petri dishes after the treatment, and here they were fed with *M.*
261 *persicae* aphids, while maintained at 25±1°C, 70±5% R.H., and 16:8 (L:D); mortality was
262 noted after 48 h.
263
264 *2.12. Toxicity on non-target Eisenia fetida earthworms*
265 The standard OECD (1984) method was followed to test the toxicity of the *C. sativa*
266 cv Felina 32 essential oil on *E. fetida* adult earthworms. In these assays, the used artificial soil
267 was characterized by the same composition and pH as described for *E. fetida* rearing; the soil
268 was prepared by adding the EOs in concentrations of 200, 100 and 50 $mg.kg^{-1}$, mixed with
269 Tween 80 (ratio 1:1 v:v), equivalent to 100, 50 and 25 mg EO a.i. per kg of dry weight basis
270 soil. We used *α*-cypermethrin at 50.0, 25.0 and 12.5 $mg.kg^{-1}$ of dry soil [i.e., Vaztak® at

271 1000, 500 and 250 μL.kg^{-1} (v/v)] as the positive control, while distilled water was used as the
272 negative control. An aqueous formulation containing the *C. sativa* cv Felina 32 essential oil,
273 pure water or *α*-cypermethrin was mixed into the soil (650 g) and 10 *E. foetida* adults were
274 added. Both the treated and control soil samples were then stored in glass pots (1 L) covered
275 with gauze in order to ensure aeration. The mortality rates of *E. foetida* were noted after 7 and
276 14 days of exposure to the treatments at 20±1 °C, R.H. 80-85%, 16:8 (L:D) and 600 lux.
277
278 *2.13. Statistical analysis*
279 When the controlled mortality reached 20%, the observed mortality was corrected
280 using Abbott's formula (Abbott, 1925). Probit analysis was conducted to estimate the $LD_{50(90)}$
281 and $LC_{50(90)}$ values, with associated 95% confidence limits for each treatment (Finney, 1971).
282 Mortality rates (%) were transformed using the arcsine√ transformation before running
283 ANOVA and Tukey's HSD test (P≤0.05).
284
285 **3. Results and Discussion**
286
287 *3.1. Composition of hemp essential oil*
288 Steam-distillation of the fresh inflorescences of industrial hemp (cv Felina 32) gave
289 0.1% of essential oil whose chemical profile is depicted in Fig. 1. A total of forty-seven
290 volatile constituents were identified by GC-MS, accounting for 99.8% of the total area
291 percentage (Table 1). The oil was dominated by two major groups of components, namely
292 monoterpene hydrocarbons (54.0%) and sesquiterpene hydrocarbons (44.2%). Overall, the
293 most abundant components exhibiting percentage values above 10% were (*E*)-caryophyllene
294 (23.8%), α-pinene (16.4%) and myrcene (14.2%), accounting together for more than half the
295 oil composition. Other components occurring at noteworthy amounts were terpinolene

296 (9.6%), α-humulene (8.3%), β-pinene (5.2%), (*E*)-β-ocimene (5.1%) and (*E*)-β-farnesene
297 (3.0%). It is worth mentioning that cannabinoids were almost missing from this oil sample;
298 they were represented only by cannabidiol (0.1%). Further, the content of caryophyllene
299 oxide, the main oxidation product of (*E*)-caryophyllene which is considered the key volatile
300 for detection of marijuana was here present in scant amounts (1.2%).

301 In the present work, we have also developed a GC-FID method for accurate
302 quantification of marker compounds of the hemp essential oil, namely α-pinene, myrcene,
303 terpinolene, (*E*)-caryophyllene and cannabidiol. The method was proven to be linear and
304 reproducible, allowing to quantitatively determine the absolute content of these oil
305 constituents. Indeed, (*E*)-caryophyllene was the most abundant compound accounting for
306 45.4% of the oil, followed by myrcene (25.0%), α-pinene (17.9%) and terpinolene (10.1%),
307 whereas cannabidiol was found at trace levels (<0.1%).

308 Actually, the chemical composition of the essential oil from hemp inflorescences is
309 affected by several factors such as cultivar, harvesting period and extraction and processing of
310 the raw material (Benelli et al., 2018a; Calzolari et al., 2017). As a consequence, several
311 chemotypes have been reported showing different levels of monoterpene and sesquiterpene
312 hydrocarbons as well as cannabinoids (Bertoli et al., 2010). When compared with the study of
313 Bertoli et al. (2010) conducted on the same cv (Felina 32), a substantial overlapping of the
314 chemical profiles can be observed. As matter of fact, Bertoli et al. (2010) reported α-pinene
315 (20.3-20.4%), (*E*)-caryophyllene (19.4-19.5%), terpinolene (15.0-19.1%) and myrcene (12.3-
316 13.6%) as the major constituents of the essential oils obtained from inflorescences of plants
317 cultivated during two years in Tuscany, Italy. Among the other constituents, also in these
318 samples β-pinene (6.3-7.9%), (*E*)-β-ocimene (5.9-6.5%) and α-humulene (5.0-6.0%) were
319 detected as the most representative ones. On the other hand, here cannabidiol (1.7-1.9%) was
320 found at higher levels than in our sample (0.1%). We assume that the slight differences

321 emerged in these studies may be due to the different methods used for the extraction of
322 essential oils, i.e. steam-distillation vs. hydrodistillation. As a matter of fact, the latter is
323 known to be more aggressive than the former and produces oxidative and hydrolytic reactions
324 to a major extent leading to decarboxylation of the cannabinoid acids into the relative alcohol
325 forms (Calzolari et al., 2017). Also, when considered the yields related to the fresh matter
326 distilled, the two studies gave convergent values (0.1%).

327 On the other hand, the chemical profile observed in this study was quite different from
328 those obtained from other cultivars and/or subjected to different processing. As a matter of
329 fact, in our previous study performed on the hemp Felina 75, we found (*E*)-caryophyllene
330 (21.4%), myrcene (11.3%), cannabidiol (11.1%), α-pinene (7.8%), terpinolene (7.6%), and α-
331 humulene (7.1%) as the most abundant essential oil constituents (Benelli et al., 2018a). We
332 imputed this variance to the different cultivar utilized as well to the extraction procedure
333 (hydrodistillation) and condition of raw material (dried vs fresh).

334

335 *3.2. Insecticidal activity and impact on non-target invertebrates*

336 Essential oils have been successfully used to manage a rather wide number of insect
337 pests and vectors (Benelli and Pavela, 2018a,b). Herein, the results from insecticidal tests
338 showed that the essential oil from inflorescences of industrial hemp cv Felina 32 was highly
339 toxic to *M. persicae* aphids ($LC_{50(90)}$ of 3.5(6.2) $mL.L^{-1}$) and *M. domestica* flies ($LD_{50(90)}$ =
340 43.3(213.5) $\mu g\ adult^{-1}$) (Table 2), while toxicity was moderate towards *S. littoralis* larvae
341 (152.3 $\mu g\ larva^{-1}$), and scarce against *C. quinquefasciatus* larvae (LC_{50} of 252.5 $mL.L^{-1}$) and
342 adults (LC_{50} > 500 $\mu g.cm^{-2}$) (Table 2). Furthermore, the toxicity of cypermethrin, tested as
343 positive control, on the four insect species, was detailed in Table 2. The hemp cv Felina 32
344 essential oil was not toxic to non-target organisms, such as 3rd instar larvae and adults of *H.*
345 *axyridis* ladybugs (Table 3) and adults of the *E. foetida* earthworms (Table 4).

346 Although the tested EO showed lower efficacy against the larvae of *S. littoralis, C.*
347 *quinquefasciatus* and against the adults of *M. domestica*, we found outstanding aphicidal
348 efficacy against *M. persicae*. As already reported, some EOs exhibit very promising effects
349 against aphids (Pavela, 2018). However, our paper provides not only the first information
350 about very good aphicidal efficacy of the EO from *C. sativa*, but also evidence of the fact that
351 this EO is very friendly to non-target organisms. This information is important as it indicates
352 environmental safety of residues of potential botanical insecticides developed based on this
353 EO. Generally, aphids are widely recognized as key pests in agriculture, including the tested
354 *M. persicae*, which is also one relevant insect vector of plant viruses (Blackman and Eastop,
355 2000; Blanc et al., 2011). In addition, this aphid species has been known for its ability to
356 develop populations showing resistance to synthetic insecticides (Bass and Field, 2011; Bass
357 et al., 2014). Botanical insecticides based on EOs from *C. sativa* can thus become a suitable
358 alternative solution for the protection against these important pests.

359 Environmental safety of EOs against natural enemies of aphids and soil organisms has
360 also been confirmed by some previous studies. For example, the EO from *Foeniculum*
361 *vulgare* Mill. showed no significant toxicity for earthworms, unlike a synthetic insecticide
362 based on *α*-cypermethrin, which killed all earthworms instantly, just like in our case (Pavela,
363 2018). Earthworms are necessary for the development and maintenance of the nutritional
364 value and structure of soil (Datta et al., 2016). As such, they play an important role in the
365 conversion of biodegradable materials and organic waste to vermicast, which is rich in
366 nutrients (Jansirani et al., 2012). Protection of these organisms thus clearly requires proper
367 attention.

368 Additionally, even though earthworms are able to consume a wide range of
369 contaminated organic materials including but not limited to sewage sludge and industrial
370 waste (Lim et al., 2016), they are very sensitive to insecticides (Datta et al., 2016; Vasantha-

371 Srinivasan et al., 2018). Generally, insecticides have a definite negative effect on the survival
372 of earthworms, especially in concentrations higher than 25 mg.kg^{-1} (Rodriguez-Campos et al.,
373 2014; Datta et al., 2016). In this respect, we have succeeded in finding a promising and
374 environmentally acceptable active substance for potential botanical insecticides.

375 The oleoresin secreted by hemp glandular trichomes has evoluted as a chemical
376 defence against insects (Potter, 2009). The volatile part of this secretion is mainly composed
377 of monoterpene and sesquiterpene hydrocarbons having variable chemical structures (e.g.,
378 linear, monocyclic, and bicyclic ones), low molecular weight and high hydrophobicity that
379 make them particularly capable of crossing easily the insect surface, diffusing through the
380 body and entering the cells. Moreover, their high lipophilicity allows them to interact with
381 behavioural, metabolic and physiological processes of insect (Jacobson, 1989). Hemp
382 essential oil is a mixture of several dozens of chemical constituents of various structure and
383 mechanism of action, having frequently a multi-target action. Indeed, synergistic effects occur
384 among the different constituents so that the crude essential oil has toxicity higher than that of
385 its major components, with none or reduced probability of inducing resistance in insects
386 (Hummelbrunner and Isman, 2001). Essential oils can produce neurotoxicity on insects by
387 interacting with different receptors such as cholinergic, gamma-aminobutyric acid (GABA)
388 and octopaminergic ones (Pavela and Benelli, 2016). In this regard, it has been reported that
389 the hemp essential oil is able to inhibit the acetylcholinesterase (AChE) enzyme in a more
390 effective manner than other oils (Benelli et al., 2018a).

391 Overall, the toxic effects of hemp essential oil on the four target insects may be
392 attributed to the main chemical constituents such as α-pinene, myrcene, (*E*)-caryophyllene
393 and terpinolene. Indeed, α-Pinene was reported as toxic against *Aedes aegypti* and *Culex*
394 *pipiens molestus* with LD_{50} of 15.4 and 47-49 ppm, respectively (Lucia et al., 2007; Traboulsi
395 et al., 2002), while myrcene is a key component in several essential oils endowed with

396 larvicidal and/or repellent effects against mosquito vectors such as those from orange (*Citrus*
397 x *aurantium* L.) peel (Govindarajan et al., 2012) and lemongrass (*Cymbopogon citratus* (DC.)
398 Stapf (Bossou et al., 2013; Suwansirisilp et al., 2013).

399 Besides, the essential oils containing high levels of (*E*)-caryophyllene, such as those
400 from *Artemisia nilagirica* (C.B.Clarke) Pamp. and *Pinus nigra* J.F. Arnold var. *italica* were
401 reported as repellent against the dengue vector *A. aegypti* (Ali et al., 2016) and toxic against
402 *C. quinquefasciatus* (Benelli et al., 2017b). Assayed as a pure compound, (*E*)-caryophyllene
403 was toxic on larvae of *Anopheles subpictus*, *Aedes albopictus* and *C. tritaeniorhynchus* with
404 LD_{50} of 41.7, 44.8 and 48.2 ppm, respectively (Govindarajan et al., 2016). More generally, it
405 has been reported that this compound is among the most active essential oil constituents with
406 larvicidal effects known so far, and its effects may be synergized by other compounds
407 occurring in mixture (Pavela, 2015). (*E*)-caryophyllene and α-pinene isolated from the *Vitex*
408 *negundo* seed extract exhibited toxicity and repellency against *Aphis gossypii* Glover along
409 with effects on fertility at sublethal doses (Liu et al., 2010).

410 Tepinolene, another main constituent of hemp essential oil, can neutralize the
411 octopaminergic system causing knockout of insect (Rattan, 2010). Terpinolene is one of the
412 major volatile constituents of *Clausena excavata* Burm. F. which showed inhibitory effects on
413 larvae of *A. aegypti* and *A. albopictus* (LD_{50} of 37 and 41 ppm, respectively) (Cheng et al.,
414 2009) and of *Tagetes patula* L. which exerted toxicity against larvae of *A. aegypti*, *C.*
415 *quinquefasciatus* and *Anopheles stephensi* (LD_{50} of 37, 22 and 12 ppm, respectively)
416 (Dharmagadda et al., 2005).

417

418 ## 4. Conclusions

419

420 Overall, taken together our results shed light into the possible utilization of the crop
421 residue of industrial hemp cv Felina 32 as a source of botanical insecticides to be used in
422 Integrated Pest Management programmes and organic agriculture. Although the hemp
423 essential oil marker compounds are already known, this research showed that their mixtures in
424 the form of a pure essential oil may act as a good biopesticide, notably against aphids and
425 houseflies, giving an added value to the by-product, i.e., inflorescences, obtained during hemp
426 cultivation. Its great availability following the harvesting and processing of hemp fibre and/or
427 seeds make it an additional resource to exploit and valorise at industrial level.
428
429 **Conflict of Interest**
430
431 Authors declare no conflict of interest.
432
433 **Acknowledgments**
434
435 The Authors thank Michele Grossi, Luigi Corradini and La Biologica Soc. Coop. Agr.
436 for kindly providing samples of hemp cv Felina 32. R. Pavela thanks Ministry of Agriculture
437 of the Czech Republic for its financial support concerning botanical pesticide and basic
438 substances research (Project No. RO0417). F. Maggi is grateful to University of Camerino
439 (Fondo di Ateneo per la Ricerca, FAR 2014/2015, FPI 000044) for supporting this work.
440
441 **References**
442
443 Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. J. Econ.
444 Entomol. 18, 265–267.

445 Adams, R.P., 2007. Identification of essential oil components by gas chromatography/mass
446 spectroscopy. Carol Stream (IL): Allured.
447 Ali, A., Tabanca, N., Amin, E., Demirci, B., Khan, I.A., 2016. Chemical composition and
448 biting deterrent activity of essential oil of *Tagetes patula* (Marigold) against *Aedes aegypti*.
449 Nat. Prod. Comm. 11, 1535-1538.
450 Bass, C., Puinean, A. M., Zimmer, C.T., Denholm, I., Field, L.M., Foster, S.P., Gutbrod, O.,
451 Nauen, R., Slater, R., Williamson, M.S., 2014. The evolution of insecticide resistance in
452 the peach potato aphid, *Myzus persicae*. Insect Biochem. Mol. Biol. 51, 41-51.
453 Bass, C., Field, L.M., 2011. Gene amplification and insecticide resistance. Pest Manag. Sci.
454 67, 886–890.
455 Bass, C., Puinean, A.M., Zimmer, C.T., Denholm, I., Field, L.M., Foster, S.P., Gutbrod, O.,
456 Nauen, R., Slater, R., Williamson, M.S., 2014. The evolution of insecticide resistance in
457 the peach potato aphid, *Myzus persicae*. Insect Biochem. Mol. Biol. 51, 41–51.
458 Bedini, S., Flamini, G., Cosci, F., Ascrizzi, R., Benelli, G., Conti, B., 2016. *Cannabis sativa*
459 and *Humulus lupulus* essential oils as novel control tools against the invasive mosquito
460 *Aedes albopictus* and fresh water snail *Physella acuta*. Ind. Crops Prod. 85, 318-323.
461 Benelli, G., Mehlhorn, H., 2016. Declining malaria, rising dengue and Zika virus: insights for
462 mosquito vector control. Parasitol. Res. 115, 1747–1754.
463 Benelli, G., Romano, D., 2017. Mosquito vectors of Zika virus. Entomologia Generalis 36,
464 309-318.
465 Benelli G, Pavela R (2018a) Repellence of essential oils and selected compounds against ticks
466 – a systematic review. Acta Trop. 179:47–54, doi: 10.1016/j.actatropica.2017.12.025
467 Benelli G, Pavela R (2018b) Beyond mosquitoes – Essential oil toxicity and repellency
468 against bloodsucking insects. Ind. Crops Prod. 117:382–392, doi:
469 10.1016/j.indcrop.2018.02.072.

470 Benelli, G., Pavela, R., Canale, A., Mehlhorn, H. 2016. Tick repellents and acaricides of
471 botanical origin: a green roadmap to control tick-borne diseases? Parasitol. Res. 115, 2545-
472 2560.
473 Benelli, G., Pavela, R., Iannarelli, R., Petrelli, R., Cappellacci, L., Cianfaglione, K., Afshar,
474 F.H., Nicoletti, M., Canale, A., 2017a. Synergized mixtures of Apiaceae essential oils and
475 related plant-borne compounds: larvicidal effectiveness on the filariasis vector *Culex*
476 *quinquefasciatus* Say. Ind. Crops Prod. 96, 186-195.
477 Benelli, G., Pavela, R., Canale, A., Cianfaglione, K., Ciaschetti, G., Conti, F., Nicoletti, M.,
478 Senthil-Nathan, S., Mehlhorn, H., Maggi, F., 2017b. Acute larvicidal toxicity of five
479 essential oils (*Pinus nigra*, *Hyssopus officinalis*, *Satureja montana*, *Aloysia citrodora* and
480 *Pelargonium graveolens*) against the filariasis vector *Culex quinquefasciatus*: Synergistic
481 and antagonistic effects. Parasitol. Int. 66, 166-171.
482 Benelli, G., Pavela, R., Lupidi, G., Nabissi, M., Petrelli, R., Kamte, S.L.N., Cappellacci, L.,
483 Fiorini, D., Sut, S., Dall'Acqua, S., Maggi, F., 2018a. The crop-residue of fiber hemp cv.
484 Futura 75: from a waste product to a source of botanical insecticides. Environ. Sci. Poll.
485 Res. doi: 10.1007/s11356-017-0635-5.
486 Benelli, G., Pavela, R., Giordani, C., Casettari, L., Curzi, G., Cappellacci, L., Petrelli, R.,
487 Maggi, F., 2018b. Acute and sub-lethal toxicity of eight essential oils of commercial
488 interest against the filariasis mosquito *Culex quinquefasciatus* and the housefly *Musca*
489 *domestica*. Ind. Crops Prod. 112, 668-680.
490 Bertoli, A., Tozzi, S., Pistelli, L., Angelini, L.G., 2010. Fiber hemp inflorescences: from crop-
491 residues to essential oil production. Ind. Crops Prod. 32, 329–337.
492 Blackman, R.L., Eastop, V.F., 2000. Aphids on the World's crops. An identification and
493 information guide, 2nd edn. John Wiley & Sons Ltd, Hoboken.

494 Blanc, S., Uzest, M., Drucker, M., 2011. New research horizons in vector transmission of
495 plant viruses. Curr. Opin. Microbiol. 14, 483–491.
496 Bossou, A.D., Mangelinckx, S., Yedomonhan, H., Boko, P.M., Akogbeto, M.C., De Kimpe,
497 N., Avlessi, F., Sohounhloue, C.K., 2013. Chemical composition and insecticidal activity
498 of plant essential oils from Benin against *Anopheles gambiae* (Giles). Parasit. Vectors 6,
499 337.
500 Calzolari, D., Magagnini, G., Lucini, L., Grassi, G., Appendino, G.B., Amaducci, S., 2017.
501 High added-value compounds from *Cannabis* threshing residues. Ind. Crops Prod. 108,
502 558-563.
503 Cheng, S.S., Chang, H.T., Lin, C.Y., Chen, P.S., Huang, C.G., Chend, W.J., Chang, S.T.,
504 2009. Insecticidal activities of leaf and twig essential oils from *Clausena excavata* against
505 *Aedes aegypti* and *Aedes albopictus* larvae. Pest Manag. Sci. 65, 339–343.
506 Datta, S., Singh, J., Singh, S., Singh, J., 2016. Earthworms, pesticides and sustainable
507 agriculture: a review. Environ. Sci. Pollut. Res. 23, 8227–8243.
508 Davies, M. P., Anderson, M., Hilton, A. C., 2016. The housefly *Musca domestica* as a
509 mechanical vector of *Clostridium difficile*. J. Hosp. Infect. 94, 263-267.
510 Desneux, N., Decourtye, A., Delpuech, J. M., 2007. The sublethal effects of pesticides on
511 beneficial arthropods. Annu. Rev. Entomol. 52, 81-106.
512 Dharmagadda, V.S.S., Naik, S.N., Mittal, P.K., Vasudevan, P., 2005. Larvicidal activity of
513 *Tagetes patula* essential oil against three mosquito species. Bioresour. Technol. 96, 1235–
514 1240.
515 FFNSC 2, 2012. Flavors and Fragrances of Natural and Synthetic Compounds. Mass spectral
516 database. Japan: Shimadzu Corps.
517 Finney, D.J., 1971. Probit Analysis. Cambridge University, London, pp. 68–78.

518 Govindarajan, M., Rajeswary, M., Bhattacharyya, A., Benelli, G., 2016. Eugenol, α-pinene
519 and β-caryophyllene from *Plectranthus barbatus* essential oil as eco-friendly larvicides
520 against malaria, dengue and Japanese encephalitis mosquito vectors. Parasitol. Res. 115,
521 807–815.
522 Govindarajan, M., Sivakumar, R., Rajeswary, M., Yogalakshmi, K., 2012. Chemical
523 composition and larvicidal activity of essential oil from *Mentha spicata* (Linn.) against
524 three mosquito species. Parasitol. Res. 110, 2023-2032.
525 Hummelbrunner, L.A., Isman, M.B., 2001. Acute, sublethal, antifeedant, and synergistic
526 effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera*
527 *litura* (Lep., Noctuidae). J. Agric. Food Chem. 49, 715-720.
528 Isman, M.B., 2015. A renaissance for botanical insecticides. Pest Manag. Sci. 71:1587–1590.
529 Isman, M.B., Machial, C.M., 2006. Chapter 2 Pesticides based on plant essential oils: from
530 traditional practice to commercialization. In M. Rai and M.C. Carpinella (eds.), Naturally
531 Occurring Bioactive Compounds, Elsevier, BV, pp 29–44.
532 Jacobson, M., 1989. Botanical Pesticides: Past, Present and Future. Insecticides of Plant In
533 Origin. Arnason, Philogene, Bjr and Morand, P. ACS Symp. Ser., vol. 387, pp. 1–10.
534 Jansirani, D., Nivethitha, S., Singh, M.V.P., 2012. Production and utilization of vermicast
535 using organic wastes and its impact on *Trigonella foenum* and *Phaseolus aureus*. Int. Res.
536 J. Biol. Sci. 2, 187–189.
537 Lim, S.L., Lee, L.H., Wu, T.Y., 2016. Sustainability of using composting and
538 vermicomposting technologies for organic solid waste biotransformation: recent overview,
539 greenhouse gases emissions and economic analysis. J. Clean. Prod. 111, 262–278.
540 Liu, Y., Xue, M., Zhang, Q., Zhou, F., Wei, J., 2010. Toxicity of β-caryophyllene from *Vitex*
541 *negundo* (Lamiales: Verbenaceae) to *Aphis gossypii* Glover (Homoptera: Aphididae) and
542 its action mechanism. Acta Entomologica Sinica 53, 396-404.

543 Lucia, A., Audino, P.G., Seccacini, E., Licastro, S., Zerba, E., Masuh, H., 2007. Larvicidal
544 effect of *Eucalyptus grandis* essential oil and turpentine and their major components on
545 *Aedes aegypti* larvae. J. Am. Mosq. Control Assoc. 23, 299-303.
546 NIST 08, 2008. National Institute of Standards and Technology. Mass spectral library
547 (NIST/EPA/NIH). Gaithersburg (MD): National Institute of Standards and Technology.
548 OEPP/EPPO, 2015. EPPO standards PM 7/124(1) diagnostic protocol for *Spodoptera*
549 *littoralis, Spodoptera litura, Spodoptera frugiperda, Spodoptera eridania.* Bull.
550 OEPP/EPPO Bull. 34: 257–270.
551 Pavela, R., Benelli, G., 2016. Essential oils as eco-friendly biopesticides? Challenges and
552 constraints. Trends in Plant Science 21, 1000-1007.
553 Pavela, R., 2015. Essential oils for the development of eco-friendly mosquito larvicides: a
554 review. Ind. Crops Prod. 76, 174-187.
555 Potter, D.J., 2009. The Propagation, Characterisation and Optimisation of *Cannabis sativa* L.
556 as a Phytopharmaceutical. Pharmaceutical Sciences, King's College, London.
557 Pavela, R., Zabka, M., Vrchotova, N., Triska, J., Kazda, J. 2013. Selective effects of the
558 extract from *Angelica archangelica* L. against *Harmonia axyridis* (Pallas)—an important
559 predator of aphids. Ind. Crops Prod. 51, 87-92.
560 Pavela, R., 2016. History, presence and perspective of using plant extracts as commercial
561 botanical insecticides and farm products for protection against insects—a review. Plant
562 Protection Science 52, 229–241.
563 Pavela, R., Maggi, F., Lupidi, G., Cianfaglione, K., Dauvergne, X., Bruno, M., Benelli, G.,
564 2017. Efficacy of sea fennel (*Crithmum maritimum* L., Apiaceae) essential oils against
565 *Culex quinquefasciatus* Say and *Spodoptera littoralis* (Boisd.). Ind. Crops Prod. 109, 603-
566 610.

567 Pavela, R., Maggi, F., Lupidi, G., Mbuntcha, H., Woguem, V., Womeni, H. M., Barboni, L.,
568 Tapondjou, L.A., Benelli, G., 2018. *Clausena anisata* and *Dysphania ambrosioides*
569 essential oils: from ethno-medicine to modern uses as effective insecticides. Environ. Sci.
570 Pollut. Res. doi: 10.1007/s11356-017-0267-9
571 Pavela, R., 2018. Essential oils from *Foeniculum vulgare* Miller as a safe environmental
572 insecticide against the aphid *Myzus persicae* Sulzer. Environ. Sci. Pollut. Res. doi:
573 10.1007/s11356-018-1398-3
574 Rattan, R.S., 2010. Mechanism of action of insecticidal secondary metabolites of plant origin.
575 Crop Prot. 29, 913–920.
576 Rodriguez-Campos, J., Dendooven, L., Alvarez-Bernal, D., Contreras-Ramos, S.M., 2014.
577 Potential of earthworm to accelerate removal of organic contaminants from soil: a review.
578 Appl. Soil Ecol. 79, 10–25.
579 Stepanycheva, E. A., Petrova, M. O., Chermenskaya, T. D., Pavela, R., 2014. Prospects for
580 the Use of *Pongamia pinnata* Oil-Based Products against the Green Peach Aphid *Myzus*
581 *persicae* (Sulzer) (Hemiptera: Aphididae). Psyche 2014, 705397
582 Stevenson, P.C., Isman, M.B., Belmain, S.R. 2017. Pesticidal plants in Africa: a global vision
583 of new biological control products from local uses. Ind. Crops Prod. 110, 2-9.
584 Sut, S., Pavela, R., Kolarčik, V., Lupidi, G., Maggi, F., Dall'Acqua, S., Benelli, G., 2017.
585 Isobutyrylshikonin and isovalerylshikonin from the roots of *Onosma visianii* inhibit larval
586 growth of the tobacco cutworm *Spodoptera littoralis*. Ind. Crops Prod. 109, 266-273.
587 Thakore, Y., 2006. The biopesticide market for global agricultural use. Ind. Biotechnol. 2,
588 194–208.
589 Traboulsi, A.F., Taoubi, K., El-Haj, S., Bessiere, J.M., Rammal, S., 2002. Insecticidal
590 properties of essential plant oils against the mosquito *Culex pipiens molestus* (Diptera:
591 Culicidae). Pest Manag. Sci. 58, 491–495

592 Van den Dool, H., Kratz, P.D., 1963. A generalization of the retention index system including
593 linear temperature programmed gas—liquid partition chromatography. J. Chromatogr. A
594 11, 463-471.
595 Vasantha-Srinivasan, P., Senthil-Nathan, S., Ponsankar, A., Thanigaivel, A., Chellappandian,
596 M., Edwin, E.S., Selin-Rani, S., Kalaivani, K., Hunter, W.B., Duraipandiyan, V., Al-
597 Dhabi, N.A., 2018. Acute toxicity of chemical pesticides and plant-derived essential oil on
598 the behavior and development of earthworms, *Eudrilus eugeniae* (Kinberg) and *Eisenia*
599 *fetida* (Savigny). Environ. Sci. Pollut. Res. doi: 10.1007/s11356-017-9236-6.
600 WHO, 1991. The Housefly. Training and Information Guide (intermediate Level). Geneva,
601 vol. 1211 Division of Control of Tropical Diseases, World Health Organization, Geneva
602 27, Switzerland (unpublished document WHO/VBC/90.987; available on request).
603 WHO, 1996. Report of the WHO informal consultation on the evaluation and testing of
604 insecticides. CTD/WHOPES/IC/96.1.
605

Table

Table 1. Chemical composition of the essential oil from the fresh inflorescences of *Cannabis sativa* cv Felina 32 cultivated in central Italy.

N.	Component[a]	RI Exp.[b]	RI Lit.[c]	(%)[d]	ID[e]
1	tricyclene	914	921	0.1±0.0	RI,MS
2	α-thujene	921	924	0.1±0.0	RI,MS
3	α-pinene	926	932	16.4±2.9	Std,RI,MS
4	camphene	939	946	0.2±0.0	Std,RI,MS
5	β-pinene	968	974	5.2±1.1	Std,RI,MS
6	myrcene	990	988	14.2±2.6	Std,RI,MS
7	α-phellandrene	1003	1002	0.3±0.1	Std,RI,MS
8	δ-3-carene	1008	1008	0.2±0.0	Std,RI,MS
9	α-terpinene	1014	1014	0.3±0.1	Std,RI,MS
10	*p*-cymene	1022	1020	tr[f]	Std,RI,MS
11	limonene	1025	1024	0.5±0.1	Std,RI,MS
12	β-phellandrene	1025	1025	1.0±0.2	RI,MS
13	1,8-cineole	1028	1025	0.1±0.0	Std,RI,MS
14	(*Z*)-β-ocimene	1037	1032	0.6±0.2	Std,RI,MS
15	(*E*)-β-ocimene	1047	1044	5.1±0.9	Std,RI,MS
16	γ-terpinene	1056	1054	0.1±0.0	Std,RI,MS
17	terpinolene	1085	1086	9.6±1.6	Std,RI,MS
18	borneol	1161	1165	tr	Std,RI,MS
19	terpinen-4-ol	1174	1174	tr	Std,RI,MS
20	7-*epi*-sesquithujene	1387	1390	tr	RI,MS
21	hexyl hexanoate	1389	1389	tr	RI,MS
22	(*Z*)-caryophyllene	1398	1399	0.2±0.0	RI,MS
23	sesquithujene	1404	1405	tr	RI,MS
24	(*E*)-caryophyllene	1411	1412	23.8±3.9	Std,RI,MS
25	α-santalene	1415	1416	0.2±0.0	RI,MS
26	γ-elemene	1428	1434	0.1±0.0	RI,MS
27	α-*trans*-bergamotene	1432	1432	2.2±0.3	RI,MS
28	α-humulene	1445	1452	8.3±1.7	Std,RI,MS
29	*allo*-aromadendrene	1451	1458	0.8±0.2	Std,RI,MS
30	(*E*)-β-farnesene	1457	1454	3.0±0.5	Std,RI,MS

31	β-chamigrene	1468	1476	tr	RI,MS
32	γ-muurolene	1470	1478	0.1±0.0	RI,MS
33	β-selinene	1477	1489	1.7±0.3	RI,MS
34	α-selinene	1486	1498	1.3±0.3	RI,MS
35	β-bisabolene	1506	1505	0.2±0.0	RI,MS
36	β-curcumene	1508	1514	0.4±0.1	RI,MS
37	δ-cadinene	1518	1522	0.1±0.0	RI,MS
38	β-sesquiphellandrene	1520	1521	0.2±0.0	RI,MS
39	selina-4(15),7(11)-diene	1526	1540	0.5±0.1	RI,MS
40	selina-3,7(11)-diene	1532	1545	0.9±0.2	RI,MS
41	(*E*)-α-bisabolene	1541	1540	0.1±0.0	RI,MS
42	germacrene B	1546	1559	0.2±0.0	RI,MS
43	(*E*)-nerolidol	1563	1561	tr	Std,RI,MS
44	caryophyllene oxide	1572	1583	1.2±0.2	Std,RI,MS
45	humulene epoxide II	1598	1608	0.2±0.0	Std,RI,MS
46	caryophylla-4(12),8(13)-dien-5-β-ol	1626	1639	tr	RI,MS
47	cannabidiol	2421	2430	0.1±0.0	Std,RI,MS
	Total identified (%)			99.8	
	Oil yield (%, w/w)			0.3	
	Grouped compounds (%)				
	Monoterpene hydrocarbons			54.0	
	Oxygenated monoterpenes			0.2	
	Sesquiterpene hydrocarbons			44.2	
	Oxygenated sesquiterpenes			1.4	
	Cannabinoids			0.1	
	Others			0.1	

[a] Compounds are listed in order of their elution from a HP-5MS column. [b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8-C_{30} alkanes. [c] Linear retention index taken from Adams (2007), or NIST 08 (2008) and FFNSC2 (2012) and literature (for compound 47). [d] Relative area percentage values are means of three determinations ± SD. [e] Identification methods: std, based on comparison with authentic compounds; RI, based on comparison of calculated RI with those reported in ADAMS, FFNSC 2 and NIST 08; MS, based on comparison with WILEY, ADAMS, FFNSC2 and NIST 08 MS databases. [f] tr, % below 0.05%.

Table 2. Toxicity of the essential oil from the fresh inflorescences of *Cannabis sativa* cv Felina 32 cultivated in central Italy on four insect pests of economic importance; α-cypermethrin was tested as positive control.

Insect species and tested instar	Unit	LC_{50}/LD_{50}	CI_{95}	LC_{90}/LD_{90}	CI_{95}	Chi-square	Mortality (%) at maximum tested dose or concentration
				Cannabis sativa cv Felina 32 essential oil			
Culex quinquefasciatus 3rd instar larva	$\mu l\ L^{-1}$	252.5	215.2-330.1	700.9	649.7-895.3	4.365	100.0±0.0 % mortality testing 1,000 $\mu l\ L^{-1}$
Culex quinquefasciatus adult female	$\mu g\ cm^{-2}$	>500	-	-		-	32.7±8.9 % mortality testing 500 $\mu g\ cm^{-2}$
Musca domestica adult female	$\mu g\ adult^{-1}$	43.3	31.6-55.7	213.5	212.1-226.7	5.844	80.1±3.5 % mortality testing 200 µg/adult
Myzus persicae adult	$ml\ L^{-1}$	3.5	3.1-4.8	6.2	5.9-6.8	0.252	100.0±0.0 % mortality testing 15 $ml\ L^{-1}$
Spodoptera littoralis 3rd instar larva	$\mu g\ larva^{-1}$	152.3	127.1-193.8	313.1	293.8-385.6	0.271	66.6±4.7 % mortality testing 200 µg/larva
				α-Cypermethrin			
Culex quinquefasciatus 3rd instar larva	$\mu l\ L^{-1}$	0.0003	0.0002-0.0004	0.0009	0.0008-0.0025	3.252	-
Culex quinquefasciatus adult female	$\mu g\ cm^{-2}$	<0.1	-	-	-	-	-
Musca domestica adult female	$\mu g\ adult^{-1}$	0.15	0.12-0.25	0.62	0.58-0.91	0.356	-
Myzus persicae adult	$ml\ L^{-1}$	0.002	0.001-0-003	0.005	0.004-0.006	1.252	-
Spodoptera littoralis 3rd instar larva	$\mu g\ larva^{-1}$	0.0025	0.0012-0-0045	0.0062	0.0058-0.0095	2.352	-

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

Table 3. Acute toxicity of the essential oil from the fresh inflorescences of *Cannabis sativa* cv Felina 32 cultivated in central Italy against larvae and adults of *Harmonia axyridis*. α-cypermethrin was tested as positive control.

Concentration of essential oil ($ml.L^{-1}$)	Mortality of larvae (% ± SD)	Mortality of adults (% ± SD)
5.5	3.3 ± 4.1^{a}	0.0 ± 0.0^{a}
2.5	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
1.25	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
0.6	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Positive control	100.0 ± 0.0^{b}	100.0 ± 0.0^{b}
Negative control	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
ANOVA	$F_{5,18}$=1,228; $P<0.0001$	$F_{5,18}$=1,128; $P<0.0001$

* 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
Positive control = 1 $ml.L^{-1}$ Vaztak (0.005 $ml.L^{-1}$ (w/v) of α-cypermethrin.

Table 4. Toxicity of the essential oil from the fresh inflorescences of *Cannabis sativa* cv Felina 32 cultivated in central Italy on *Eisenia fetida* eartworms. α-cypermethrin was tested as positive control.

Treatment and concentration ($mg.kg^{-1}$)	7th day* (mortality % ± SD)	14th day* (mortality % ± SD)
EO 100.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{b}
EO 50.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
EO 25.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
A-CM 50.0	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}
A-CM 25.0	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}
A-CM 12.5	89.5 ± 2.5^{b}	95.5 ± 2.5^{b}
Control	0.0 ± 0.0^{a}	2.5 ± 5.0^{a}
ANOVA	$F_{6,21}$=391.05; P<0.0001	$F_{6,21}$=559.92; P<0.0001

*Average mortality of *E. fetida* (± SD) achieved on the 7th and 14th day after application of essential oil from *Cannabis sativa* cv Felina 34 (EO) and α-cypermethrin (A-CM)
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

Fig. 1. TIC-GC/MS chromatogram of the essential oil from the fresh inflorescences of industrial hemp, *Cannabis sativa*, cv Felina 32.